**ORIGINAL RESEARCH**

# Metabolic Adaptation in Methicillin-Resistant *Staphylococcus aureus* Pneumonia

Stanislaw J. Gabryszewski1, Tania Wong Fok Lung1, Medini K. Annavajhala2,3, Kira L. Tomlinson1, Sebastian A. Riquelme1, Ibrahim N. Khan1, Loreani P. Noguera1, Matthew Wickersham1, Alison Zhao1, Arielle M. Mulesnos4, David Peaper5, Jonathan L. Koff4, Anne-Catrin Uhlemann2,3, and Alice Prince1

1Department of Pediatrics, 2Department of Medicine, and 3Microbiome and Pathogen Genomics Core, Department of Medicine, Columbia University Irving Medical Center, New York, New York; and 4Department of Internal Medicine and 5Department of Laboratory Medicine, Yale University School of Medicine, New Haven, Connecticut

ORCID ID: 0000-0002-7399-9295 (A.P.).

# Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a versatile human pathogen that is associated with diverse types of infections ranging from benign colonization to sepsis. We postulated that MRSA must undergo specific genotypic and phenotypic changes to cause chronic pulmonary disease. We investigated how MRSA adapts to the human airway to establish chronic infection, as occurs during cystic fibrosis (CF). MRSA isolates from patients with CF that were collected over a 4-year period were analyzed by whole-genome sequencing, transcriptional analysis, and metabolic studies. Persistent MRSA infection was associated with staphylococcal metabolic adaptation, but not changes in immunogenicity. Adaptation was characterized by selective use of the tricarboxylic acid cycle and generation of biofilm, a means of limiting oxidant stress. Increased transcription of specific metabolic genes was conserved in all host-adapted strains, most notably a 10,000-fold increase in *fumC*, which catalyzes the interconversion of fumarate and malate. Elevated fumarate levels promote biofilm production in clinical isolates. Host-adapted strains preferred to assimilate glucose polymers and pyruvate, which can be metabolized to generate N-acetylglucosamine polymers that comprise biofilm. MRSA undergoes substantial metabolic adaptation to the human airway to cause chronic pulmonary infection, and selected metabolites may be useful therapeutically to inhibit infection.

**Keywords:** bacterial metabolic adaptation; chronic pulmonary infection; *Staphylococcus aureus*; cystic fibrosis

# Clinical Relevance

This study should be of substantial interest to the cystic fibrosis community, as well as to critical care, pulmonary, and infectious disease physicians who deal with the treatment of *Staphylococcus aureus* pneumonia. Our findings indicate that “host-adapted” *S. aureus* strains retain their immunogenicity and do not necessarily become less virulent over time. In addition, these findings lend support to the concept of targeting bacterial metabolism as a mechanism to prevent bacterial metabolic reprogramming, biofilm formation, and the development of chronic airway infection.

---

*Staphylococcus aureus* is a frequent cause of pulmonary infection targeting individuals with underlying pulmonary conditions, including intubation, chronic obstructive pulmonary disease (COPD), and especially cystic fibrosis (CF). *S. aureus* pulmonary infections, particularly those caused by methicillin-resistant *S. aureus* (MRSA), are refractory to antibiotic treatment and often portend a poor prognosis (1–3). Exactly how *S. aureus* transitions from a nasal commensal to a pulmonary pathogen...
remains unclear. Moreover, the impact of adaptive changes to the human airway on the virulence of these organisms is poorly understood.

Both the host and infecting bacteria undergo metabolic adjustments to cope with the demands of infection. Immune cells (e.g., macrophages) switch from oxidative phosphorylation to aerobic glycolysis (4) to generate ATP while regulating the release of reactive oxygen species and immunoreactive metabolites. Concurrently, bacteria adjust their metabolic activity to fuel the production of surface and secreted proteins required to thwart immune clearance mechanisms, including reactive oxygen species, while also limiting the generation of endogenous oxidants. Bacterial metabolic reprogramming (5) is a widely conserved mechanism by which bacteria preferentially use metabolic pathways to generate antioxidant NADPH while supporting gluconeogenesis to provide substrates for extracellular polysaccharides, which are important for biofilm formation (6). Although staphylococcal biofilm production has been best characterized in the setting of skin/soft tissue- and catheter-associated infections (7), it is likely that biofilms also play an important role in the setting of lung infection.

The prolonged course of bacterial colonization and infection in CF allows for a longitudinal study of the commensal-to-pathogen transition of S. aureus. Murine studies have identified specific toxins, especially α-toxin, as critical for the establishment of an acute infection (8). However, identification of the bacterial determinants of chronic infection has been challenging. Epidemiological studies have not revealed major genomic differences between nasal colonizers and organisms that cause severe infections (9). Transcripitomic studies of CF-associated S. aureus have implicated genes involved in immune evasion, toxin production, iron scavenging, and intercellular adhesion (10, 11) in adaptation to the host.

In this work, we used sequential isolates of MRSA obtained from chronically infected adult patients with CF to study how clinical isolates of S. aureus adapt to the airway, as compared with the standard laboratory strain LAC USA300 MRSA. Using genomic, metabolic, and gene expression studies, we demonstrate that host-adapted MRSA undergoes substantial metabolic reprogramming, allowing for the use of available carbon sources to simultaneously promote biofilm production and limit oxidant generation.

Some of the results of these studies have been previously reported in the form of an abstract (12).

Methods

**Bacterial Isolation from Patients**

MRSA isolates were obtained from the sputum, blood, or BAL fluid (BALF) of three patients with CF who were being cared for at a CF center as part of routine care and ongoing epidemiological surveillance. The fumC transposon mutant from the NTML transposon library was in the JE2 background (13).

**Bacterial Culture**

Bacteria were cultured at 37°C in Luria-Bertani broth, with shaking at 250 rpm. Overnight cultures were subinoculated at 1:100 and grown to an OD600 of 1. Growth curves were performed in 96-well plates inoculated with a 1:100 dilution of overnight cultures and monitored using a BioTek Synergy H1 microplate reader with Gen5 software. Bacterial growth was similarly determined in the presence or absence of 1.2 mM pararquat (Sigma-Aldrich) or select carbon sources (5–500 mM sodium acetate, sodium citrate, disodium succinate; Sigma-Aldrich).

**Whole-Genome Sequencing and Hybrid Assembly**

Genomic DNA was extracted using a Qiagen DNeasy UltraClean Microbial DNA Isolation Kit. A Nextera XT DNA Library Prep Kit was used to prepare barcoded libraries, which were sequenced using an Illumina MiSeq v3 sequencing kit. Multilocus sequence typing and characterization of antibiotic-resistance genes was performed using SRST2 (14). To generate accurate reference genomes for isolate series, we also conducted long-read sequencing of strains A1, B1, and C1 on a MinION (Oxford Nanopore Technologies) after library preparation with the Rapid Barcoding Sequencing Kit. Reads were basecalled using MinKnow and Epi2ME (Metrichor), and hybrid de novo assembly of Nanopore and Illumina reads was performed using SPAdes v3.10.1 (6). The genome was annotated using Prokka v1.12 (15), and mobile genetic elements and prophage regions were identified by IslandViewer 4 (16) and PHASTER (17), respectively. We used Snippy software (Snippy v3, https://github.com/tseemann/snippy) to identify core genome concatenated SNPs.

**Phylogenetic Analysis**

We constructed phylogenetic trees by mapping individual reads from each isolate against the curated S. aureus sequence type 105 (ST105) genome using Snippy v3 after excluding mobile genetic elements and prophage regions. Maximum-likelihood phylogenetic analysis based on core genome concatenated SNPs was performed using RAxML v8.0.0 with 100 bootstrap inferences and the GTR GAMMA model. Phylogenetic trees were visualized using iTOL v3 (18).

**RNA Isolation and qRT-PCR**

For bacterial RNA isolation, bacteria were grown statically for 24 hours in tryptic soy broth (TSB) containing 0.5% glucose. The bacteria were harvested by centrifugation, stored in RNAProtect (QIAGEN), and normalized based on OD600. Bacterial pellets were incubated in lysis buffer (50 μM Tris-EDTA [pH 7.5], 8 U/ml mutanolysin [Sigma], 0.018 mg/ml lysostaphin [Sigma], 0.05 g/ml lysozyme [Sigma]) at 37°C for 45 minutes, and TRK lysis buffer (Omega Bio-tek) was added. After 10 minutes at room temperature, 70% ethanol was added and the samples were transferred to EZNA RNA isolation kit columns (Omega Bio-tek). RNA was isolated according to the manufacturer’s instructions and treated with a DNA-free DNase treatment and removal kit (Invitrogen). RNA quality was verified using a NanoDrop spectrophotometer (NanoDrop Technologies). A SuperScript III RT kit (Invitrogen) was used to generate cDNA for qRT-PCR reactions with SYBR green reagents (Applied Biosystems). qRT-PCR was performed using a StepOne Plus thermal cycler (Applied Biosystems), and data were analyzed using the ΔΔCt method. The qRT-PCR primers are listed in Table 1. Primers directed against the 16S rRNA
gene were used as the housekeeping gene control. Additional controls included "no reverse transcriptase" and "no template" controls.

**Biofilm Assays**

Bacterial cultures were normalized to an OD<sub>600</sub> of 1.0 and inoculated at 1:100 into TSB supplemented with 0.5% glucose in a 96-well plate. Soy fumarate (Sigma-Aldrich) and L-malic acid (Sigma-Aldrich) were used at 62.5 mM, 125 mM, 250 mM, and 500 mM concentrations. Bacterial cultures were grown statically at 37°C. After 24 hours, OD<sub>600</sub> was measured and plates were washed twice with water, dried, and fixed with methanol. They were then stained with 1% crystal violet (wt/vol) and again washed twice with water and dried. After solubilization of the well contents with 33% acetic acid (vol/vol), OD<sub>540</sub> values were determined.

**Extracellular DNA Quantification**

 Supernatants from the biofilm experiments were transferred to a new 96-well V-bottom plate and centrifuged (2,500 rpm, 5 min). The supernatants were then transferred to new wells and stained for 10 minutes with 1 μM (final concentration) SYTOX Blue stain (Invitrogen). Fluorescence was determined using a Tecan Infinite M200 plate reader, with excitation and emission spectra of 444 nm and 480 nm, respectively.

**Congo Red Susceptibility Assays**

Bacterial strains were grown in Luria-Bertani broth to an OD<sub>600</sub> of 1 and serially diluted before they were plated on tryptic soy agar plates with or without 0.08% Congo Red stain. The plates were incubated at 37°C overnight and colonies were enumerated after 24 hours.

**Carbon Source Use Assays**

Carbon use was assessed via Biolog PM1 assays according to the manufacturer’s instructions.

**Cell Culture and Infections**

Human bronchial epithelial (16HBE) cells were cultured in BronchiaLife medium (Lifeline Cell Technology) supplemented with 1% FBS and 1% penicillin/streptomycin. THP-1 cells were grown in RPMI 1640 medium (Corning) supplemented with 10% FBS and 1% penicillin/streptomycin. All cells were incubated at 37°C in 5% CO<sub>2</sub>.

**Gentamicin Protection Assays**

THP-1 cells were infected at a multiplicity of infection of 10. For time points exceeding 30 minutes, the cells were washed 1 hour after infection and replaced with RPMI containing gentamicin at a final concentration of 0.5 mg/ml. The cells were washed with PBS at the desired time point, harvested by disassociating them with TrypLE Express cell dissociation enzyme (Life Technologies), serially diluted, and plated.

**Mouse Infections and Sample Collection**

Mouse care, anesthesia, and infections were performed in accordance with an institutionally approved protocol (AAAR9403). For infections, 6-week-old C57BL/6 mice were anesthetized with ketamine/xylazine and intranasally inoculated with 4 × 10<sup>7</sup> cfu (50 μl total volume) of S. aureus strains or PBS vehicle control. The mice were killed 24 hours after infection with pentobarbital, followed by collection of BALF and lungs. Lung cell homogenates were generated using 40 μm nylon cell strainers (Falcon). Bacteria in the BALF and lung cell homogenates were enumerated after serial dilution and plating on CHROMagar plates (BD).

**Cytokine Analysis**

After centrifugation to remove cellular content, samples were analyzed by multiplex cytokine array assays (Eve Technologies). IFN-β levels were assessed by ELISA (R&D) according to the manufacturer’s instructions.

**Flow Cytometric Analysis**

BALF and lung cell suspensions were centrifuged (1,500 rpm, 6 min), and red blood cells were lysed with ammonium-chloride-potassium lysis buffer. Cells were resuspended in staining buffer and transferred to a V-bottom 96-well plate treated with DAPI and stained with a panel of antibodies (CD45-AF700, CD11b-
Figure 1. Characterization of cystic fibrosis (CF) host-adapted methicillin-resistant Staphylococcus aureus (MRSA) strains. (A) Timeline and sequence type (ST) of MRSA isolation from patients A, B, and C. All samples were harvested from sputum, with the exception of A6 (*, blood) and B3 (**, pleural fluid).
AF594, Ly6G-PerCPCy5.5, Ly6C-BV421, CD11c-BV605, MHCIi-APC-Cy7, SiglecF-APC/AF647, and Pro-IL1β-PercPCy5.5, Ly6C-BV421, neutrophils (CD451, Ly6Ghi, Ly6Clo), and monocytes (CD451, Ly6Glo, Ly6Chi). Cells were fixed in 10% paraformaldehyde solution and analyzed using a BD LSRII flow cytometer and FlowJo v10 software. Cell surface staining to identify cell types included alveolar macrophages (CD451, CD11bhi, SiglecFlo), eosinophils (CD451, CD11bhi, SiglecFhi), neutrophils (CD451, CD11bhi, SiglecFhi), CD11c- MHCIi (Ly6Ghi, Ly6Clo), and monocytes (CD451, CD11bhi, SiglecFhi, CD11c-MHCIi (Ly6Glo, Ly6Chi)). For a given condition, the absolute numbers of recruited immune cells represent the product of the percentage of viable cells that exhibited a specific set of cell surface markers and the viable cell count, as determined using a Countess automated cell counter (Invitrogen).

**Data Analysis and Statistics**

Data were plotted with SE of mean, and statistical significances were determined using GraphPad Prism 7 software.

**Results**

**Host-adapted MRSA Strains Acquire Mutations Predominantly in Genes Affecting Metabolism**

MRSA isolates obtained from three patients with CF (patients A, B, and C) over a period of 2–4 years, with the most recent isolate taken at the time of an acute exacerbation, associated with bacteremia, lobar consolidation, or empyema, were studied by whole-genome sequencing (Figure 1A). The patients had distinct Cftr genotypes, with clinical severity ranging from a forced expiratory volume in 1 second (FEV1) of 20% to 70% at the time of acute exacerbation, and had been coinfected with methicillin-sensitive *S. aureus* (MSSA), *Pseudomonas aeruginosa*, and *Haemophilus influenzae* species in the recent past (Table 2). We observed patient-specific clonality with distinct STs: ST105 (A), ST109 (B), and ST5 (C). To compare the evolution of SNPs among the patients’ MRSA isolates, we *de novo* assembled the genomes of each initial isolate (A1, B1, and C1). The serial isolates from each patient differed by a substantial number of SNPs over the 2- to 4-year collection period. There are inherent difficulties in determining true insertion or deletion of genome sequences, or the presence of allele-specific insertions or deletions. In this study, we have used whole-genome sequencing data to demonstrate significant differences in SNPs associated with *S. aureus* infection.

**Figure 1.** (Continued). A2–A6 were one-SNP variants of ST105 (**`). (B) Phylogenetic tree of MRSA isolates from the A series, with an ST-matched non-CF isolate (CP011147) serving as a reference. Branch lengths represent the number of nucleotide substitutions per site. (C) Schematic diagram showing the number of nonsynonymous mutations (NSMs) observed among A-series MRSA isolates as compared with a non-CF reference strain, and their associated genotypes, with differentiation in clonality with respect to each patient. (D) Growth curves of the laboratory strain of MRSA USA300 and the isolates from the A, B, and C series in Luria-Bertani broth. Data shown are from four biological replicates in triplicate. (D) Intracellular bacterial persistence of the MRSA isolates from patients A, B, and C in THP-1 cells. Data shown are from two independent experiments with each sample in triplicate. (F) Cytokines from 16HBE cells infected with MRSA isolates. (G) Cytokines from THP-1 cells infected with MRSA isolates. (H) IFN-β production by 16HBE cells 24 hours after *S. aureus* infection. Data are shown for three independent experiments in triplicate. (I) Impact of the superoxide generator paraquat on growth of the MRSA isolates. Data shown are from two independent experiments in triplicate. Statistical significance was determined by one-way ANOVA, **`P < 0.0001, *P < 0.001, **P < 0.01, and ***P < 0.05. OD = optical density; Ref = reference non-CF isolate CP011147 strain.

**Table 2.** Clinical Characteristics of the Patients from Whom MRSA Isolates were Collected

| Patients’ Clinical Characteristics | Patient A | Patient B | Patient C |
|-----------------------------------|-----------|-----------|-----------|
| Age (years)                       | 45        | 27        | 41        |
| Sex                               | Female    | Male      | Male      |
| Sweat chloride values, mmol/L     | 97        | 87        | 34/36     |
| CFTR genotype                     | ΔF508/ΔF508| R1162X/R347P | c.273+1G>A and c.3718-2477 C>T with 7T/7T poly-T variants |
| FEV1, Most recent, L, % predicted | 1.88 L (69%) | 3.24 L (76%) | 0.99 L (23%) |
| At admission, L, % predicted      | 1.37 L (51%) | 2.93 L (70%) | 0.87 L (20%) |
| CF-related complications           | CF-related diabetes, pancreatic insufficiency | Pancreatic insufficiency | Pancreatic insufficiency |
| Acute presentation, 02/2017       | Pneumonia with bacteremia | Pneumonia with right pleural effusion | Lobar pneumonia |
| Site of specimen collection       | Sputum (A1–A5), blood (A6) | Sputum (B1–B2), BAL fluid (B3) | Sputum (C1–C3) |
| Period of MRSA collection         | 2013–2017 | 2013–2017 | 2015–2017 |
| Bacteriology, others (2017)       | MSSA, *Haemophilus parainfluenzae*, *P. aeruginosa* | MSSA, *Haemophilus influenzae*, *Escherichia coli* |

**Definition of abbreviations:** CF = cystic fibrosis; FEV1 = forced expiratory volume in 1 second; MRSA = methicillin-resistant *Staphylococcus aureus*; MSSA = methicillin-sensitive *S. aureus*;
deletion of bases (indels) from short read sequencing, as these often occur in regions of a gene with numerous repetitive sequences that could also arise due to mapping difficulties. For this reason, indels were not included in our analyses. The six isolates from patient A differed by 44 SNPs in total and 27 nonsynonymous mutations (NSMs), the three isolates from patient B differed by 24 SNPs in total and 17 NSMs, and the three isolates from patient C differed by 50 SNPs in total and 27 NSMs (Table 3 and Figure 1A). When compared with an ST-matched (ST105), non-CF reference strain (CP011147), the A-series isolates differed by 192 SNPs overall and 80 NSMs, 10 of which represented polymorphic sites among the A-series isolates themselves (Table 3 and Figure 1B). Based on pathway categorization, most of these mutations were in genes involved in metabolism and transport (Figure 1C). No mutations were observed in the virulence-associated agr locus, although mutations in saeR, which encodes part of a two-component regulatory system involved in virulence gene expression, were observed in the A series.

The accumulated mutations had minimal effects on growth rates (Figure 1D), as all strains achieved comparable densities by the stationary phase despite differences in lag and log phases in some isolates. Uptake and persistence within immune cells (THP-1, a human monocyte-macrophage cell line) were variable and, in most instances, comparable to those observed in the USA300 MRSA control (Figure 1E). Immunogenicity was assessed by quantifying cytokine expression induced in either human airway cells (16HBE) (Figure 1F) or THP-1 cells (Figure 1G). No global differences in immunogenicity were identified, but strain-dependent trends were observed. The later A-series isolates stimulated more IFN-β secretion in 16HBEs than the initial isolate (Figure 1H). The clinical strains appeared to become less resistant to oxidant stress over time, as assessed by susceptibility to the superoxide-generating agent paraquat (Figure 1I).

**Host-adapted MRSA Strains Produce Biofilm**

The A-series isolates developed mutations in *dacA* and *gpdP*, which encode a diadenylate cyclase required for the synthesis of cyclic-di-AMP (c-di-AMP) and a phosphodiesterase responsible for its hydrolysis, respectively (Figure 2A). c-di-AMP is a multifunctional bacterial secondary metabolite that controls the expression of many genes in response to environmental conditions, including those involved in biofilm formation. Increasing amounts of biofilm production was noted in later A and C isolates (Figure 2B).

Transcriptional analysis of *dacA* and *gpdP* (Figure 2C) suggested a trend toward increased expression of both genes over time, roughly comparable to the biofilm phenotype (Figure 2B). As this was not a linear relationship, there are likely multifactorial means of regulation of c-di-AMP metabolism in the setting of CF host adaptation (Figure 2C). This highlights the contribution of both genetic and transcriptional changes in c-di-AMP metabolism to biofilm formation by host-adapted strains. In addition, the A6 isolate harbored an NSM mutation in the *saeR* locus, which is responsive to cellular metabolism and involved in biofilm formation (19).

**Table 3. List of Nonsynonymous Mutations that Differed between at Least One of the A-Series Strains and a Sequence Type–matched, Non–Cystic Fibrosis Strain, or among Strains Harvested from a Single Patient**

| Isolate | Number of NSMs | Implicated Genes and Pathways |
|---------|----------------|-------------------------------|
| A vs. reference | 80 | Adhesion (sdrC, fnbB) DNA replication and repair ( gyrA, addA, recN) Metabolism ( adhE, sufS, trgP, ilvB, hisA, deoD, purl, my, malX, araB, tktA, pckA, nagB, rpoC, mvaK2, malA, dagK) Post-translational processing and proteolysis ( pepF, pepA2, pepX, aur, ureE) Redox homeostasis (hmp, ndhF) Structural homeostasis (tagE, tagH, fmt, ebh, pepB3, lytH) Transcriptional regulation (rmtS, malR, kdpD, licR) Translation (prfB, miaA, leuS, rumA, rplX, rpmH) Transport (uhtP, oppA, ykoD, potA, kdpA, kdpB, corA, nark, copA, rarD_2) Other (hemC, hssS) Unknown (ybaB, comFC) |
| A | 27 | Adhesion (fnbB, sdrE, ebhA) Metabolism (robo, mvaK2, malA, dagK, argF, gdpP, dacA, phoA, carB, bgk, tagE, glmM, lqo) Post-translational processing (ureE) Transcriptional regulation (licF, saeR, rsbV, bgI) Translation (rpmH, rpsJ) Transport (gscO) fadN, metG, hisC, nikE, lysC, recD2, acsA, lidA, smc, leuA, rpsJ |
| B | 17 | hutH, ggt, oxyR, tagO, urvA, mecA, purS, carB, pyrF, lapB, mmaA, ezrA, mrcA, artQ, ebh, rplC, rpsJ, sarR, dapF, gntT, oatA |
| C | 27 | |

**Definition of abbreviation:** NSMs = nonsynonymous mutations.
Figure 2. Biofilm-forming ability of the MRSA isolates. (A) Summary of NSMs observed in gdpP and dacA in MRSA isolates and schematic of the role of these genes in c-di-AMP metabolism. (B) Biofilm formation of the MRSA clinical isolates, MRSA strain USA300, and Pseudomonas aeruginosa PAO1. Data shown are from at least three independent experiments in triplicate. Statistical significance was determined by one-way ANOVA, ****P < 0.0001, ***P < 0.001, **P < 0.01, and *P < 0.05. (C) Transcription of dacA and gdpP from MRSA isolates grown in biofilm-inducing conditions. Data are shown for four biological replicates. Statistical significance was determined by Mann-Whitney t test, *P < 0.05. (D) Effect of Congo Red (CR) on the growth of the MRSA isolates. The transcription of ltaS, which controls the synthesis of the cell wall component leipoteichoic acid, is also shown. (E) Quantification of extracellular DNA from the MRSA isolates. Data represent two independent experiments performed in triplicate. Statistical significance was determined by one-way ANOVA, ****P < 0.0001, ***P < 0.001, **P < 0.01, and *P < 0.05. (F) Transcription of the intercellular adhesion locus (icaA/B/C/D) from the clinical MRSA strains. For C, D, and F, RQ = relative quantification to wild-type (WT) LAC; TS = tryptic soy.
Metabolic adaptation of the host-adapted MRSA isolates. (A and B) Carbon source assimilation of the clinical strains relative to USA300 (A) and USA300 alone (B). The heatmap color intensity corresponds to the absorbance (OD$_{600}$) of the indicated strain in the presence of the indicated carbon source, normalized to the absorbance (OD$_{600}$) of USA300 in the same carbon source. Data represent three independent experiments. (C) Schematic diagram showing the major metabolic pathways in S. aureus and the production of polysaccharide intercellular adhesin (PIA). (D) Transcription of bacterial genes involved in glycolysis, the pentose phosphate pathway, and the tricarboxylic acid (TCA) cycle. Data shown are from four biological replicates. Statistical significance was determined by Mann-Whitney t test, $^*P < 0.05$ and $^{**}P < 0.01$. 

**Figure 3.** Metabolic adaptation of the host-adapted MRSA isolates. (A and B) Carbon source assimilation of the clinical strains relative to USA300 (A) and USA300 alone (B). The heatmap color intensity corresponds to the absorbance (OD$_{600}$) of the indicated strain in the presence of the indicated carbon source, normalized to the absorbance (OD$_{600}$) of USA300 in the same carbon source. Data represent three independent experiments. (C) Schematic diagram showing the major metabolic pathways in S. aureus and the production of polysaccharide intercellular adhesin (PIA). (D) Transcription of bacterial genes involved in glycolysis, the pentose phosphate pathway, and the tricarboxylic acid (TCA) cycle. Data shown are from four biological replicates. Statistical significance was determined by Mann-Whitney t test, $^*P < 0.05$ and $^{**}P < 0.01$. 

192 American Journal of Respiratory Cell and Molecular Biology Volume 61 Number 2 | August 2019
Figure 4. Role of *fumC* in *S. aureus* pathogenesis in vivo. (A) Bacterial enumeration from the BAL fluid (BALF) and lungs of mice infected with WT USA300 or the MRSA isolates A1, A5, and A6. Data are from three independent experiments done at least in triplicate. (B) Immune cell recruitment in the lungs of
Host-adapted MRSA Strains Exhibit Altered Carbon Metabolism

We further explored how the metabolic activity of the clinical isolates differed from that of the USA300 MRSA control by quantifying the assimilation of single carbon sources by each strain (Figure 3A). None of the MRSA strains consumed the tricarboxylic acid (TCA) cycle substrates citrate, fumarate, and malate. However, the host-adapted strains showed increased avidity for the glucose polymers trehalose, maltose, and sorbitol (A and C series), as well as pyruvate (A strains). The A and C series showed several similarities in preferred substrate assimilation, differing from the B series and the USA300 control, which had a marked preference for glucose use above all of the substrates offered (Figure 3B). The increased consumption of pyruvate by the A- and C-series strains and the minimal consumption of TCA cycle intermediates suggest shunting of substrates away from the TCA cycle, which generates prooxidant NADH. Instead, increased consumption of pyruvate and glucose polymers would fuel glycolysis to generate N-acetyl-glucosamine for biofilm production, which is inherently antioxidant (5).

To further determine differences in carbon source use, we monitored the expression of representative genes that are important for TCA cycle activity and gluconeogenesis by qRT-PCR (Figures 3C and 3D). Relative to the USA300 control strain, expression of gapR, which encodes a central glycolytic regulator (25), was increased in both the A and C series of isolates (Figure 3D). Expression of zwf, which is involved in the pentose phosphate pathway, was also increased in the later isolate A5. Transcription of selected TCA cycle genes was significantly altered in host-adapted MRSA strains, with a trend toward upregulation of TCA cycle enzymes as compared with USA300. This is consistent with reports that have linked sdh upregulation with biofilm production (26). B-series isolates showed a different pattern of TCA cycle enzyme use, with increased expression of mgoA, which encodes a malate:quinone oxidoreductase converting malate to oxaloacetate, yet decreased expression of glnA. Most impressively, in all three lineages of host-adapted strains, we observed up to 10,000-fold increases in the expression of fumC, which codes for the enzyme fumarate hydratase (Figure 3D), suggesting that the metabolism of fumarate and/or malate is a critical component of staphylococcal adaptation.

Participation of fumC Expression in the Pathogenesis of Pneumonia

Focusing on the A series of strains, which included simultaneous sputum (A5) and blood isolates (A6), we monitored MRSA clearance and the nature of the immune response elicited in a murine model of acute pneumonia, as compared with the USA300 control (Figure 4A). There was a statistically significant, but perhaps biologically less meaningful, decrease in bacterial recovery of the A-series strains from BALF and lung relative to USA300, although without discernible differences among the A-series strains themselves. The nature of the immune responses elicited was comparable for all of the MRSA strains (Figures 4B and 4C). Thus, despite the significant differences in fumC expression noted earlier (Figure 3D), USA300 and the A-series strains behaved comparably in an acute infection model. To appreciate how fumC might contribute to pathogenesis in vivo, we compared the clearance (Figure 4D) and immune responses (Figure 4E) associated with acute infection using a MRSA strain and fumC transposon mutant. Notably, there was a 2- to 3-log decrease in retention of fumC bacteria in murine BALF and lung, despite equivalent growth rates of WT and fumC bacteria in vitro (Figure 4E). The lower organism burden in fumC-infected mice was associated with a trend toward decreased induction of proinflammatory cytokines (TNF, IL-6, keratinocyte chemoattractant, and IL-1β), which supports a role for fumC in pulmonary infection.

Discussion

This analysis of CF-adapted MRSA isolates highlights notable differences in the metabolism of clinical strains compared with the standard USA300 LAC control, a prototype for community-acquired MRSA infection. Strain-dependent mutations were noted in each of the lineages, with evidence of ongoing diversification and adaptation.
There were differences in gene expression in the CF isolates over time, but no definitive changes were associated with acute exacerbation. Among the genomic and transcriptional changes we documented were mutations and altered expression of dacA and gdpP, which affect turnover of the global regulator c-di-AMP. We note that these differences correlated with increased biofilm formation in some, but not all, of the host-adapted strains. In light of the recent documentation of increased expression of dacA and its link to biofilm formation (27), our findings confirm the clinical relevance of c-di-AMP regulation in the setting of bacterial adaptation to the CF host. Biofilm not only protects the bacteria from oxidant stress but also impedes phagocytic clearance, factors that promote the selection of these organisms from the infecting population (28, 29). In addition to its role in directing biofilm production, c-di-AMP is also involved in the induction of type I IFN signaling (27, 30, 31). IFN-β was one of the few cytokines that increased over the course of infection in the A-series isolates, consistent with the pathogenicity attributed to S. aureus activation of IFN-β signaling (32).

In contrast to studies of S. aureus strains associated with acute pneumonia in other patient populations (33), we did not observe consistent mutations in the agr locus, which regulates the expression of many virulence factors in the host-adapted CF isolates (34). Although we used numerous assay systems with both in vitro and in vivo outcome measurements, we were unable to identify a virulence phenotype associated with the CF strains that was significantly different from the USA300 control, beyond a 0.5-log decrease in acute infectivity. Many of the changes in gene expression that we observed were at

Figure 5. Fumarate enhances biofilm formation. (A and B) Bacterial growth and biofilm formation of CF host-adapted strains (WT USA300, WT JE2, and fumC mutant) in the presence of increasing concentrations of (A) fumarate and (B) malate. Data represent at least three independent experiments done in triplicate. Purple asterisks denote statistical difference respective to WT USA300 at the given concentration of fumarate or malate. Colored asterisks show statistical difference within the given strain across different concentrations of fumarate or malate respective to the untreated condition. Statistical significance was determined by one-way ANOVA; ****p < 0.0001, ***p < 0.001, **p < 0.01, and *p < 0.05. (C) Growth and (D) biofilm-forming ability as determined by crystal violet staining of MRSA strains in the presence of various concentrations of the TCA intermediates citrate, acetate, and succinate, as assessed in three independent experiments.
the level of transcription and could be readily reversed depending on environmental conditions.

Our transcriptional and metabolic studies provided more definitive evidence of MRSA adaptive changes that were not reflected in the genomic analysis. In contrast to USA300, the host-adapted MRSA strains preferentially consumed glucose polymers and selectively upregulated the portion of the TCA cycle that regulates the abundance of fumarate, indicating increased \( \text{fumC} \) gene expression in addition to elevated \( \text{fumC} \) expression. Most striking of all the adaptive changes we observed was the uniform upregulation of \( \text{fumC} \) in all three lineages of strains from the respiratory tract of patients with CF. Staphylococcal production of fumarate appears to be important for bacterial persistence within the CF lung. This may be due to fumarate’s ability to act as a terminal electron acceptor in a setting of low oxygenation, which is often present within a biofilm (35–37), as well as its observed effects in promoting biofilm production and growth.

The unique airway environment in CF drives extensive bacterial metabolic reprogramming to generate ATP for ongoing metabolic needs, biofilm production, and immunoevasion (38). These selective processes generate a diverse bacterial community that is protected from the penetration of complement, antibody, and phagocytes, and thus is able to sustain chronic infection. Our analysis of the properties of CF host-adapted MRSA suggests a role for exogenous carbon substrates in therapy for MRSA pulmonary infection, as has been suggested to potentiate the activity of aminoglycosides (39). In the absence of a murine model of chronic \( S. aureus \) pneumonia, our \textit{in vitro} and acute infection models indicate that host-adapted strains become increasingly susceptible to oxidants generated by the TCA cycle. Accordingly, the use of cell-permeable carboxylates as adjunctive therapy for infection in CF warrants further investigation.

**Author disclosures** are available with the text of this article at www.atsjournals.org.

---

**References**

1. Dasenbrook EC, Merlo CA, Diener-West M, Lechtzin N, Boyle MP. Persistent methicillin-resistant \( \text{Staphylococcus aureus} \) and rate of FEV1 decline in cystic fibrosis. \textit{Am J Respir Crit Care Med} 2008;178:814–820.
2. Junge S, Görlisch D, den Reijs M, Wiedemann B, Tümmler B, Ellemunter H, \textit{et al.} Factors associated with worse lung function in cystic fibrosis patients with persistent \( \text{Staphylococcus aureus} \). \textit{PLoS One} 2016;11: e0166220.
3. Dasenbrook EC, Checkley W, Merlo CA, Konstan MW, Lechtzin N, Boyle MP. Association between respiratory tracts' methicillin-resistant \( \text{Staphylococcus aureus} \) and survival in cystic fibrosis. \textit{JAMA} 2010; 303:2386–2392.
4. O’Neill LA, Pearce EJ. Immunometabolism governs dendritic cell and macrophage function. \textit{J Exp Med} 2016;213:15–23.
5. Lemire J, Alhasawi A, Appanna VP, Tharmalingam S, Appanna VD. Metabolic defence against oxidative stress: the road less travelled so far. \textit{J Appl Microbiol} 2017;123:798–805.
6. Gaupp R, Schlag S, Liebeke M, Lalk M, Gotz F. Advantage of upregulation of succinate dehydrogenase in \( \text{Staphylococcus aureus} \) and selectively upregulated the portion of the TCA cycle that regulates the abundance of fumarate.

---

**American Journal of Respiratory Cell and Molecular Biology Volume 61 Number 2 | August 2019**
23. Arciola CR, Campoccia D, Ravaoli S, Montanaro L. Polysaccharide intercellular adhesin in biofilm: structural and regulatory aspects. Front Cell Infect Microbiol 2015;5:7.

24. Cramton SE, Gerke C, Schnell NF, Nichols WW, Götz F. The intercellular adhesion (ica) locus is present in Staphylococcus aureus and is required for biofilm formation. Infect Immun 1999;67:5427–5433.

25. Purves J, Cockayne A, Moody PC, Morrissey JA. Comparison of the regulation, metabolic functions, and roles in virulence of the glyceraldehyde-3-phosphate dehydrogenase homologues gapA and gapB in Staphylococcus aureus. Infect Immun 2010;78:5223–5232.

26. Gaupp R, Schlag S, Liebeke M, Lalk M, Götz F. Advantage of upregulation of succinate dehydrogenase in Staphylococcus aureus biofilms. J Bacteriol 2010;192:2385–2394.

27. Gries CM, Bruger EL, Moormeier DE, Scherr TD, Waters CM, Kilian T. Cyclic di-AMP released from Staphylococcus aureus biofilm induces a macrophage type I interferon response. Infect Immun 2016;84:3564–3574.

28. Domenech M, Ramos-Sevillano E, Garcia E, Moscoso M, Yuste J. Biofilm formation avoids complement immunity and phagocytosis of Streptococcus pneumoniae. Infect Immun 2013;81:2606–2615.

29. Thurlow LR, Hanke ML, Fritz T, Angle A, Aldrich A, Williams SH, et al. Staphylococcus aureus biofilms prevent macrophage phagocytosis and attenuate inflammation in vivo. J Immunol 2011;186:6585–6596.

30. Commmichau FM, Dickmanns A, Gundlach J, Fenrer R, Stülke J. A jack of all trades: the multiple roles of the unique essential second messenger cyclic di-AMP. Mol Microbiol 2015;97:189–204.

31. Devaux L, Kaminski PA, Trieu-Cuot P, Firon A. Cyclic di-AMP in host-pathogen interactions. Curr Opin Microbiol 2018;41:21–28.

32. Martin FJ, Gomez MI, Wetzel DM, Memmi G, O’Seaghdha M, Soong G, et al. Staphylococcus aureus activates type I IFN signaling in mice and humans through the Xr repeated sequences of protein A. J Clin Invest 2009;119:1931–1939.

33. Altman DR, Sullivan MJ, Chacko KL, Balasubramanian D, Pak TR, Sause WE, et al. Genome plasticity of agr-defective Staphylococcus aureus during clinical infection. Infect Immun 2018;86 pii:e00331-18.

34. 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.

35. Leys D, Tsapin AS, Nealson KH, Meyer TE, Cusanovich MA, Van Beeumen JJ. Structure and mechanism of the flavocytochrome c fumarate reductase of Shewanella putrefaciens MR-1. Nat Struct Biol 1999;6:1113–1117.

36. Uribe-Alvarez C, Chiquete-Felix N, Contreras-Zentella M, Guerrero-Castillo S, Peña A, Uribe-Carvajal S. Staphylococcus epidermidis: metabolic adaptation and biofilm formation in response to different oxygen concentrations. Pathog Dis 2016;74:ftv111.

37. Van Hellemont JJ, Tielens AG. Expression and functional properties of fumarate reductase. Biochem J 1994;304:321–331.

38. Foster TJ. Immune evasion by staphylococci. Nat Rev Microbiol 2005;3:948–958.

39. Meylan S, Porter CBM, Yang JH, Belenky P, Gutierrez A, Lobritz MA, et al. Carbon sources tune antibiotic susceptibility in Pseudomonas aeruginosa via tricarboxylic acid cycle control. Cell Chem Biol 2017;24:195–206.