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

Xin Tan,¹ Mathieu Coureuil,¹ Elodie Ramond,¹ Daniel Euphrasie,¹ Marion Dupuis,¹ Fabiola Tros,¹ Julie Meyer,¹ Ivan Nemazany,² Cerina Chhuon,³ Ida Chiara Guerrero,⁴ Agnes Ferroni,⁵ Isabelle Sermet-Gaudelus,⁶ Xavier Nassif,¹ Alain Charbit,¹ and Anne Jamet^{1,6}

¹Université Paris Descartes, INSERM U1151 - CNRS UMR 8253, Institut Necker-Enfants Malades. Team: Pathogenesis of Systemic Infections, ²Plateforme d'étude du métabolisme, Structure Fédérative de Recherche INSERM US24/CNRS UMS3633, ³Plateforme Protéome Institut Necker-Enfants Malades, PPN, Structure Fédérative de Recherche SFR Necker, University Paris Descartes, ⁴Proteomics platform 3P5-Necker, Université Paris Descartes - Structure Fédérative de Recherche Necker, INSERM US24/CNRS UMS3633, ⁵Laboratoire de Microbiologie de l'hôpital Necker, University Paris Descartes, and ⁶Université Paris Descartes, INSERM U1151 - CNRS UMR 8253, Institut Necker-Enfants Malades. Team: Canalopathies épithéliales: la mucoviscidose et autres maladies, Paris, France

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.

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Correspondence: A. Jamet, Bâtiment Leriche, 14 Rue Maria Helena Vieira Da Silva CS 61431 - 75993 Paris, France (anne.jamet@inserm.fr).

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 metabolomics, 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.

For 2-sample comparisons, statistical significance was measured using unpaired 2-tail Student *t* test or Wilcoxon rank sum test as indicated in the figure legends. For comparisons between more than 2 groups, statistical significance was measured using 1-way analysis of variance with multiple comparisons (Dunnett's correction) performed, with each value compared to that of the reference strain. *P* values < .05 were considered to be statistically significant.

Ethics Statement

All experiments were performed in accordance with the guidelines and regulations described by the Declaration of Helsinki and the low Huriet-Seruscot on human research ethics, and informed consent was obtained for all participating patients. Serial isolates of *S. aureus* were obtained from airway secretions from 4 patients with chronic lung infection at the Necker-Enfants Malades University Hospital, Paris, France. Sputum sampling is part of routine standard care. The research procedure is validated by Ile de France 2 IRB (ID-RCB/Eudract: 2016 A00309-42).

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 haemin 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

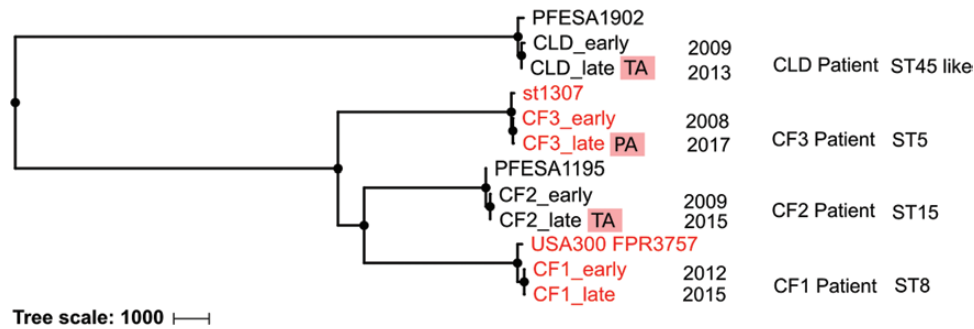


Figure 1. Selection of 4 pairs of *Staphylococcus aureus* isolates belonging to 4 sequence types (STs) in 4 patients. Dendrogram generated by wgsa.net from the genomes of the 8 clinical isolates retrieved from the respiratory samples of 3 patients with cystic fibrosis (CF), 1 patient with non-CF chronic 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 *S. aureus*. Reference strains included are PFESA1902 (ERR554197), st1307 (ERR158691), and PFESA1195 (ERR554722). Abbreviations: CF, cystic fibrosis; CLD, chronic lung disease.

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 *thyA*, *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

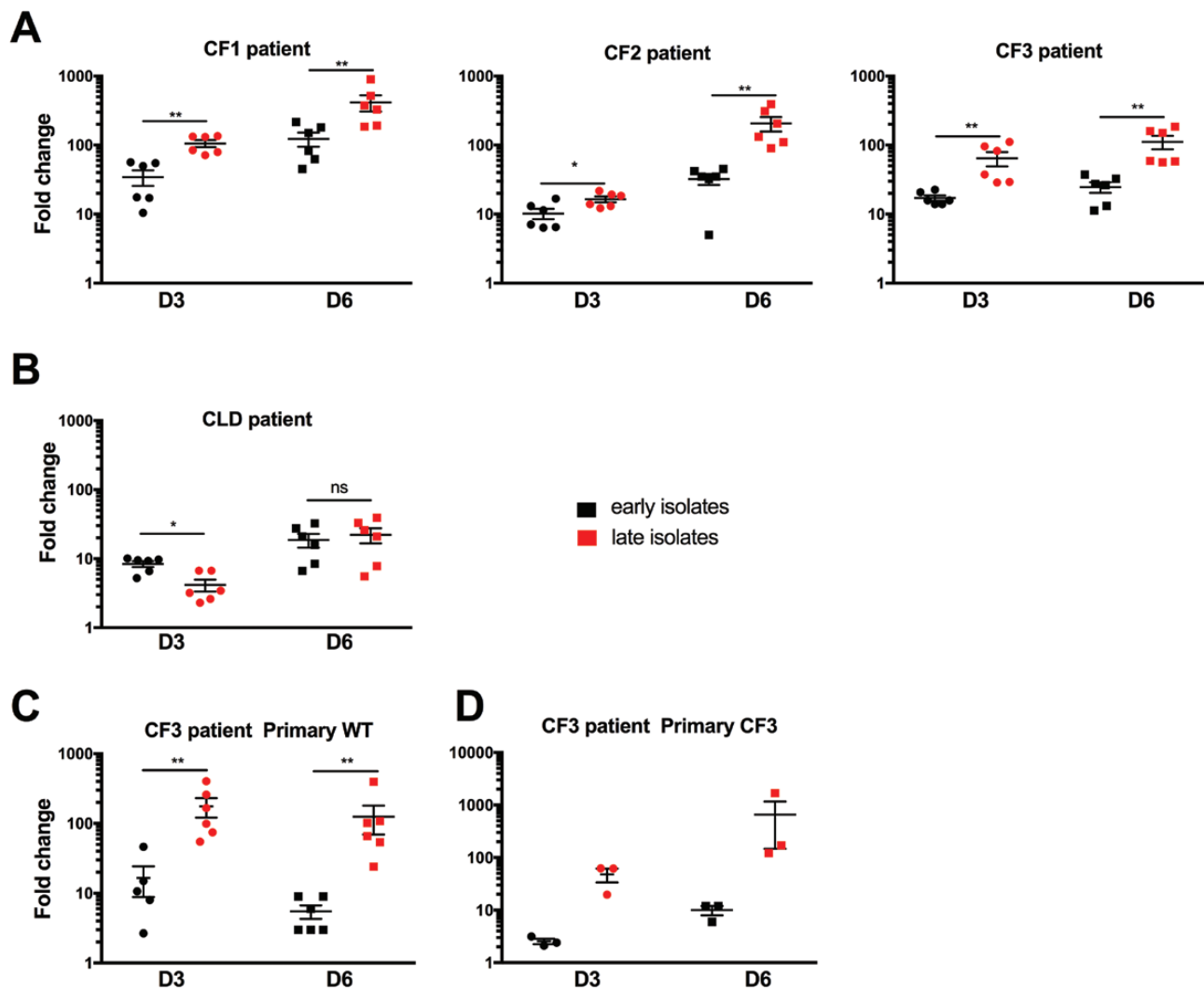


Figure 2. Intracellular persistence of *Staphylococcus aureus* clinical isolates in cystic fibrosis bronchial epithelial (CFBE)-F508del epithelial cell line and within primary patient cells. (A and B) Bronchial CFBE epithelial cell line (CFBE-F508del homozygous for the F508del-cystic fibrosis transmembrane 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: * $P < .05$; ** $P < .01$; $P > .05$.

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

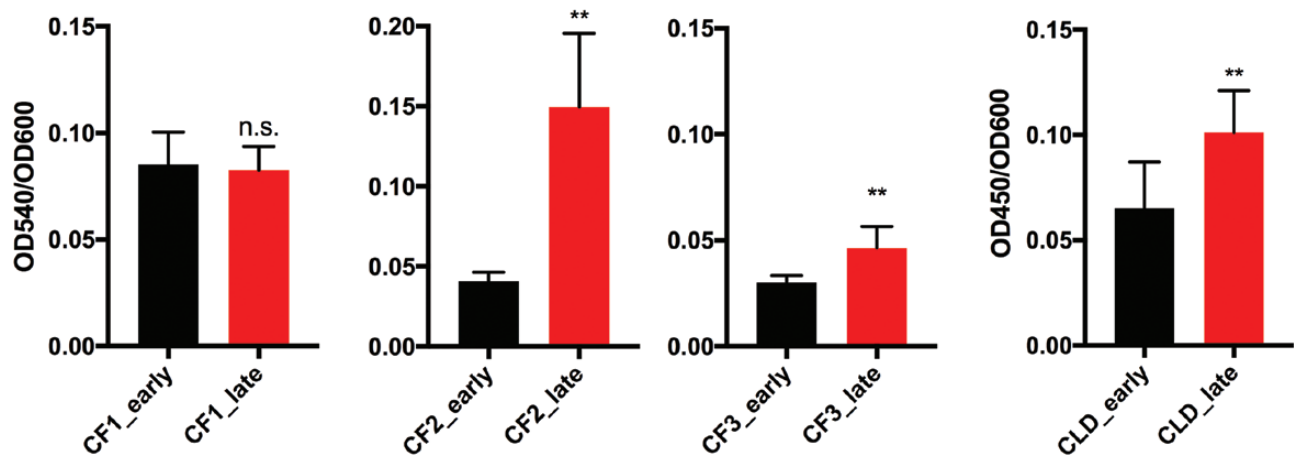


Figure 3. Quantification of biofilm formation of *Staphylococcus aureus* clinical isolates. Biofilm formation quantification was performed using the crystal violet microtiter assay in brain–heart infusion medium with 1% glucose. Results shown are the mean \pm standard deviation for 3 independent experiments 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 $P > .05$.

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*, *fmbA*, *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

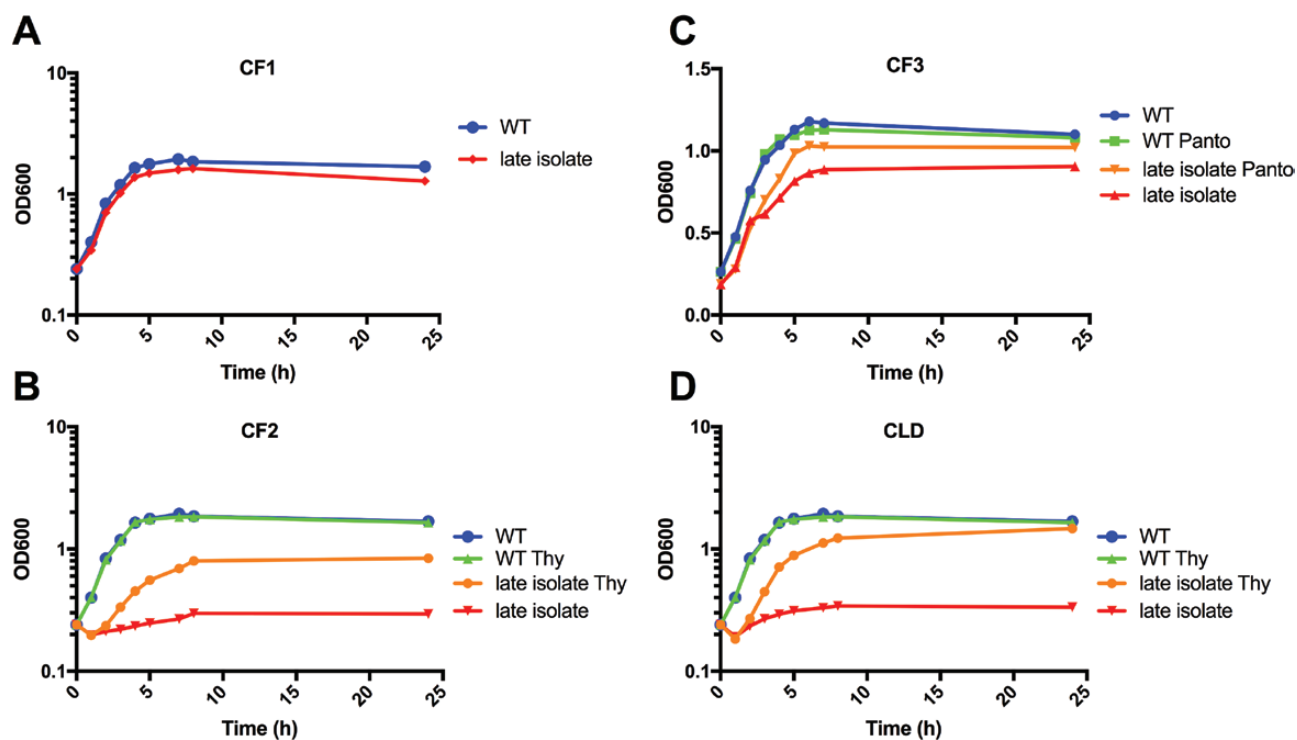


Figure 4. Growth of late *Staphylococcus aureus* clinical isolates in cystic fibrosis sputum medium. Growth curves were carried out in medium that mimics the respiratory fluid of cystic fibrosis patients, with or without the addition of thymidine or pantothenate. The results shown correspond to a representative 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.

Table 1. Mutations Likely Linked to Phenotypic Changes in Clinical Isolates

Isolate	Gene	Product	Mutation	Putative Associated Phenotype
CF1_late	<i>agrC</i>	Accessory gene regulator protein C	Premature stop codon (L193X)	Downregulation of toxins and upregulation of adhesins
CF1_late	<i>saeR</i>	<i>Staphylococcus</i> exoprotein expression protein R	Missense G179L	Upregulation of SdrD adhesin
CF2_late	<i>thyA</i>	Thymidylate synthase	Premature stop codon (W88X)	SXT resistance and thymidine auxotrophy
CF2_late	<i>rpsJ</i>	30S ribosomal protein S10	Missense K57M	Cyclines resistance
CF2_late	<i>thiM</i>	Hydroxyethylthiazole kinase	Frameshift	Decrease in adenosine diphosphate
CF2_late	<i>fakA</i>	Fatty acid kinase A	Missense G187D	Increased biofilm formation and intracellular persistency
CF3_late	<i>panB</i>	3-methyl-2-oxobutanoate hydroxymethyltransferase	Frameshift	Pantothenate auxotrophy
CF3_late	<i>fakA</i>	Fatty acid kinase A	Frameshift	Increased biofilm formation and intracellular persistency
CF3_late	<i>gyrB</i>	DNA topoisomerase subunit B	Missense F226S	Fluoroquinolones resistance
CF3_late	<i>saeR</i>	<i>Staphylococcus</i> exoprotein expression protein R	Missense A190T	Upregulation of SdrD adhesin
CLD_late	<i>thyA</i>	Thymidylate synthase	Missense P48R	SXT resistance and thymidine auxotrophy

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

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

Table 2. General Features of Detected Mutations

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 (77.8)	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.

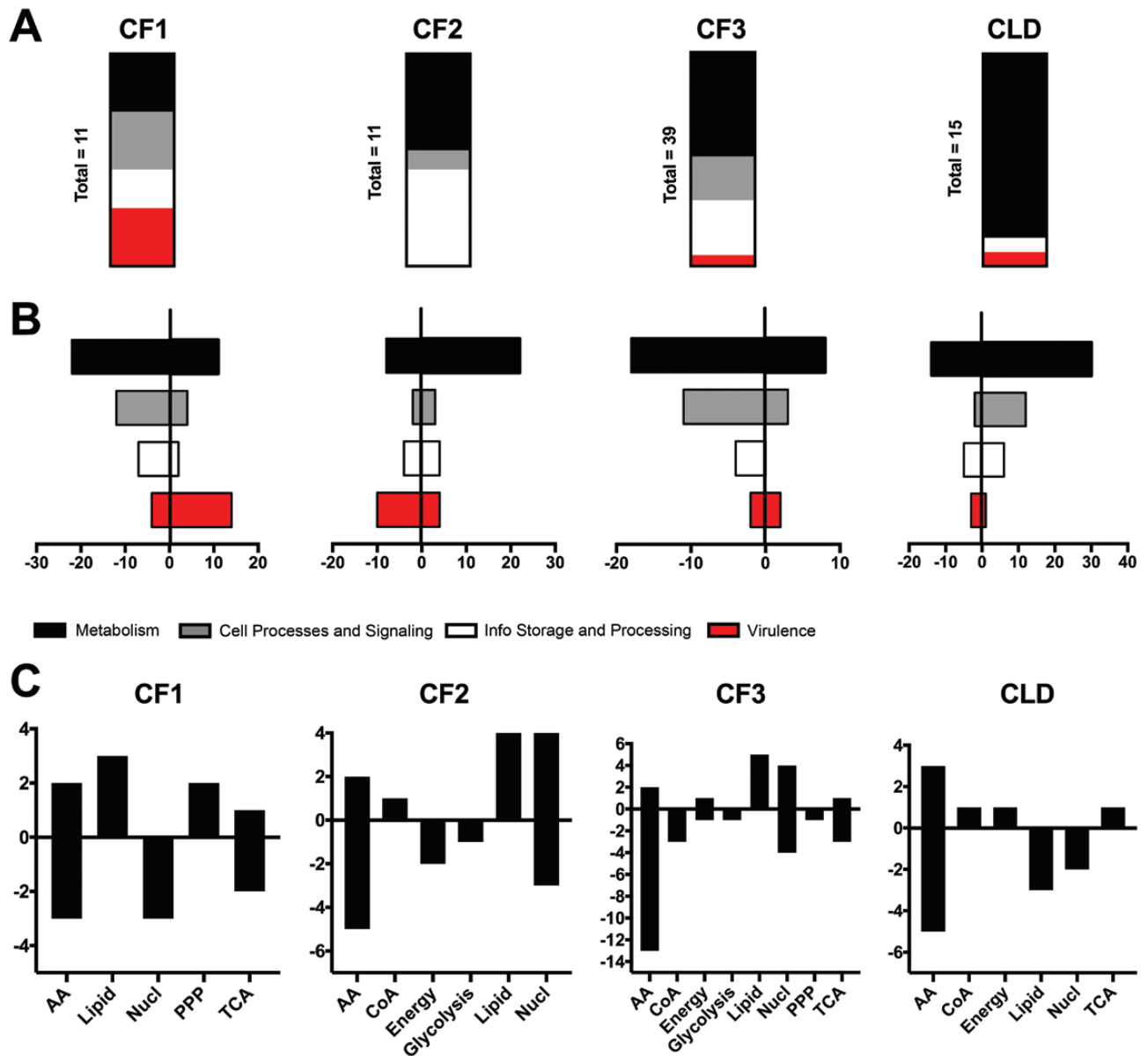


Figure 5. Proteogenomic and metabolomic analysis of the 4 pairs of *Staphylococcus aureus* isolates. (A) Vertical histograms show the functional classification of proteins encoded by genes with nonsynonymous mutations in the genomes of late isolates of *S. aureus* compared to early isolates. (B) Horizontal histograms show the functional classification of differentially expressed annotated proteins in late compared to early isolates of each patient. For each category, histograms represent the number of down- and upregulated proteins from proteomic analysis using the threshold of <2 and >2 , respectively. Only genes and proteins with functional annotation available are included. The “cellular processes and signaling” category encompasses regulatory proteins and proteins involved in cell wall and capsule synthesis. The “information storage and processing” category encompasses proteins involved in replication, translation, and repair processes. The “metabolism” category encompasses proteins involved in metabolism and transport. The “virulence” category encompasses exotoxins and proteins involved in adhesion, biofilm formation, and immunomodulation. (C) Categorization in 8 categories of an altered amount of metabolites in late compared to early isolates. Metabolites were detected by carrying out 2 independent experiments performed in triplicate. Abbreviations: AA, amino acids; CF, cystic fibrosis; CLD, chronic lung disease; CoA, coenzyme A; Nucl, nucleotides; PPP, pentose phosphate pathway; TCA, tricarboxylic acid cycle.

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 *saeR/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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