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**Chronic *Staphylococcus aureus* Lung Infection Correlates With Proteogenomic and Metabolic Adaptations Leading to an Increased Intracellular Persistence**

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**Background.** Chronic lung infection in cystic fibrosis (CF) patients by *Staphylococcus aureus* is a well-established epidemiological fact. Indeed, *S. aureus* is the most commonly identified pathogen in the lungs of CF patients. Improving our understanding of the mechanisms associated with the persistence of *S. aureus* is therefore an important issue.

**Methods.** We selected pairs of sequential *S. aureus* isolates from 3 patients with CF and from 1 patient with non-CF chronic lung disease. We used a combination of genomic, proteomic, and metabolomic approaches with functional assays for in-depth characterization of *S. aureus* long-term persistence.

**Results.** In this study, we show that late *S. aureus* isolates from CF patients have an increased ability for intracellular survival in CF bronchial epithelial-F508del cells compared to ancestral early isolates. Importantly, the increased ability to persist intracellularly was confirmed for *S. aureus* isolates within the own-patient F508del epithelial cells. An increased ability to form biofilm was also demonstrated. Furthermore, we identified the underlying genetic modifications that induce altered protein expression profiles and notable metabolic changes. These modifications affect several metabolic pathways and virulence regulators that could constitute therapeutic targets.

**Conclusions.** Our results strongly suggest that the intracellular environment might constitute an important niche of persistence and relapse necessitating adapted antibiotic treatments.

**Keywords.** cystic fibrosis; *Staphylococcus aureus*; intracellular persistence; biofilm; proteogenomics.

*Staphylococcus aureus* and *Pseudomonas aeruginosa* are the most common pathogens that infect the lungs of patients with a chronic lung disease (CLD) including cystic fibrosis (CF) [1, 2]. Furthermore, *S. aureus* is one of the earliest bacteria detected in infants with CF. However, very few studies have addressed the adaptations *S. aureus* has undergone in this context [3–6].

*Staphylococcus aureus* has the ability to form biofilm [7–9] and to survive within a wide range of eukaryotic host cells [10–19]. These abilities are likely to contribute to the persistence of *S. aureus* in airways of patients with CLDs despite appropriate antimicrobial treatments [20, 21]. *Staphylococcus aureus* persistence is associated with a drastic decrease in metabolism [22], a decrease in the expression of virulence factors, and an increase in the expression of bacterial adhesins [23]. Such a profile is typical of small-colony variants (SCVs) that are defined by small-sized colonies [17, 24, 25]. In addition to SCVs, strains with normal colony morphology can exhibit similar patterns of “low toxicity” that allow them to persist intracellularly without being cleared by host cell defense mechanisms [23]. A low-toxicity pattern can be achieved either transiently, following changes in the expression of genes encoding toxins and/or regulators, or permanently by mutations in global regulators [26–28].

The alternative sigma B factor (SigB) has pleiotropic roles in gene regulation and has been shown to be a master regulator of intracellular persistence [29, 30]. SigB was shown to downregulate the *agr* system, which is responsible for enhanced inflammatory activity. Hence, the expression of SigB during chronic persistence is linked to its capacity to downregulate proinflammatory virulence factors and to increase the expression of factors that promote persistence [28, 30]. SigB enables the bacteria to downregulate its aggressive phenotype and to switch toward a silent SCV phenotype that allows for long-term intracellular persistence.
By studying serial isolates, we show that during long-term lung infection, *S. aureus* adaptation occurs through genomic modifications that accumulate over time and lead to major metabolic modifications and protein expression changes. We also reveal that persistence of *S. aureus* is associated with convergent phenotypic evolution responsible for an increased ability to form biofilm as well as to survive within host cells. These observations should be taken into account in therapeutic decisions aimed at eradicating *S. aureus* chronic infections by choosing drugs that specifically target biofilm-embedded and intracellular bacteria.

**METHODS**

Whole-genome sequencing was performed on an Illumina MiSeq instrument (2 × 150 bp), and the sequences were processed using the Nullarbor bioinformatic pipeline software v1.20 and RAST server. The sequences reported here are available at the National Center for Biotechnology Information's BioProject database under accession PRJNA446073.

Quantification of biofilm formation was assessed with crystal violet staining in polystyrene 96-well plates. CF bronchial epithelial cell line CFBE41o- and primary nasal epithelial cell were infected with a multiplicity of infection of 100 using an inoculum taken from cultures of *S. aureus* grown in brain-heart infusion (BHI) until the exponential growth phase. Infected cells were kept for 6 days in a medium that contained 50 μg/mL gentamicin to kill extracellular bacteria. E-tests were used to determine minimal inhibitory concentrations (MICs) to gentamicin. Gentamicin has a high bactericidal effect on *S. aureus* USA300-LAC (MIC = 2 μg/mL) and a very poor penetration inside eukaryotic cells. In addition, the measured MICs to gentamicin of all tested clinical strains were similar to that of USA300 reference strain (≤2 μg/mL). Hence, an accumulation of gentamicin within epithelial cells should impact equally all tested strains and is unlikely to explain differences between isolates.

For proteomics, proteins were digested and analyzed using liquid chromatography (LC) coupled with tandem mass spectrometry (nanoLC-MS/MS). For metabonomics, metabolite profiling of *S. aureus* isolates was performed using LC–MS. The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [31] partner repository with the dataset identifier PXD011281. The metabolomics raw data are provided in Supplementary Table S1. A full description of methods is available in the Supplementary Methods.

**Statistical Analyses**

Data were analyzed using R or GraphPad Prism software. Results are presented either with 1 representative experiment for clarity or as means ± standard deviation. The number of biological and technical replicates is indicated per figure.

**RESULTS**

Selection of *S. aureus* Sequential Isolates From Patients With Chronic Lung Infection

Three patients with CF (CF1, CF2, and CF3) and, for comparison purposes, 1 patient with non-CF CLD were chosen. For each patient, we selected 1 early and 1 late isolate separated by 3- to 9-year intervals. By definition, in a pair of isolates that belong to the same clone, the “early isolate” corresponds to the isolate collected earlier than the second isolate that had been collected later and that corresponds to the “late isolate.” Whole-genome sequencing confirmed that each pair of isolates belonged to 4 distinct clones (Figure 1). Patient diseases and treatments are detailed in the Supplementary Methods.

*S. aureus* Clinical Isolates From CF Patients Evolved an Increased Persistence Ability Within CFBE-F508del Epithelial Cell Line

Numerous studies have shown that *S. aureus* has the ability to survive within human cells [10–19]. We subsequently aimed at determining if during the course of within-lung adaptation, *S. aureus* isolates have evolved a greater ability to persist within epithelial cells. We infected the bronchial CFBE epithelial cell line (F508del +/- cystic fibrosis transmembrane conductance regulator [CFTR] mutation) with clinical isolates, the control strain USA300-LAC, and a stable SCV mutant altered in the haem biosynthetic pathway (hereafter, named Δhem). As expected, wild-type bacteria were not able to persist, whereas the Δhem mutant was able to persist intracellularly during the whole course of the experiment (Supplementary Figure S1) [15]. All early and late clinical isolates were able to persist at least 2.6-fold, and up to 900-fold, more than the USA300-LAC reference strain at day 3 and day 6 post-infection (Figure 2A, 2B). Furthermore, at day 3 and day 6 post-infection, all the late
isolates recovered from CF patients exhibited an improved ability to persist intracellularly within CFBE-F508del epithelial cells compared to cognate early isolates (Figure 2A). Interestingly, the CLD\_late isolate recovered from the non-CF patient did not exhibit an improved ability to persist within CFBE epithelial cells compared to CLD\_early isolate (Figure 2B). These data suggest that S. aureus adaptation within CF lungs correlates with an improved ability to persist intracellularly in cells with a CFTR dysfunction.

**Late S. aureus Isolate of CF3 Patient Exhibits an Increased Persistence Within Primary F508del Epithelial Own-patient Cells**

To confirm the relevance of the results obtained with the bronchial CFBE epithelial cell line, we first assessed the persistence of CF3 isolates within primary epithelial cells isolated from the nose of a healthy donor (Figure 2C). In addition, we performed an infection assay with the CF3 primary epithelial own-patient cells (F508del +/+ CFTR mutation) to verify the specific within-patient adaptation of S. aureus recovered from long-term infection (Figure 2D). These experiments confirmed that the late isolate persistence ability is improved compared to the early isolate at day 3 and day 6 within both primary nasal epithelial cells retrieved from a healthy donor and from the CF3 patient.

**S. aureus Clinical Isolates From Chronically Infected Patients Evolved High Biofilm Formation Ability**

Assuming that isolates retrieved from chronic infections might have a high biofilm-forming capacity, we studied the biofilm formation ability of the pairs of isolates. Remarkably, all isolates displayed a greater capacity to form biofilms compared to that of the weak biofilm-producer USA300-LAC reference strain ($P < .001$; Supplementary Figure S2). Furthermore, for 3 chronically infected patients, the late isolates formed more biofilm than the early isolates, revealing that long-term adaptation within lungs had improved their biofilm formation capacity ($P < .001$; Figure 3).

**Late S. aureus Clinical Isolates From Chronically Infected Patients Acquired Auxotrophies**

Compared to that of USA300-LAC, all patients’ early isolates and the CF1 late isolate displayed similar colony morphology on BHI agar plates and a wild-type growth in a liquid broth that mimics sputum (CF sputum medium [CFSM]; Supplementary Figures S3 and 4A). In contrast, the late isolate of CF2 and CLD patients displayed a typical stable SCV phenotype with very small colonies on BHI agar (Supplementary Figure S4) and CF2\_late, CF3\_late, and CLD\_late isolates exhibited a growth defect in CFSM broth (Figure 4B–4D). Thymidine-dependent SCVs are frequently isolated from patients treated with sulfamethoxazole-trimethoprim (SXT) [32]. Indeed, supplementation with thymidine restored most wild-type growth for CF2\_late and CLD\_late isolates (Figure 4B, 4D). We used genomic data to determine the auxotrophy of CF3\_late isolate and identified a frameshift in panB gene, which is involved in de novo biosynthesis of pantothenic acid (Table 1). Accordingly, growth of CF3\_late isolate in the presence of pantothenate restored wild-type growth (Figure 4C). Thus, isolates from 3 of 4 patients with S. aureus chronic lung infection acquired auxotrophy during the course of the disease.

**Late S. aureus Clinical Isolates From Chronically Infected Patients Acquired Antibiotic Resistance**

Genome analysis evidenced mutations in thvA, gyrB, and rpsJ genes, which are likely to explain SXT, fluoroquinolones, and cyclines resistance, respectively (Table 1). However, in the case of point mutations, a causal relation with phenotypic changes has to be experimentally verified. Thus, isolates from 3 of 4 patients with S. aureus chronic lung infection acquired antibiotic resistance consistent with administration of the corresponding drugs during the course of the disease.

**Genomic, Proteomic, and Metabolomic Modifications Associated With S. aureus Adaptation During Chronic Lung Infection**

In order to investigate the underlying genomic, proteomic, and metabolic modifications associated with the observed

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**Figure 1.** Selection of 4 pairs of *Staphylococcus aureus* isolates belonging to 4 sequence types (STs) in 4 patients. Dendrogram generated by wgss.net from the genomes of the 8 clinical isolates retrieved from the respiratory samples of 3 patients with cystic fibrosis (CF), 1 patient with non-CF chronic lung infection, and 4 reference genomes from public databases. Branch length is proportional to the number of variant nucleotide sites within the core genes. For each patient, the isolate taken first is termed “early” and the isolate taken later is termed “late.” The dates of sampling and the ST of the isolates are indicated. “TA” and “PA” mean that the isolate is auxotrophic for thymidine or pantothenate, respectively. The name of the isolate is indicated in red when it is methicillin-resistant. Reference strains included are PFESA1902 (ERR554197), st1307 (ERR158691), and PFESA1195 (ERR554722). Abbreviations: CF, cystic fibrosis; CLD, chronic lung disease.
phenotypic changes, we compared genomes, proteomes, and metabolomes of late compared to early isolates. The differences in proteomic and metabolic profiles between early and late isolates of patients are highlighted by heat maps shown in Supplementary Figure S5.

Genomes of all clinical isolates were de novo assembled, and coding DNA sequences (CDSs) were annotated. Most of the single-nucleotide polymorphisms were missense variants occurring in CDSs (Table 2). Nonsynonymous mutations acquired by late isolates were found mainly in genes involved in metabolic processes (Figure 5A) and, more specifically, in the following functional categories: “amino acid transport and metabolism” and “carbohydrate transport and metabolism”. In addition, the largest category of proteins to be differentially expressed for all pairs also comprised proteins related to metabolism processes (and more specifically to the “amino acid transport and metabolism” category) (Figure 5B). Concordant with genomic and proteomic results, the category “amino acids” was the most altered metabolite category in the late isolate of all patients compared to their cognate early isolates (Figure 5C). Of note, our proteomics data also indicated that there is no category in which the overall direction of change is the same in all patients (Figure 5B).

Many regulatory proteins were differentially expressed. Indeed, proteins of the Agr, Rot, Sae, Sar, or Fur regulatory

Figure 2. Intracellular persistence of Staphylococcus aureus clinical isolates in cystic fibrosis bronchial epithelial (CFBE)-F508del epithelial cell line and within primary patient cells. (A and B) Bronchial CFBE epithelial cell line (CFBE-F508del homozygous for the F508del-cystic fibrosis transmembrane conductance regulator [CFTR] mutation) was infected with the control strain USA300-LAC and clinical isolates from cystic fibrosis (CF) patients (A) or the chronic lung disease (CLD) patient (B). (C and D) Primary nasal epithelial cells retrieved from a healthy donor (“Primary WT”) (C) and from the CF3 patient (“Primary CF3” with F508del +/- CFTR mutation) (D) were infected with the control strain USA300-LAC and CF3 isolates. For all experiments, gentamicin was present throughout the experiment to prevent extracellular bacterial growth and new infection. Bacterial loads inside cells were evaluated by colony-forming unit (CFU) enumeration at 3 and 6 days after infection. Results are normalized with USA300-LAC strain as a reference and expressed as a fold change of CFUs. Results have been obtained from 2 independent experiments performed in triplicate for A, B, and C and 1 experiment for D. Abbreviations: CF, cystic fibrosis; CLD, chronic lung disease; ns, not significant; WT, wild type. Statistical analysis was performed by Wilcoxon rank sum test: * P < .05; ** P < .01; P > .05.
networks were differently expressed in all late isolates. In CF1_late isolate, the premature stop codon identified in agrC (Table 1) is likely to profoundly impact the agr regulon and to explain a pleiotropic effect on the proteome (eg, downregulation of delta hemolysin and PSMb1 and upregulation of proteins encoded by spa, sbi, fnbA, rot, and coa genes). In addition, a missense mutation in saeR regulatory gene (Table 1) could also affect sae regulon and modify the proteome. In CF2_late isolate, the agr regulon is also downregulated, suggesting an evolution toward low virulent and highly adhesive properties. The metabolite profiling of CF2_late isolate showed a decrease in adenosine diphosphate, which is well correlated with the

Figure 3. Quantification of biofilm formation of Staphylococcus aureus clinical isolates. Biofilm formation quantification was performed using the crystal violet microtiter assay in brain–heart infusion medium with 1% glucose. Results shown are the mean ± standard deviation for 3 independent experiments performed in triplicate. Statistical significance was measured using a 2-tail Student t test when biofilm production of a late isolate was compared with biofilm production of a cognate early isolate from the same patient. Abbreviations: CF, cystic fibrosis; CLD, chronic lung disease. ** indicates \( P<.001 \) and ns indicates \( P>.05 \).

Figure 4. Growth of late Staphylococcus aureus clinical isolates in cystic fibrosis sputum medium. Growth curves were carried out in medium that mimics the respiratory fluid of cystic fibrosis patients, with or without the addition of thymidine or pantothenate. The results shown correspond to a representative experiment. The orange and green curves correspond to bacterial growth in media supplemented with either thymidine or pantothenate; the red and blue curves correspond to bacterial growth in medium without thymidine or pantothenate. WT, USA300-LAC. Abbreviations: CF, cystic fibrosis; CLD, chronic lung disease; Panto, pantothenate; Thy, thymidine; WT, wild type.
lack of hydroxyethylthiazole kinase expression found in proteomic analysis due to a frameshift in thiM gene (Table 1). In CF3_late isolate, frameshifts in fakA and panB genes (Table 1) were associated with a lack of cognate protein expression in CF3_late isolate. In addition, adhesins encoded by sdrD and sasF were upregulated. Interestingly, an overproduction of penicillin-binding protein 2 encoded by mecA is correlated with the saeR mutation (Table 1) [33]. The metabolite profiling of CF3_late isolate revealed a drastic reduction in pantothenate, coenzyme A, and dephospho-coenzyme A, which is in line with the lack of expression of PanB and PanC proteins [34].

Of note, the non-CF control clone displays a different evolutionary trajectory. Indeed, AgrA and AgrC were upregulated in CLD_late isolate, suggesting that it has retained virulent properties.

SCVs have typically decreased ribosomal activity and DNA and amino acid synthesis. Indeed, we noticed that in CLD_late isolate, 2 ribosomal proteins as well as a ribosomal protein methyltransferase were less expressed compared to early isolate. In CF2_late, 1 ribosomal protein and a DNA topoisomerase were also less expressed compared to early isolate. Also, metabolic profiling suggests that all late isolates have evolved a reduced citric acid cycle activity compared to cognate early isolates.

**DISCUSSION**

Here, we show that during chronic lung infection, *S. aureus* adapts through the acquisition of common adaptive traits including antibiotic resistances, auxotrophies, reduced citric acid cycle activity, increased biofilm, and intracellular persistence abilities that occurred irrespective of the clone type. Of particular interest, we report mutations in 2 master regulatory systems, Agr and Sae, likely to impact multiple protein expression and metabolite amounts.

The agr-defective mutants, such as CF1_late isolate, have been shown to arise during chronic infections and are better adapted to persistence within the infected host [27, 35, 36]. Genetic alterations that directly or indirectly target SaeR regulon were identified in the 3 CF patients. SaeR is directly involved in the regulation of more than 20 virulence factor genes [37] but is likely involved in the regulation of additional proteins by indirect mechanisms [38]. For instance, Cassat et al showed that 31 proteins, including SdrD, significantly increase

| Variable                  | CF1_late | CF2_late | CF3_late | CLD_late |
|---------------------------|----------|----------|----------|----------|
| Time since early isolate  | 2.8 years| 6.7 years| 9 years  | 4.4 years|
| Total polymorphisms       | 21       | 34       | 79       | 30       |
| Single-nucleotide polymorphism | 19   | 25       | 73       | 26       |
| Insertion–deletion        | 2        | 9        | 6        | 4        |
| Coding sequence           | 18       | 21       | 62       | 23       |
| Nonsynonymous mutation (%)| 14 (77%) | 16 (47.1)| 51 (64.6)| 17 (56.7)|
| Frameshift variant        | 2        | 4        | 4        | 2        |
| Missense variant          | 10       | 11       | 47       | 14       |
| Premature stop codon      | 1        | 1        | 0        | 1        |
| Other                     | 1        | 0        | 0        | 0        |
| Synonymous mutation       | 4        | 5        | 11       | 6        |
| Intergenic                | 3        | 13       | 17       | 7        |

Abbreviations: CF, cystic fibrosis; CLD, chronic lung disease; SXT, sulfamethoxazole-trimethoprim.

**Table 2. General Features of Detected Mutations**

| Isolate | Gene   | Product                          | Mutation                  | Putative Associated Phenotype |
|---------|--------|----------------------------------|---------------------------|------------------------------|
| CF1_late| agrC   | Accessory gene regulator protein C| Premature stop codon (L193X)| Downregulation of toxins and upregulation of adhesins |
| CF1_late| saeR   | *Staphylococcus* exprotein expression protein R | Missense G179L | Upregulation of SdrD adhesin |
| CF2_late| thyA   | Thymidylate synthase              | Premature stop codon (W88X) | SXT resistance and thymidine auxotrophy |
| CF2_late| thiM   | Hydroxyethylthiazole kinase       | Frameshift                | Decrease in adenosine diphosphate |
| CF2_late| fakA   | Fatty acid kinase A               | Missense G187D            | Increased biofilm formation and intracellular persistency |
| CF3_late| panB   | 3-methyl-2-oxobutanoate hydroxymethyltransferase | Frameshift               | Pantothenate auxotrophy |
| CF3_late| fakA   | Fatty acid kinase A               | Frameshift                | Increased biofilm formation and intracellular persistency |
| CF3_late| gyrB   | DNA topoisomerase subunit B       | Missense F226S            | Fluoroquinolones resistance |
| CF3_late| saeR   | *Staphylococcus* exprotein expression protein R | Missense A190T | Upregulation of SdrD adhesin |
| CLD_late| thyA   | Thymidylate synthase              | Missense P48R             | SXT resistance and thymidine auxotrophy |

Abbreviations: CF, cystic fibrosis; CLD, chronic lung disease.
in abundance upon inactivation of *sae* [38]. Furthermore, SaeRS-deficient bacteria are less infective in animal models [39].

In the 3 CF\_late isolates, we observed an increase in the expression of the SdrD adhesin belonging to the SaeR regulon and involved in adhesion to human nasal epithelial cells and to human keratinocytes [38, 40]. Our results suggest that SdrD is also important for long-term lung colonization.

In patients with chronic lung infections, SCV detection is most often the consequence of a long-term SXT treatment [41]. Mutations in the *thyA* gene, as found in CF2 and CLD\_late
isolates, are known to lead to stable clinical SCVs that are no longer susceptible to SXT and are thymidine-auxotrophic (TA-SCV) [32, 41]. Since thymidine is assumed to be abundant during lung inflammation, TA-SCVs can still grow in the inflamed alveolar environment.

In CF3 late isolate, we observed a pantothenate auxotrophy, which has been previously associated with persistence in Mycobacterium tuberculosis [42]. The acquisition of pantothenate auxotrophy suggests that pantothenate could also be present in CF lungs. Thus, our data confirm that metabolic specialization is a common phenomenon among long-term colonizers [43].

Other striking traits of phenotypic convergent evolution of S. aureus identified in this work were the increased ability to form biofilm and to persist in the intracellular niche. For CF2 and CF3 patients, the increased biofilm ability of late isolates could be linked to a mutation in the fakA gene, encoding fatty acid kinase A (FakA). Indeed, several studies showed that FakA-null strains were proficient in biofilm formation [8] and deficient in the expression of virulence factors controlled by the SaeRS system [44]. Overexpression of adhesins detected in proteomic analysis could also ultimately lead to increase biofilm formation in clinical isolates.

Numerous studies have demonstrated the ability of S. aureus to persist within host cells [10–19]. Strikingly, for the 3 CF patients, the S. aureus late isolates showed a greater ability to persist within CFBE-F508del epithelial cells compared to the early ones at day 3 and day 6 post-infection. Of note, the late isolate of the CLD patient did not present an improved ability to persist intracellularly within CFBE-F508del epithelial cells, possibly due to the fact that it has adapted to a non-CF patient. However, it is not possible to draw conclusions on lung adaptation of S. aureus among patients with non-CF lung dysfunction based on a single patient.

In a previous study, Ziebandt and coworkers [45] reported that the protein patterns recorded within each pair of S. aureus isolates were very similar, whereas the pattern recorded between pairs of isolates belonging to different clonal groups were highly variable. Strikingly, in our study, marked differences in proteomic profiles between isolates belonging to the same clone were observed. These proteomic changes are likely the consequence of within-host adaptation. The fact that each patient’s isolates exhibited specific proteomic changes could be correlated to host-specific factors or to clone-specific factors.

Our multi-omics approach allowed both confirmation of previously known mechanisms and identification of novel candidate genes and pathways involved in the persistence ability of clinical isolates. We now provide evidence that the saeRS/fakA regulon and the pantothenate pathway could also be promising therapeutic targets to fight persistent S. aureus infections.

Supplementary Data
Supplementary materials are available at Clinical Infectious Diseases online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes
Author contributions. X. T., M. C., X. N., A. C., and A. J. conceived and designed the study: X. T., E. R., M. D., D. E., F. T., J. M., and A. J. performed the experiments and analysis. I. N., C. C., I. C. G., A. F., and I. S. G. contributed with data and analysis. A. J., A. C., E. R., and X. T. wrote the manuscript, with contributions and comments from all authors.

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References
1. Wijers CD, Chmiel JF, Gaston BM. Bacterial infections in patients with primary ciliary dyskinesia: comparison with cystic fibrosis. Chron Respir Dis 2017; 14:392–406.
2. Jesenak M, Banovcin P, Jesenakova B, Babusikova E. Pulmonary manifestations of primary immunodeficiency disorders in children. Front Pediatr 2014; 2:77.
3. McAdam PR, Holmes A, Templeton KE, Fitzgerald JR. Adaptive evolution of Staphylococcus aureus during chronic endobronchial infection of a cystic fibrosis patient. PLoS One 2011; 6:e24301.
4. López-Collazo E, Jurado T, de Dios Caballero J, et al. In vivo attenuation and genetic evolution of a ST247-SCCmecI MRSA clone after 13 years of pathogenic bronchopulmonary colonization in a patient with cystic fibrosis: implications of the innate immune response. Mucosal Immunol 2015; 8:362–71.
5. Treffon J, Block D, Moche M, et al. Adaptation of Staphylococcus aureus to airway environments in patients with cystic fibrosis by upregulation of superoxide dismutase M and iron-scavenging proteins. J Infect Dis 2018; 217:1453–61.
6. Langhanki L, Berger P, Treffon J, Catania F, Kahl BC, Mellmann A. In vivo competition and horizontal gene transfer among distinct Staphylococcus aureus lineages as major drivers for adaptional changes during long-term persistence in humans. BMC Microbiol 2018; 18:152.
7. Zapotoczna M, O’Neill E, O’Garra J. Untangling the diverse and redundant mechanisms of Staphylococcus aureus biofilm formation. PLoS Pathog 2016; 12:e1005671.
8. Sabirova JS, Hernalsteens JP, De Backer S, et al. Fatty acid kinase A is an important determinant of biofilm formation in Staphylococcus aureus USA300. BMC Genomics 2015; 16:861.
9. Schwartzbeek B, Birtel J, Treffon J, et al. Dynamic in vivo mutations within the ica operon during persistence of Staphylococcus aureus in the airways of cystic fibrosis patients. PLoS Pathog 2016; 12:e1006024.
10. Garzoni C, Francois P, Huegle A, et al. A global view of Staphylococcus aureus whole genome expression upon internalization in human epithelial cells. BMC Genomics 2007; 8:171.
11. Garzoni C, Kelley WL. Staphylococcus aureus: new evidence for intracellular persistence. Trends Microbiol 2009; 17:59–65.
12. Kalinka J, Hachmeister M, Geraci J, et al. Staphylococcus aureus isolates from chronic osteomyelitis are characterized by high host cell invasion and intracellular adaptation, but still induce inflammation. Int J Med Microbiol 2014; 304:1038–49.
13. Mitchell G, Grondin G, Bûdeau G, Cantin AM, Malouin F. Infection of polarized airway epithelial cells by normal and small-colony variant strains of Staphylococcus aureus is increased in cells with abnormal cystic fibrosis transmembrane conductance regulator function and is influenced by NF-kB. Infect Immun 2011; 79:3541–51.
Proctor RA, von Eiff C, Kahl BC, et al. Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. Nat Rev Microbiol 2006; 4:295–305.

Rollin G, Tan X, Tros F, et al. Intracellular survival of Staphylococcus aureus in endothelial cells: a matter of growth or persistence. Front Microbiol 2017; 8:1354.

Sendi P, Proctor RA. Staphylococcus aureus as an intracellular pathogen: the role of small colony variants. Trends Microbiol 2009; 17:54–8.

Tuchscherr I, Heitmann V, Hussain M, et al. Staphylococcus aureus small-colony variants are adapted phenotypes for intracellular persistence. J Infect Dis 2010; 202:1031–40.

Tuchscherr L, Medina E, Hussain M, et al. Staphylococcus aureus phenotype switching: an effective bacterial strategy to escape host immune response and establish a chronic infection. EMBO Mol Med 2011; 3:129–41.

von Eiff C, Becker K, Metze D, et al. Intracellular persistence of Staphylococcus aureus small-colony variants within keratinocytes: a cause for antibiotic treatment failure in a patient with Darier's disease. Clin Infect Dis 2001; 32:1643–7.

Branger C, Gardye C, Lambert-Zechovsky N. Persistence of Staphylococcus aureus strains among cystic fibrosis patients over extended periods of time. J Med Microbiol 1996; 45:294–301.

Kahl BC, Duesbery A, Lubritz G, et al. Population dynamics of persistent Staphylococcus aureus isolated from the airways of cystic fibrosis patients during a 6-year prospective study. J Clin Microbiol 2003; 41:4424–7.

Kriegeskorte A, Grubmüller S, Huber C, et al. Staphylococcus aureus small colony variants show common metabolic features in central metabolism irrespective of the underlying auxotrophy. Front Cell Infect Microbiol 2014; 4:141.

García-Betancur JC, Goní-Moreno A, Horger T, et al. Cell differentiation defines acute and chronic infection cell types in Staphylococcus aureus. eLife 2017; 6:e28023.

Tuchscherr I, Kreis CA, Hoerr V, et al. Staphylococcus aureus develops increased resistance to antibiotics by forming dynamic small colony variants during chronic osteomyelitis. J Antimicrob Chemother 2016; 71:438–48.

Sadowska B, Bonar A, von Eiff C, et al. Characteristics of Staphylococcus aureus isolated from airways of cystic fibrosis patients, and their small colony variants. FEMS Immunol Med Microbiol 2002; 32:191–7.

Das S, Lindemann C, Young BC, et al. Natural mutations in a Staphylococcus aureus virulence regulator attenuate cytotoxicity but permit bacteremia and abscess formation. Proc Nat Acad Sci U S A 2016; 113:E101–10.

Suligoy CM, Lattar SM, Noto Llanà M, et al. Mutation of Agr is associated with the adaptation of Staphylococcus aureus to the host during chronic osteomyelitis. Front Cell Infect Microbiol 2018; 8:18.

Tuchscherr L, Löffler B. Staphylococcus aureus dynamically adapts global regulators and virulence factor expression in the course from acute to chronic infection. Curr Genet 2016; 62:15–7.

Mäder U, Nicolas P, Depke M, et al. Staphylococcus aureus transcriptome architecture: from laboratory to infection-mimicking conditions. PLoS Genet 2016; 12:e1005962.

30. Tuchscherr L, Bischoff M, Lattar SM, et al. Sigma factor SigB is crucial to mediate Staphylococcus aureus adaptation during chronic infections. PLoS Pathog 2015; 11:e1004870.

31. Deutsch EW, Csordas A, Sun Z, et al. The ProteomeXchange Consortium in 2017: supporting the cultural change in proteomics public data deposition. Nucleic Acids Res 2017; 45:D1100–6.

32. Chatterjee I, Kriegeskorte A, Fischer A, et al. In vivo mutations of thymidylate synthase (encoded by thyA) are responsible for thymidine dependency in clinical small-colony variants of Staphylococcus aureus. J Bacteriol 2008; 190:834–42.

33. Nygaard TK, Pallister KB, Ruzevich P, Griffith S, Vuong C, Voyich JM. SaeR binds a consensus sequence within virulence gene promoters to advance USA300 pathogenesis. J Infect Dis 2010; 201:241–54.

34. Spry C, Kirk K, Saliba KJ. Coenzyme A biosynthesis: an antimicrobial drug target. FEMS Microbiol Rev 2008; 32:56–106.

35. Painter KL, Krishna A, Wigneshwaran S, Edwards AM. What role does the quorum-sensing accessory gene regulator system play during Staphylococcus aureus bacteremia? Trends Microbiol 2014; 22:676–85.

36. Shopsin B, Eaton C, Wasserman GA, et al. Mutations in agr do not persist in natural populations of methicillin-resistant Staphylococcus aureus. J Infect Dis 2010; 202:1593–9.

37. Liu Q, Yeo WS, Bae T. The SaeRS two-component system of Staphylococcus aureus. Genes 2016; 7:81.

38. Cassat JE, Hammer ND, Campbell JP, et al. A secreted bacterial protease tailors the Staphylococcus aureus virulence repertoire to modulate bone remodeling during osteomyelitis. Cell Host Microbe 2013; 13:759–72.

39. Montgomery CP, Boyle-Vavra S, Daum RS. Importance of the global regulators Agr and SaeRS in the pathogenesis of CA-MRSA USA300 infection. PLoS One 2010; 5:e15177.

40. Askarian F, Ajayi C, Hanssen AM, et al. The interaction between Staphylococcus aureus SdrD and desmoglein 1 is important for adhesion to host cells. Sci Rep 2017; 7:22134.

41. Kriegeskorte A, Lorè NI, Bragonzi A, et al. Thymidine-dependent Staphylococcus aureus small-colony variants are induced by trimethoprim-sulfamethoxazole (SXT) and have increased fitness during SXT challenge. Antimicrob Agents Chemother 2015; 59:7265–72.

42. Sambandamurthy VK, Wang X, Chen B, et al. A pantothenate auxotroph of Mycobacterium tuberculosis is highly attenuated and protects mice against tuberculosis. Nat Med 2002; 8:1171–4.

43. La Rosa R, Johansen HK, Molin S. Convergent metabolic specialization through distinct evolutionary paths in Pseudomonas aeruginosa. mBio 2018; 9.

44. Ericson ME, Subramanian C, Frank MW, Rock CO. Role of fatty acid kinase in cellular lipid homeostasis and SaeRS-dependent virulence factor expression in Staphylococcus aureus. mBio 2017; 8.

45. Ziebandt AK, Kusch H, Degner M, et al. Proteomics uncovers extreme heterogeneity in the Staphylococcus aureus exoproteome due to genomic plasticity and variant gene regulation. Proteomics 2010; 10:1634–44.