Chronic *Staphylococcus aureus* lung infection correlates with proteogenomic and metabolic adaptations leading to an increased intracellular persistence

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40-word summary:

S. aureus persists for years in the lungs of patients with cystic fibrosis despite antibiotic therapies. We demonstrate that *S. aureus* adaptation leads to increased intracellular persistence suggesting a key role for intracellular niche during *S. aureus* chronic lung infection.

Abstract (198 words)

Background: Chronic lung infection of 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. Strikingly the molecular mechanisms underlying *S. aureus* persistency are not understood.

Methods: We selected pairs of sequential *S. aureus* isolates from 3 patients with CF and from one 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: For the first time, we show that late *S. aureus* isolates from CF patients have an increased ability for intracellular survival in CFBE-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 inducing 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.

Introduction

Staphylococcus aureus and *Pseudomonas aeruginosa* are the most common pathogens infecting the lungs of patients with a chronic lung disease 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 undergone by *S. aureus* in this context [3, 4].

S. aureus has the ability to form biofilm [5-7] and to survive within a wide range of eukaryotic host cells [8-17]. These abilities are likely to contribute to the persistence of *S. aureus* in airways of patients with chronic lung diseases despite appropriate antimicrobial treatments [18, 19]. *S. aureus* persistence is associated with a drastic decrease in metabolism [20], a decrease in the expression of virulence factors and an increase in the expression of bacterial adhesins [21]. Such profile is typical of small-colony variants (SCVs) that are defined by small-sized colonies [15, 22, 23]. Beside SCVs, strains with normal colony morphology can exhibit similar patterns of "low toxicity" which allow them to persist intracellularly without being cleared by host cell defense mechanisms [21]. 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 [24-26].

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 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 aiming at eradicating *S. aureus* chronic infections by choosing drugs specifically targeting biofilm-embedded and intracellular bacteria.

Methods

106 Whole genome sequencing was performed on an Illumina MiSeq instrument (2x150
107 bp) and the sequences were processed using the Nullarbor bioinformatic pipeline software
108 v1.20 and RAST server. The sequences reported in this paper are available at NCBI's
109 BioProject database under accession number PRJNA446073
110 (http://www.ncbi.nlm.nih.gov/bioproject/446073).

111 Quantification of biofilm formation was assessed with crystal violet staining in
112 polystyrene 96-well plates. Cystic Fibrosis Bronchial Epithelial cell line CFBE41o- and
113 primary nasal epithelial cell were infected with a multiplicity of infection of 100 using an
114 inoculum taken from cultures of *S. aureus* grown in Brain Heart Infusion until exponential
115 growth phase. Infected cells were kept for 6 days in a medium containing 50 µg/mL
116 gentamycin to kill extracellular bacteria. For proteomics, proteins were digested and analyzed
117 by liquid chromatography coupled with tandem mass spectrometry (nanoLC-MS/MS). For
118 metabolomics, metabolite profiling of *S. aureus* isolates was performed by liquid
119 chromatography–mass spectrometry (LC-MS). The mass spectrometry proteomics data have
120 been deposited to the ProteomeXchange Consortium via the PRIDE [27] partner repository
121 with the dataset identifier PXD011281.

122 A full description of methods is available in Supplementary Methods.

123 **Statistical analysis**

124 Data were analyzed using R or GraphPad Prism softwares. Results are presented either
125 with one representative experiment for clarity or as means ± standard deviation (SD). The
126 number of biological and technical replicates is indicated per figure.

127 For two-sample comparisons statistical significance was measured using unpaired two-tail
128 Student's t-test or Wilcoxon rank sum test as indicated in the figure legends. For comparisons
129 between more than two groups, statistical significance was measured using one-way analysis

130 of variance (ANOVA) with multiple comparisons (Dunnett's correction) performed, with
131 each value compared to that of the reference strain.
132 P values of <0.05 were considered to indicate statistical significance.
133 **Ethics statement**
134 All experiments were performed in accordance with the guidelines and regulations
135 described by the Declaration of Helsinki and the low Huriet-Serusclat on human research
136 ethics and informed consent was obtained for all participating subjects. Serial isolates of *S.*
137 *aureus* were obtained from airway secretions from four patients with chronic lung infection at
138 the Necker-Enfants Malades University Hospital, Paris, France. Sputum sampling is part of
139 routine standard care. The research procedure is validated by Ile de France 2 IRB (ID-
140 RCB/Eudract: 2016 A00309-42).
141
142 **Results**
143 **Selection of *S. aureus* sequential isolates from patients with chronic lung infection**.
144 Three patients with CF (CF1, CF2 and CF3) and, for comparison purpose, one patient with
145 non-CF chronic lung disease (CLD) were chosen. For each patient we selected one early and
146 one late isolate separated by 3 to 9 years intervals. Whole-genome sequencing confirmed that
147 each pair of isolates belonged to four distinct clones (**Figure 1**). Patient diseases and
148 treatments are detailed in supplementary Methods.
149 ***S. aureus* clinical isolates from CF patients evolved an increased persistence ability**
150 **within CFBE-F508del epithelial cell line.**
151 Numerous studies have shown that *S. aureus* has the ability to survive within human cells [8-
152 17]. We subsequently aimed at investigating if during the course of within-lung adaptation, *S.*
153 *aureus* isolates have evolved a greater ability to persist within epithelial cells. We infected
154 bronchial CFBE epithelial cell line (F508del +/+ CFTR mutation) with clinical isolates, the

155 control strain USA300-LAC and a stable SCV mutant altered in the haemin biosynthetic
156 pathway (hereafter named Δ*hem*). As expected, wild-type bacteria were not able to persist
157 whereas the Δ*hem* mutant was able to persist intracellularly during the whole course of the
158 experiment (**Figure S1**) [13]. All early and late clinical isolates were able to persist at least
159 2.6-fold, and up to 900-fold, more than the USA300-LAC reference strain at day 3 and 6 post-
160 infection (**Figure 2AB**). Furthermore, at day 3 and 6 post-infection, all the late isolates
161 recovered from CF patients exhibited an improved ability to persist intracellularly within
162 CFBE-F508del epithelial cells compared to cognate early isolates (**Figure 2A**). Interestingly,
163 the CLD_late isolate recovered from the non-CF patient did not exhibit an improved ability to
164 persist within CFBE epithelial cells compared to CLD_early isolate (**Figure 2B**). These data
165 suggest that *S. aureus* adaptation within CF-lungs correlates with an improved ability to
166 persist intracellularly in cells with a CFTR dysfunction.

167 **Late *S. aureus* isolate of CF3 patient exhibits an increased persistence within primary**
168 **F508del epithelial own patient cells.**

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

177 ***S. aureus* clinical isolates from chronically infected patients evolved high biofilm**
178 **formation ability.**

179 Assuming that isolates retrieved from chronic infections might have a high biofilm-forming

180 capacity, we studied the biofilm formation ability of the pairs of isolates. Remarkably, all
181 isolates displayed a greater capacity to form biofilms compared to that of the weak biofilm-
182 producer USA300-LAC reference strain (p-value of <0.001, **Figure S2**). Furthermore, for
183 three chronically infected patients, the late isolates formed more biofilm than the early
184 isolates, revealing that long-term adaptation within lungs had improved their biofilm
185 formation capacity (p-value of <0.001, **Figure 3**).

186 **Late *S. aureus* clinical isolates from chronically infected patients acquired auxotrophies.**

187 Compared to that of USA300-LAC, all patients early isolates and the CF1 late isolate display
188 similar colony morphology on brain heart infusion (BHI) agar plates and a wild-type growth
189 in a liquid broth mimicking sputum (Cystic Fibrosis Sputum Medium or CFSM) **(Figure S3**
190 **and Figure 4A)**. In contrast, the late isolate of CF2 and CLD patients displayed a typical
191 SCV phenotype with very small colonies on BHI agar (**Figure S4**) and CF2_late, CF3_late
192 and CLD_late isolates exhibited a growth defect in CFSM broth **(Figure 4BCD)**. Thymidine-
193 dependent SCVs are frequently isolated from patients treated with sulfamethoxazol (SXT)
194 [28]. Indeed, supplementation with thymidine restored almost wild-type growth for CF2_late
195 and CLD_late isolates (**Figure 4BD**). We used genomic data to determine the auxotrophy of
196 CF3_late isolate and identified a frameshift in *panB* gene, which is involved in de novo
197 biosynthesis of pantothenic acid (**Table 1**). Accordingly, growth of CF3_late isolate in the
198 presence of pantothenate restored wild-type growth (**Figure 4C**). Thus, isolates from three out
199 of four patients with *S. aureus* chronic lung infection acquired auxotrophy during the course
200 of the disease.

201 **Late *S. aureus* clinical isolates from chronically infected patients acquired antibiotic**
202 **resistance.**

203 Genome analysis evidenced mutations in *thyA*, *gyrB,* and *rpsJ* genes, which were associated
204 with SXT, fluoroquinolones and cyclines resistance, respectively (**Table 1**). Thus, isolates

205 from three out of four patients with *S. aureus* chronic lung infection acquired antibiotic
206 resistance consistent with the administration of the corresponding drugs during the course of
207 the disease.
208
209 **Genomic, proteomic and metabolomic modifications associated with *S. aureus***
210 **adaptation during chronic lung infection.**
211 In order to investigate the underlying genomic, proteomic and metabolic modifications
212 associated with the observed phenotypic changes we compared genomes, proteomes and
213 metabolomes of late compared to early isolates. The differences in proteomic and metabolic
214 profiles between early and late isolates of patients are highlighted by heatmaps shown in
215 **Figure S5**.
216 Genomes of all clinical isolates were de novo assembled and coding DNA sequences (CDSs)
217 were annotated. Most of the SNPs were missense variants occurring in CDSs (**Table 2)**.
218 Nonsynonymous mutations acquired by late isolates were found mainly in genes involved in
219 metabolic processes (**Figure 5A**) and more specifically in "amino acid transport and
220 metabolism" and "carbohydrate transport and metabolism" functional categories. In addition,
221 the largest category of proteins to be differentially expressed for all pairs also comprised
222 proteins related to metabolism processes (and more specifically to the "amino acid transport
223 and metabolism" category) (**Figure 5B**). Concordant with genomic and proteomic results, the
224 category "amino acids" was the most altered metabolites category in the late isolate of all
225 patients compared to their cognate early isolates (**Figure 5C**).
226 Many regulatory proteins were differentially expressed. Indeed, proteins of the Agr, Rot, Sae,
227 Sar or Fur regulatory networks were differently expressed in all late isolates. In CF1_late
228 isolate, the *agrC* and the *saeR* genes mutations **(Table 1)** had a pleiotropic effect on the
229 proteome (down-regulation of delta hemolysin and PSMb1 and upregulation of proteins
230 encoded by *spa, sbi, fnbA, rot* and *coa* genes). In addition, adhesins encoded by *sasG, efb,*

231 *sdrD* and *ecb* were up-regulated. In CF2_late isolate, the *agr* regulon is also downregulated
232 suggesting an evolution toward low virulent and highly adhesive properties. The metabolite
233 profiling of CF2_late isolate, showed a decrease in ADP, which is well correlated with the
234 lack of ThiM (hydroxyethylthiazole kinase) expression found in proteomic analysis due to a
235 frameshift in *thiM* gene **(Table 1)**. In CF3_late isolate, frameshifts in *fakA* and *panB* genes
236 **(Table 1)** were associated with a lack of cognate proteins expression in CF3_late isolate. In
237 addition, adhesins encoded by *sdrD* and *sasF* were upregulated. Interestingly, an over-
238 production of penicillin-binding protein 2 encoded by *mecA* is correlated with the *saeR*
239 mutation **(Table 1)** [29]. The metabolite profiling of CF3_late isolate, revealed a drastic
240 diminution of pantothenate, coenzyme A and dephospho-coenzyme A, which is in line with
241 the lack of expression of PanB and PanC proteins [30].
242 Of note, the non-CF control clone displays a different evolution trajectory. Indeed, AgrA and
243 AgrC were up-regulated in CLD_late isolate, suggesting that it has retained virulent
244 properties.
245 Altogether, the proteogenomic data suggest that all the late isolates recovered from CF
246 patients (but not CLD patient) have evolved toward highly adhesive and low virulent
247 properties. Besides, metabolic profiling suggests that all late isolates have evolved a reduced
248 citric acid cycle activity compared to cognate early isolates.
249

250 **Discussion**

251 Our study showed that during chronic lung infection, *S. aureus* adapts through the
252 acquisition of common adaptive traits including antibiotic resistances, auxotrophies, reduced
253 citric acid cycle activity, increased biofilm and intracellular persistence abilities that occurred
254 irrespective of the clone type.

255 Of particular interest, we report mutations in two master regulatory systems, Agr and Sae,
256 likely to impact multiple proteins expression and metabolites amounts.
257 *agr*-defective mutants, such as CF1 late isolate, have been shown to arise during chronic
258 infections and are better adapted to persistence within the infected host [25, 31, 32].
259 Genetic alterations directly or indirectly targeting SaeR regulon were identified in the 3 CF
260 patients. Since SaeR is involved in the regulation of over 20 virulence factor genes [33] and
261 SaeRS-deficient bacteria are less infective in animal models [34], it is likely that SaeRS is a
262 key factor in long-term colonization. In the three CF late isolates, we observed an increased in
263 the expression of the SdrD adhesin belonging to the SaeR regulon and involved in adhesion to
264 human nasal epithelial cells and to human keratinocytes [35][36]. Our results suggest that
265 SdrD is also important for long-term lung colonization.
266 In patients with chronic lung infections, SCVs detection is most often the consequence of a
267 long-term SXT treatment [37]. Mutations in the *thyA* gene, as found in CF2 and CLD late
268 isolates, lead to stable clinical SCVs that are no longer susceptible to SXT and are thymidine-
269 auxotrophic (TA-SCV) [28, 37]. Since thymidine is assumed to be abundant during lung
270 inflammation, TA-SCVs can still grow in this environment.
271 In CF3 late isolate, we observed a pantothenate auxotrophy, which has been previously
272 associated with persistency in *Mycobacterium tuberculosis* [38]. The acquisition of
273 pantothenate auxotrophy suggests that pantothenate could also be present in CF lungs. Thus,
274 our data confirm that metabolic specialization is a common phenomenon among long-term
275 colonizers [39].
276 Other striking traits of phenotypic convergent evolution of *S. aureus* identified in this work
277 were the increased ability to form biofilm and to persist in the intracellular niche. For CF2
278 and CF3 patients, the increased biofilm ability of late isolates could be linked to a mutation in
279 the *fakA* gene, encoding fatty acid kinase A (FakA). Indeed, several studies showed that

280 FakA-null strains were proficient in biofilm formation [6] and deficient in the expression of
281 virulence factors controlled by the SaeRS system [40]. Overexpression of adhesins detected in
282 proteomic analysis could also ultimately lead to increase biofilm formation in clinical isolates.
283 Numerous studies have demonstrated *S. aureus* ability to persist within host cells [8-17].
284 Strikingly, for the three CF patients, the *S. aureus* late isolates showed a greater ability to
285 persist within CFBE-F508del epithelial cells compared to the early ones at day 3 and 6 post-
286 infection. Of note, the late isolate of CLD patient did not present an improved ability to
287 persist intracellularly within CFBE-F508del epithelial cells possibly due the fact that it has
288 adapted to a non-CF patient.
289 Our multi-omics approach allowed both confirmation of previously known mechanisms
290 and identification of novel candidate genes and pathways involved in the persistence ability of
291 clinical isolates. We now provide evidence that the *saeR*/*fakA* regulon and the pantothenate
292 pathway could also be promising therapeutic targets to fight persistent *S. aureus* infections.
293 Our study suggests that the use of antibiotic with a good intracellular penetration should be
294 the best therapeutic option in order to eradicate *S. aureus* from chronically infected lungs.
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Author contributions:

XT, MC, XN, AC and AJ conceived and designed the study. XT, ER, MD, DE, FT, JM and AJ made the experiments and analysis. IN, CC, ICG, AF and ISG contributed with data and analysis. AJ, AC and XT wrote the manuscript, with contributions and comments from all authors.

No conflicts exist for the authors

317 **Tables**

318 **Table 1. Mutations linked to phenotypic changes in clinical isolates**

Isolate	Gene	Product	Mutation	Associated phenotype
CF1_late	*agrC*	Accessory gene regulator protein C	Premature stop codon (L193X)	Down-regulation of toxins and up-regulation of adhesins
CF1_late	*saeR*	Staphylococcus exoprotein expression protein R	Missense G179L	Up-regulation of adhesins
CF2_late	*thyA*	Thymidylate synthase	Premature stop codon (W88X)	SXT resistance and thymidine auxotrophy
CF2_late	*rpsJ*	30S ribosomal protein S10	Missense K57M	Cyclines resistance
CF2_late	*thiM*	Hydroxyethylthiazole kinase	Frameshift	Decrease in ADP
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 exoprotein expression protein R	Missense A190T	Over-production of penicillin-binding protein 2
CLD_late	*thyA*	Thymidylate synthase	Missense P48R	SXT resistance and thymidine auxotrophy

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322

323 **Table 2. General features of detected mutations**

	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
SNP[a]	19	25	73	26
INDEL[b]	2	9	6	4
CDS[c]	18	21	62	23
NON-SYN[d] (%)	14 (77.8)	16 (47.1)	51 (64.6)	17 (56.7)
FR[e]	2	4	4	2
MS[f]	10	11	47	14
STOP gained[g]	1	1	0	1
Other	1	0	0	0
SYN[h]	4	5	11	6
IG[i]	3	13	17	7

324 [a]SNP, single nucleotide polymorphism; [b]INDEL, insertion-deletion; [c]CDS, coding sequence;

325 [d]NON-SYN, nonsynonymous mutation; [e]FR, frameshift variant; [f]MS, missense variant;

326 [g]STOP gained, premature stop codon; [h]SYN, synonymous mutation; [i]IG, intergenic

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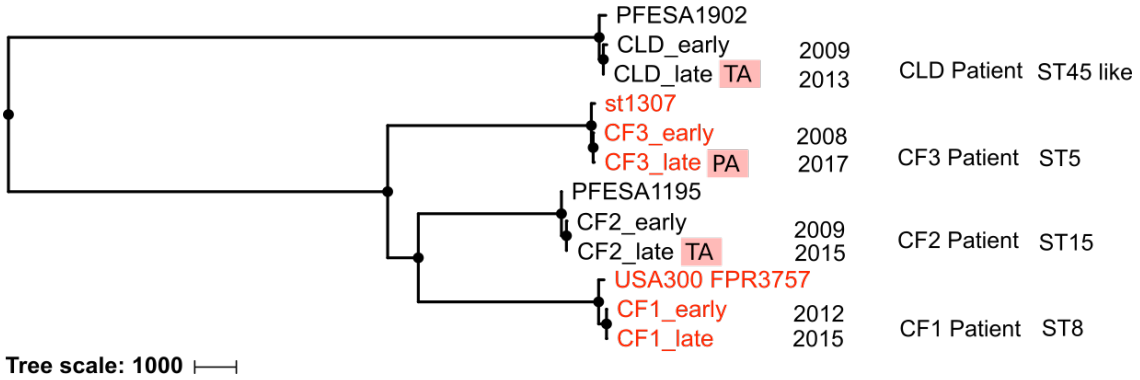
329

330 **Figures**



331

332 **Fig 1. Selection of four pairs of *S. aureus* isolates belonging to four different STs in four**

333 **patients.**

334 Dendrogram generated by wgsa.net from the genomes of the eight clinical isolates retrieved

335 from the respiratory samples of three patients with CF, one patient with non-CF chronic lung

336 infection and four reference genomes from public databases. Branch length is proportional to

337 the number of variant nucleotide sites within the core genes. For each patient, the isolate

338 taken first is called "early" while the isolate taken later is named "late". The dates of sampling

339 and the sequence type (ST) of the isolates are indicated. "TA" and "PA" mean that the isolate

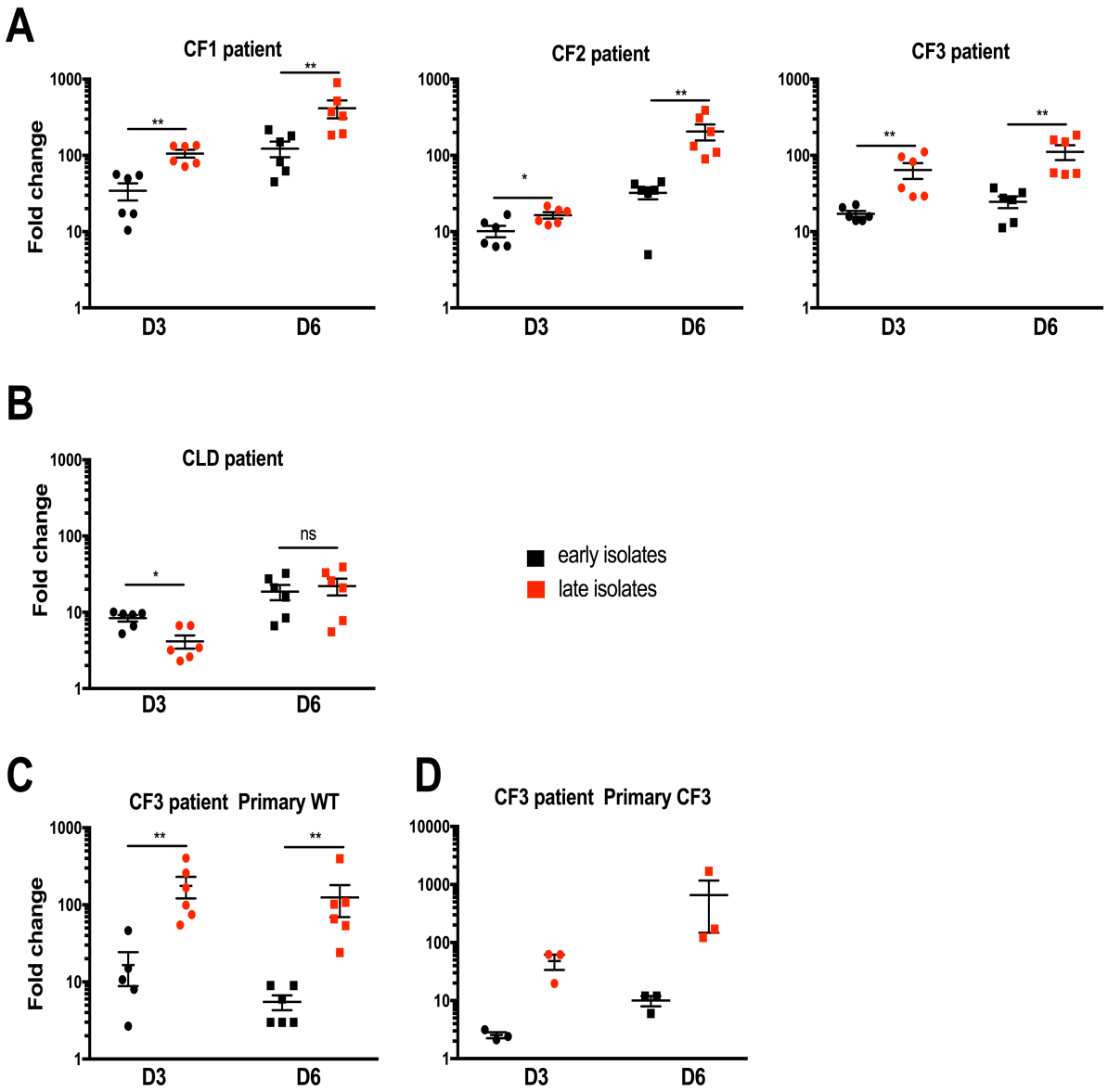
340 is auxotrophic for thymidine or pantothenate respectively. The name of the isolate is indicated

341 in red when it is resistant to methicillin (MRSA).

342 Reference strains included are PFESA1902 (ERR554197), st1307 (ERR158691), PFESA1195

343 (ERR554722).

344



345

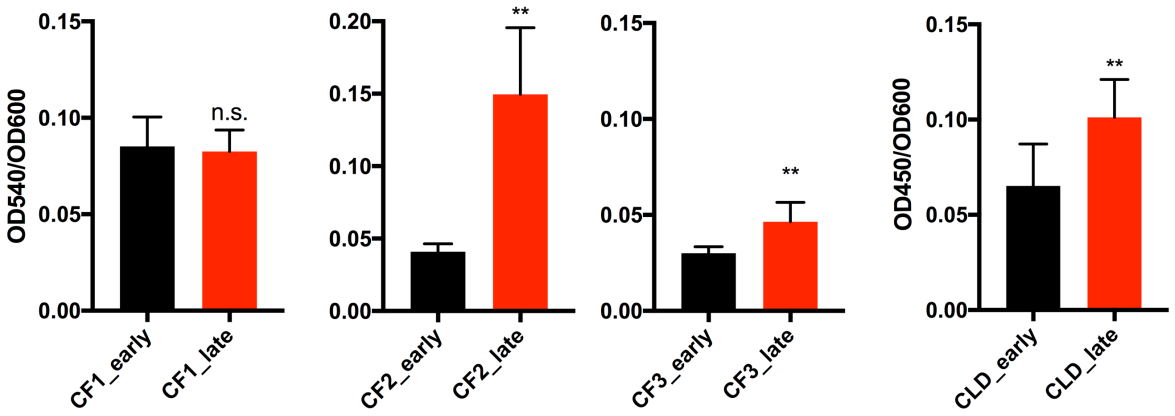
346 **Fig 2. Intracellular persistence of *S. aureus* clinical isolates in CFBE-F508del epithelial**
347 **cell line and within primary patient cells. A and B)** Bronchial CFBE epithelial cell line
348 (CFBE-F508del homozygous for the F508del-CFTR mutation) was infected with the control
349 strain USA300-LAC and clinical isolates from CF patients (A) or CLD patient (B). **C and D)**
350 Primary nasal epithelial cells retrieved from a healthy donor ("Primary WT") (C) and from
351 the CF3 patient ("Primary CF3" with F508del +/+ CFTR mutation) (D) were infected with the
352 control strain USA300-LAC and CF3 isolates.

353 For all experiments, gentamicin was present throughout the experiment to prevent

354 extracellular bacterial growth and new infection. Bacterial loads inside cells were evaluated
355 by CFU enumeration at 3 and 6 days after infection. Results are normalized with USA300-
356 LAC strain as a reference and expressed as a fold change of CFUs.

357 Results have been obtained from two independent experiments performed in triplicate for
358 ABC and one experiment for D. Statistical analysis was performed by Wilcoxon rank sum test
359 *P < 0.05; **P<0.01; ns P>0.05.

360



361

362 **Fig 3. Quantification of biofilm formation of *S. aureus* clinical isolates.** Biofilm formation
363 quantification was performed using the crystal violet microtiter assay in BHI medium with
364 1% glucose. Results shown are the mean ±SD for three independent experiments performed in
365 triplicate. Statistical significance was measured using a two-tail Student's t-test when biofilm
366 production of a late isolate was compared with biofilm production of cognate early isolate
367 from the same patient. ** indicates p-value of <0.001 whereas ns indicates p-values>0.05.

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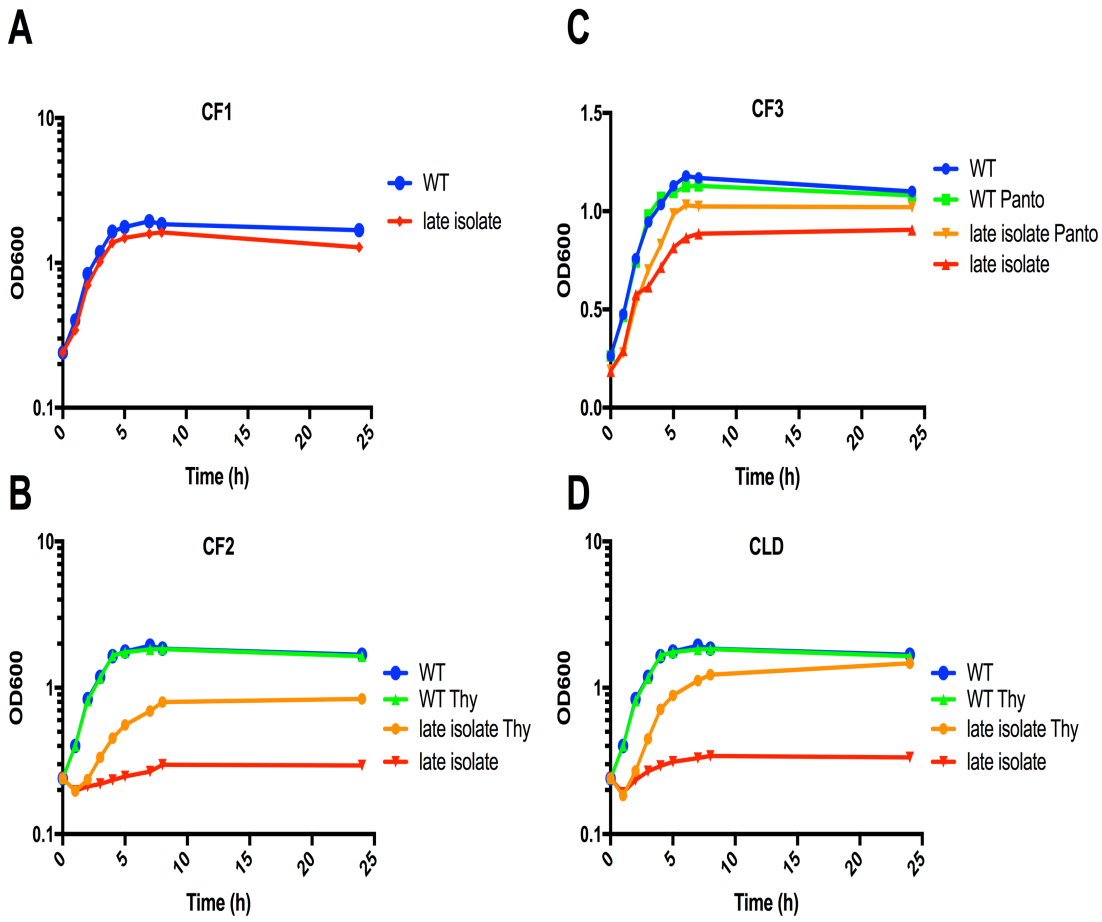

369

370 **Fig 4. Growth of late *S. aureus* clinical isolates in CFSM.** Growth curves were carried out
371 in medium mimicking the respiratory fluid of cystic fibrosis patients (Cystic Fibrosis Sputum
372 Medium or CFSM), with or without the addition of thymidine or pantothenate. The results
373 shown correspond to a representative experiment. The orange and green curves correspond to
374 bacterial growth in media supplemented with either thymidine or pantothenate; the red and
375 blue curves, to bacterial growth in medium without thymidine or pantothenate. WT, USA300-
376 LAC.

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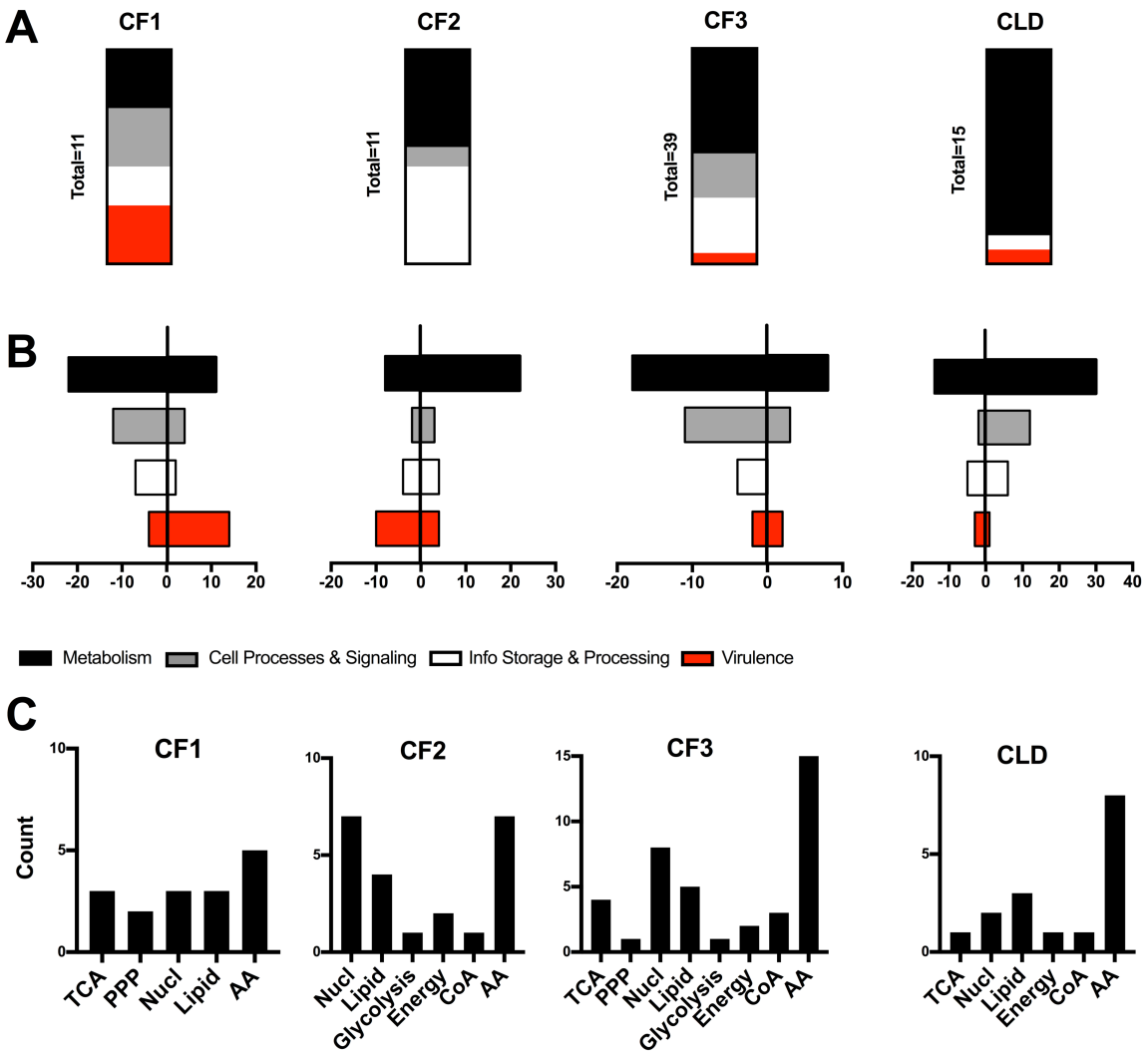

Fig 5. Proteogenomic and metabolomic analysis of the four pairs of *S. aureus* isolates. A) Vertical histograms show the functional classification of proteins encoded by genes with nonsynonymous mutations in the genomes of late isolates of *S. aureus* compared to early isolates. **B)** Horizontal histograms 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 up-regulated 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

389 in replication, translation and repair processes. The "metabolism" category encompasses
390 proteins involved in metabolism and transport. The "virulence" category encompasses
391 exotoxins, proteins involved in adhesion, biofilm formation and immunomodulation. **C)**
392 Categorization in 8 categories of altered amount of metabolites in late compared to early
393 isolates. Metabolites were detected by carrying out 2 independent experiments performed in
394 triplicate. TCA, Tricarboxylic acid cycle; PPP, Pentose phosphate pathway; Nucl,
395 Nucleotides; CoA, Coenzyme A; AA, Amino acids.
396

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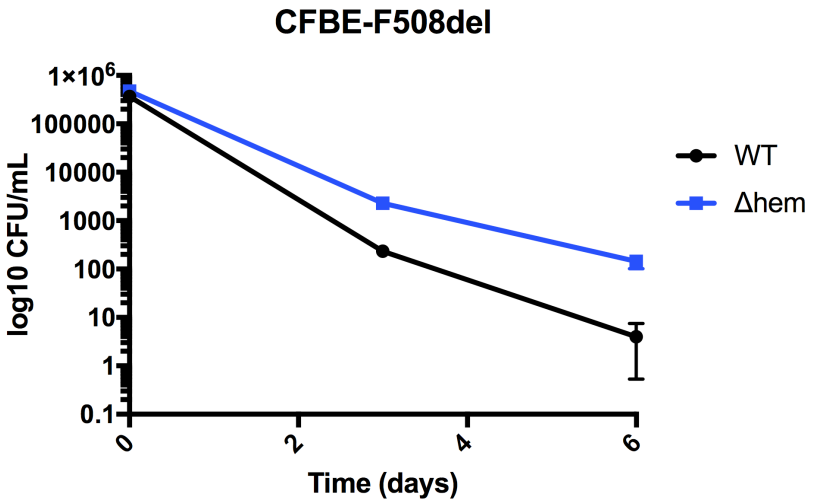
512 **Supplementary figure**



513

514 **Figure S1. Intracellular growth curves of reference *S. aureus* isolates in CFBE**
515 **epithelial cell line.** Bronchial CFBE epithelial cell line (CFBE-F508del homozygous
516 for the F508del-CFTR mutation) was infected with the control strain USA300-LAC
517 (WT) and a stable SCV mutant altered in the haemin pathway (Δ*hem*). Gentamicin
518 was present throughout the experiment to prevent extracellular bacterial growth and
519 new infection. Bacterial load inside cells were evaluated by CFU enumeration at 3
520 and 6 days after infection. Results shown are the mean ±SD for four experiments
521 performed in triplicate.

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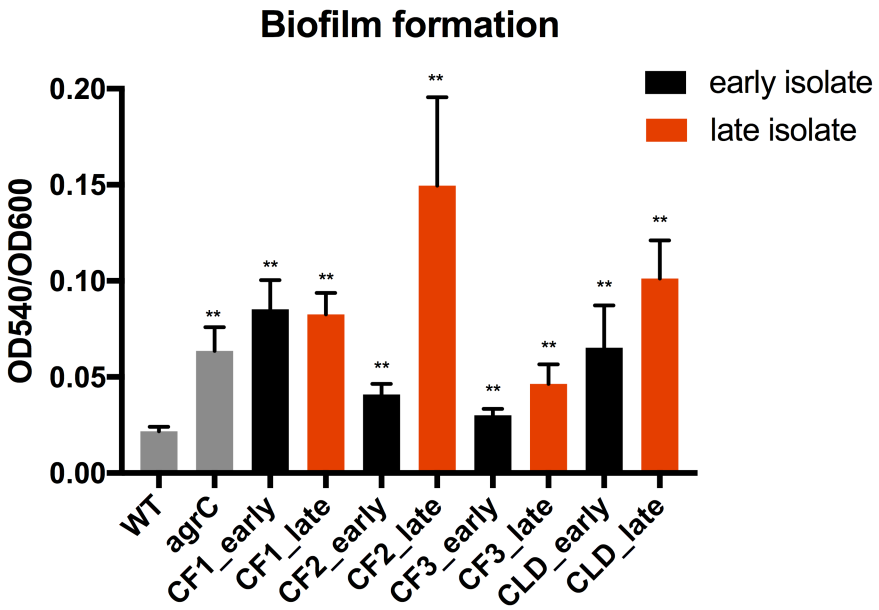

524

525 **Figure S2. Quantification of biofilm formation of *S. aureus* clinical isolates.**
526 Biofilm formation was monitored using the crystal violet microtiter assay in BHI
527 medium with 1% glucose. USA300-LAC strain (WT) was used as a reference for
528 weak biofilm production whereas its *agrC* derivative obtained from the Nebraska
529 Transposon Mutant Library was used as a reference for strong biofilm production.
530 Results shown are the mean ±SD for three independent experiments performed in
531 triplicate. Statistical significance was measured using one-way ANOVA with multiple
532 comparisons (Dunnett's correction) performed on the dataset as a whole, with each
533 value compared to the WT. ** indicates p-value of <0.001.

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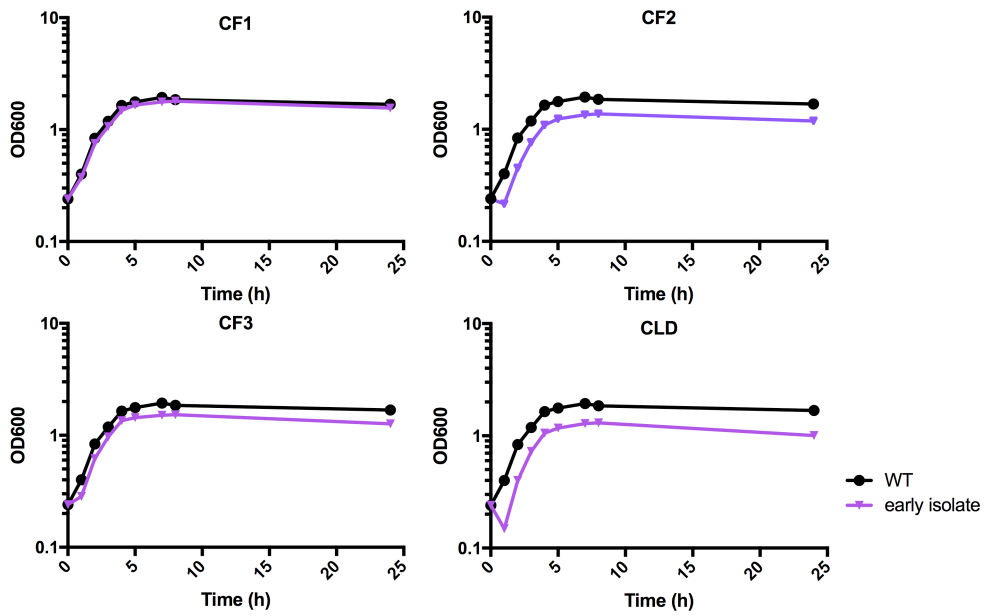

536

537 **Figure S3. Growth curves of early *S. aureus* clinical isolates in CFSM.** Growth

538 curves were carried out in CFSM. The results shown correspond to a representative

539 experiment. WT, USA300-LAC.

540

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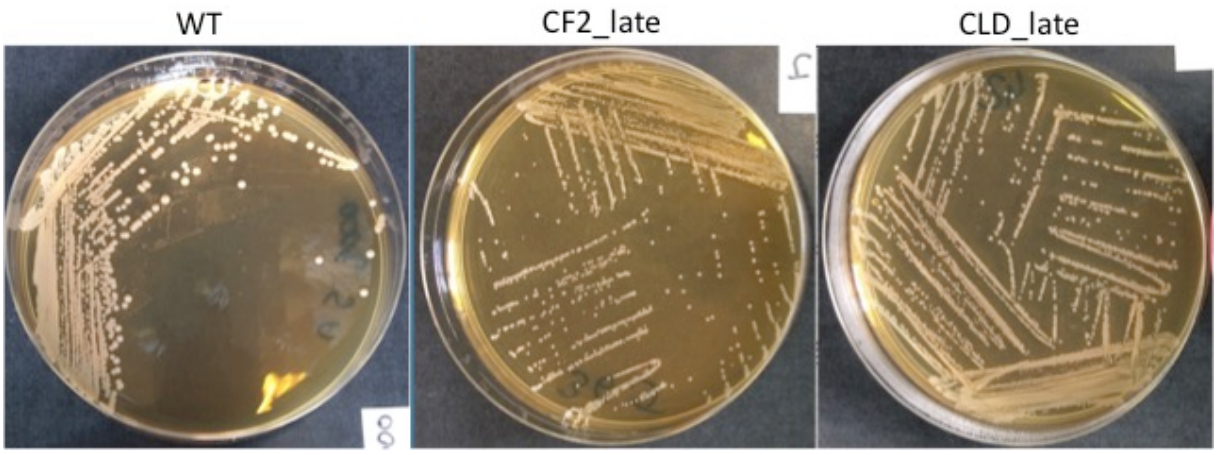

Figure S4. Colony morphology of USA300-LAC (WT), CF2_late and CLD_late isolates. Bacteria were grown on BHI agar incubated 12h at 37°C.

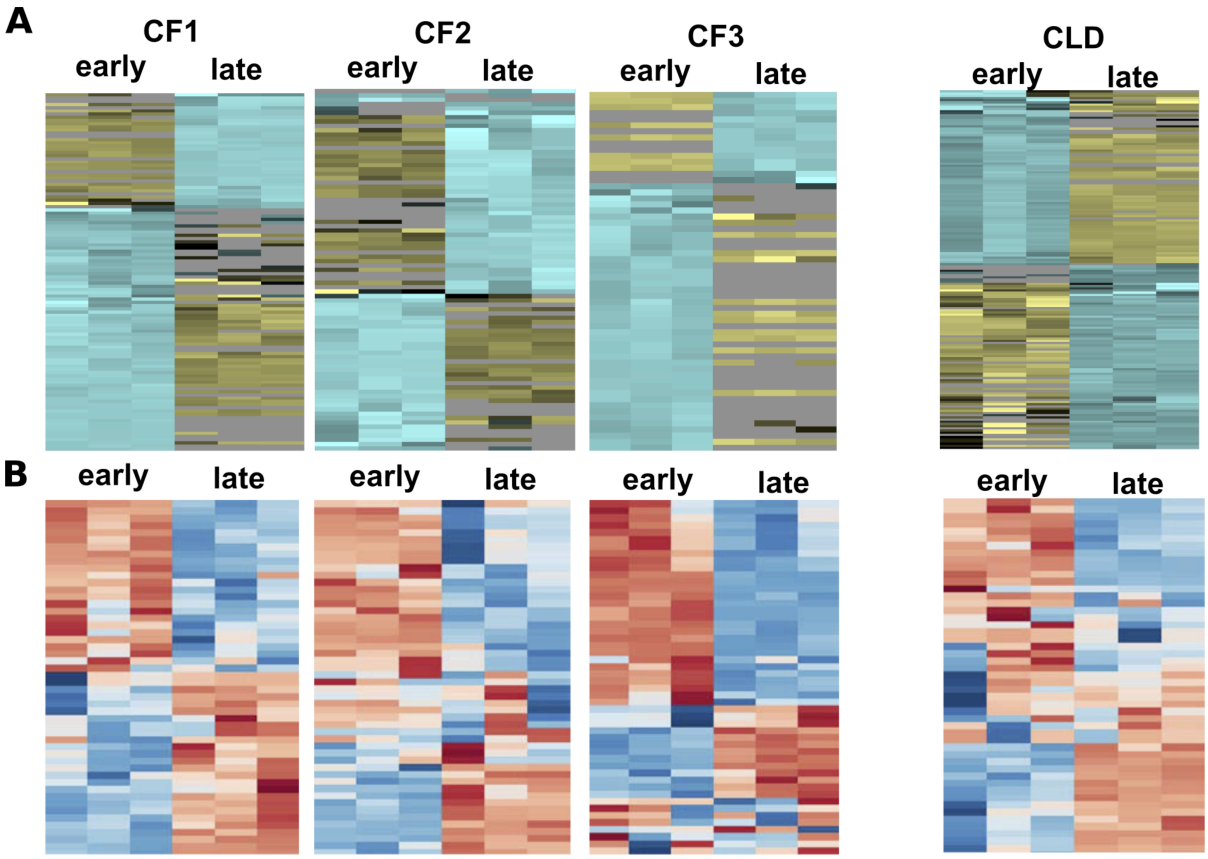

Figure S5. Heatmaps showing comparison of protein and metabolite profiles of early/late *S. aureus* isolates. The isolates were cultured to the stationary phase in medium mimicking the respiratory fluid of cystic fibrosis patients (CFSM) with the addition of thymidine. **A)** Heatmap visualization and hierarchical clustering analysis of the proteomic profiling in the late isolate compared to the early isolate of each patient. One experiment with three biological replicates was performed for each isolate. Rows: proteins; columns: samples; color key indicates protein relative concentration value (yellow: lowest; blue: highest). **B)** Heatmap visualization and hierarchical clustering analysis of the metabolite profiling in the late isolate compared to the early isolate of each patient. The top 50 most changing compounds are presented. Two independent experiments with three biological replicates were performed for each isolate. Rows: metabolites; columns: samples; color key indicates metabolite relative concentration value (blue: lowest; red: highest).