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Investigating a Defined Minimal Medium for Systems Analyses of MDR *Staphylococcus aureus*

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

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Bioengineering

by

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The Thesis of Liam Lingyan Weng is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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ABSTRACT OF THE THESIS

Investigating a Defined Minimal Medium for Systems Analyses of MDR *Staphylococcus aureus*

by

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Staphylococcus aureus is a gram-positive pathogenic bacterium that has colonized an estimated one-third of the human population and such infections can often become fatal. The adaptive mechanisms of *S. aureus*, however, still remain obscure partially due to a lack of knowledge in its metabolic requirements. Systems biology approaches can be extremely useful in predicting and interpreting metabolic phenotypes through genome-scale modeling and bioinformatics approaches. However, there is a need for a validated chemically defined minimal medium to further investigate the requirements of the cell. Identifying the nutritional requirements will

provide mechanistic insights into the functional states of the cell and its pathogenicity. In this work, a chemically defined minimal medium formulation, termed synthetic minimal medium (SMM), was investigated, modified, and validated to enable growth of three *S. aureus* strains, and enable systems analyses of this important pathogen. The formulated SMM was utilized in an adaptive laboratory evolution (ALE) experiment to further probe the ideal capabilities of the targeted strains and uncover mechanisms underlying the optimized states. The evolved strains were phenotypically characterized for their physiological characteristics and antimicrobial susceptibility. The genome of each strain was sequenced to examine the genetic basis for the observed phenotypes. The resulting SMM and the evolved strains will serve as important reagents for studying the resistance phenotypes of *S. aureus*.

Chapter 1

Introduction

Staphylococcus aureus is a gram-positive pathogenic bacterium that has colonized an estimated one-third of the human population [1]. Due to its association with antibiotic resistance, *S. aureus* infections are difficult to treat [2] and are associated with a high mortality rate [1]. Methicillin-resistant *S. aureus* (MRSA) is especially concerning due to its prevalence in hospital settings and the paucity of available treatment options [3]. The adaptive mechanisms of MRSA, however, still remain obscure partially due to a lack of knowledge of its metabolic requirements. Systems biology approaches can be extremely useful in predicting and interpreting metabolic phenotypes through genome-scale modeling and bioinformatics approaches [4,5]. However, there is a need for a validated chemically defined minimal medium to further investigate the constraints of the cell. Such constraints and requirements can provide information on functional states of the cell and unveil the underlying mechanisms for growth and pathogenicity.

Susceptibility testing is one of the critical steps in determining proper measures to treat, control and prevent *S. aureus* infections [6]. The major susceptibility testing approach of determining the Minimum Inhibitory Concentration (MIC) is typically performed in rich media such as Mueller-Hinton Broth. However, previous reports have illustrated media dependent alterations in antibiotic activity, bringing into question the *in vivo* relevance of *in vitro* susceptibility testing. The observed media-dependent variations in *S. aureus* antibiotic susceptibility highlights the need for a defined minimal

medium to provide a more consistent nutritional environment [7]. Understanding the relationship between the nutritional environment and antibiotic susceptibility may allow for a better *in vitro* prediction of clinical drug activity.

Several efforts have been undertaken to reveal the nutritional requirements for growth of *S. aureus*. Prior studies by Gladstone have demonstrated the essentiality of ammonia as the main source of nitrogen and suggested that prior cultivation conditions affect future nutritional requirements between *S. aureus* strains [8]. Additionally, prior work has shown the importance of members of the vitamin B complex [9], carbohydrates [10], and the essentiality of various amino acids [11,12] in promoting *S. aureus* growth. AAM, a defined formulation originally implemented for the isolation of amino acid auxotrophs [13], was recently analyzed using bioinformatics [14]. A reduced formulation, AAM^-, was created based on a metabolomic essentiality analysis which removed 5 L-amino acids and 1 vitamin from the original recipe [14]. Although this work has laid the foundation for identifying the metabolic requirements of *S. aureus*, a further reduced defined medium is required for fully understanding the metabolic network. In this work, a chemically defined minimal medium formulation, termed synthetic minimal medium (SMM), adapted from AAM^-, was investigated, modified, and validated to enable growth of three *S. aureus* strains, TCH1516, LAC, and D592 (all MRSA strains). The formulated SSM was utilized in an adaptive laboratory evolution (ALE) experiment to further probe the optimal capabilities of the targeted strains in this nutritional environment and uncover mechanisms underlying the optimized states. The resulting SMM and the evolved strains will serve as important tools for mapping the metabolic network and identification of core metabolic functions of pathogenic *S. aureus*, and consequently will enable improved drug design to tackle the pathogenicity.

Chapter 2

Results

2.1 Development of a Synthetic Minimal Medium for *S. aureus*

A synthetic minimal medium (SMM) was generated through the addition of a suspected growth promoting amino acid to a previously proposed formulation. AMM^-, the reduced defined medium demonstrated to support growth of *S. aureus*, was used as the starting point for the development of the SMM. Valid growth was defined as the capability of a medium to support growth for at least three consecutive transfers in the same conditions, with approximately 3 generations per flask, and reaching a final OD_{600} of 0.25 (Tecan Sunrise plate reader, see Methods). The passaging criterion is essential as maladapted strains will often still display positive growth in the first flask due to nutrient carryover from the starter cultures. Initially, AMM^- alone was found to insufficiently support the growth of multiple MDR USA300 *S. aureus* strains (TCH1516, LAC) beyond the first flask. Further analysis revealed that supplemented L-arginine, which was removed from AAM^-, was essential for growth. Prior studies have proposed an interaction between arginine and proline which facilitates protein synthesis and growth in the presence of glucose, which may serve as the basis for the essentiality of L-arginine in AMM^- [15]. Additional screening for growth of the two USA300 strains in AAM^- plus L-arginine (termed, AAM^{++}) was performed to validate growth. Five independent replicate cultures of both USA300 strains remained viable after three transfers, with an average growth rate of 0.35 hr^{-1} in the final flask.

The essentiality of the components of the modified AAM^{++} formulation was investigated to determine if the medium could be further reduced. Citric acid, L-glutamic acid, L-leucine, L-cysteine, thiamine, nicotinic acid, and calcium pantothenate were selected for further essentiality analysis. Each component was added individually or in combination to the base AAM^{-} media to create a potential candidate medium. This approach yielded a total of 13 possible candidate media formulations (**Table 1**). Of the 13 candidate media formulations, only 4 supported TCH1516 growth. Media that excluded citric acid, L-glutamic acid, and L-Leucine, individually or in combination, demonstrated the capacity to support growth based on the aforementioned selection criteria for validity. Therefore, citric acid, L-glutamic acid, and L-Leucine were determined to be non-essential and removed. The medium (AAM^{++} without the non-essential compounds) was further validated and utilized to characterize growth of TCH1516 in triplicate. Growth was seen in all replicates and an average growth rate of ~0.2 hr^{-1} was observed in the final flask after being passaged three times. The minimal composition which supported the reproducible growth of TCH1516 was termed SMM and is listed in **Table 1**. The USA300 LAC and the USA100 D592 strains were also similarly tested and shown to grow reproducibly in SMM.

Table 1: Composition of the synthetic minimal medium (SMM) and components tested and removed from original recipe.

Ingredient	Amount/Liter
Base:	
KCl	3 g
NaCl	9.5 g
MgSO4·7H2O	1.3 g
(NH4)2SO4	4 g
Tris	12.1 g
Salts:	
CaCl2·2H2O	22 mg
KH2PO4	140 mg
FeSO4·7H2O	6 mg
MnSO4·H2O	10 mg
Supplements:	
Glucose	5 g
L-Arginine	125 mg
L-Proline	200 mg
L-Cysteine*	80 mg
Thiamine*	2 mg
Nicotinic acid*	2 mg
Calcium pantothenate*	2 mg
Components removed:	
Citric Acid**	6 mg
L-Glutamic acid**	250 mg
L-Leucine**	150 mg

*Components tested and confirmed for essentiality
**Components tested and confirmed for nonessentiality

2.2 Adaptive Laboratory Evolution

Adaptive Laboratory Evolution (ALE) utilities inherited selective advantages in a given growth environment to uncover underlying growth-promoting molecular mechanisms. Owing to this capability of cells to naturally adapt to a defined growth environment, robust strains can be produced for use in a number of application areas [16–19]. Thus, an automated cell culture platform was employed [20] to evolve *S. aureus* on SMM in order to generate strains with advanced growth capabilities (i.e.,

faster growth, increased reproducibility) and to explore growth-promoting mutations. Briefly, in this approach, the cultures were consecutively passed in mid-exponential phase for over 30 days (see Methods for more details).

At the beginning of the ALE experiment, there was a surprisingly high degree of variability in the ability of identical replicates to grow on SMM for the three different *S. aureus* strains tested. Multiple replicates were used for each strain as the utility of ALE increases when evolved "endpoint" replicates can be compared on a genetic level to find commonly mutated genes. Specifically, when 10, 10, and 15 replicates were started on SMM for USA300 TCH1516, USA300 LAC, and USA100 D592, only 8, 7, and 3 of the strains grew reproducibly after initial passage attempts, some passed multiple times from long stationary phases. This was a surprising degree of stochasticity that was observed, but not further pursued. The replicates that did grow reproducibility were continued on in the ALE experiment to select for mutants with increased growth rates.

The ALE experiment resulted in observed fitness increases for evolved *S. aureus* strains that grew reproducibly in the SMM culturing conditions. The overall fitness trajectories for the approximately month-long experiment for three replicates of each strain are shown in **Figure 1** (see **Figure S1** for all experiments). The average initial growth rates for the evolved replicates were 0.196 +/- 0.041 hr^{-1} (TCH1516), 0.161 +/- 0.039 (LAC), and 0.013 +/- 0.004 (D592). At the end of the evolutions, the average growth rates for the evolved populations were 0.494 +/- 0.080 (TCH1516), 0.454 +/- 0.075 (LAC), and 0.246 +/- 0.021 (D592), for an increase of 153%, 182%, and 1839%, respectively. Thus, there was a significant increase in growth rate for each of the evolved populations and it appears that most replicates underwent one or two

major jumps in fitness. Clones were isolated from each of the evolved populations from the final flask, i.e., endpoint flask, for further testing and whole genome sequencing.

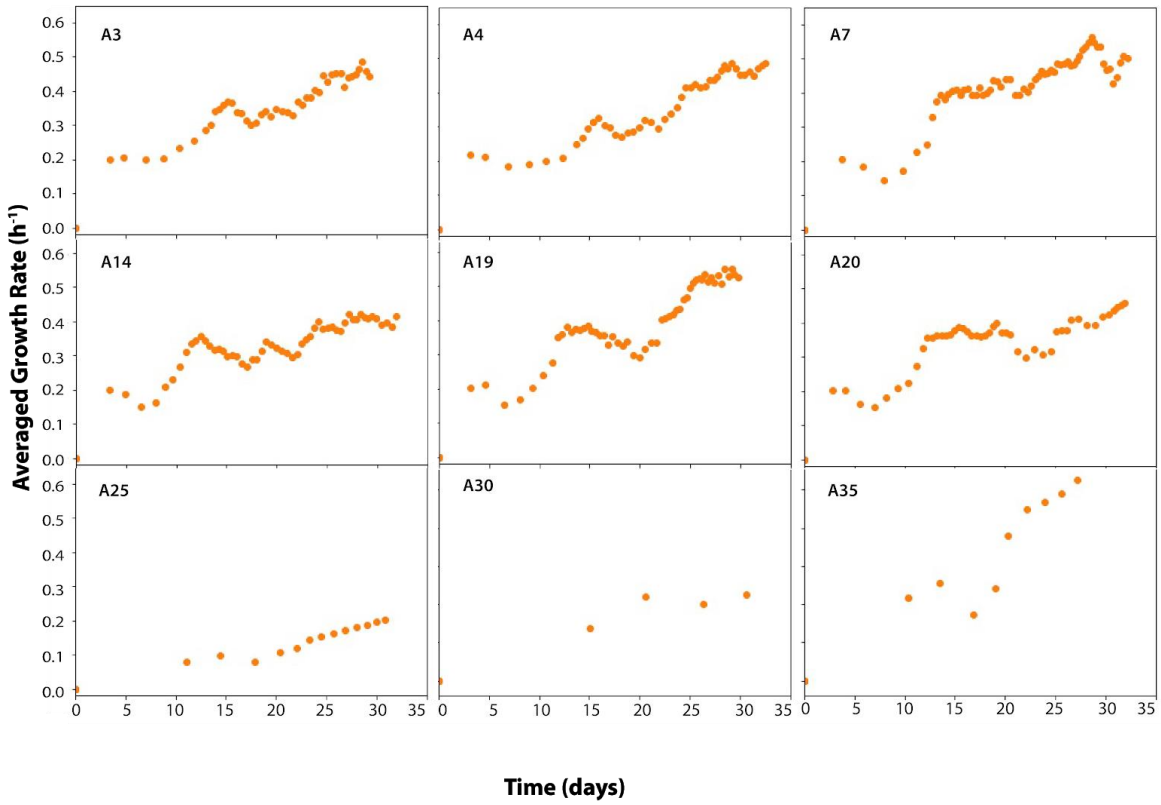


Figure 1. Fitness trajectories of representative replicates for the ALE experiment on SMM. A3, A4, and A7 are three biological replicates of *S. aureus* USA300 LAC. A14, A19, and A20 are three biological replicates of USA300 TCH1516. A25, A30, A35 are three biological replicates of USA100 D592. All strains demonstrated fitness increase over the course of the experiment. The endpoint clones were used for physiological characterizations and both clones and populations were sequenced to understand the genetic basis for increased growth.

2.3 Phenotypic Characterization of the ALE Adapted Strains

Representative isolates from the endpoint flasks were selected for further phenotypic and physiologic characterization. This process included determining the growth rate, glucose uptake rate (GUR), acetate secretion rate (ASR), and the lactase secretion rate (LSR). Three independently evolved isolates of MDR *S. aureus* strains LAC and TCH1516 were grown and characterized in duplicate. The averaged growth rates were 0.53 +/- 0.004, 0.60 +/- 0.02 and 0.54 +/- 0.01 h^{-1} for the LAC strains (A3,

A4, A7, respectively), and 0.61 +/- 0.10, 0.56 +/- 0.01, and 0.50 +/- 0.06 h^{-1} for the TCH1516 (A14, A19, A20 respectively) (**Figure 2**). Compared with the averaged endpoint population growth rates, the clonal growth rates closely match with 8% and 14% relative standard deviation (RSD) for TCH1516 and LAC, respectively. The GURs, ASRs, and LSRs were calculated and the corresponding GURs, ASRs, and LSRs are reported in **Figure 3**. The averaged GUR was 5.99 +/- 0.38 mmol gDW^{-1} h^{-1} for the LAC strains and 4.89 +/- 0.60 mmol gDW^{-1} h^{-1} for the TCH1516 strains. The averaged ASR is 5.42 +/- 0.25 mmol gDW^{-1} h^{-1} for the LAC strains and 4.84 +/- 0.61 mmol gDW^{-1} h^{-1} for the TCH1516 strains. The averaged LSR is 0.53 +/- 0.23 mmol gDW^{-1} h^{-1} for the LAC strains and 0.36 +/- 0.10 mmol gDW^{-1} h^{-1} for the TCH1516 strains.

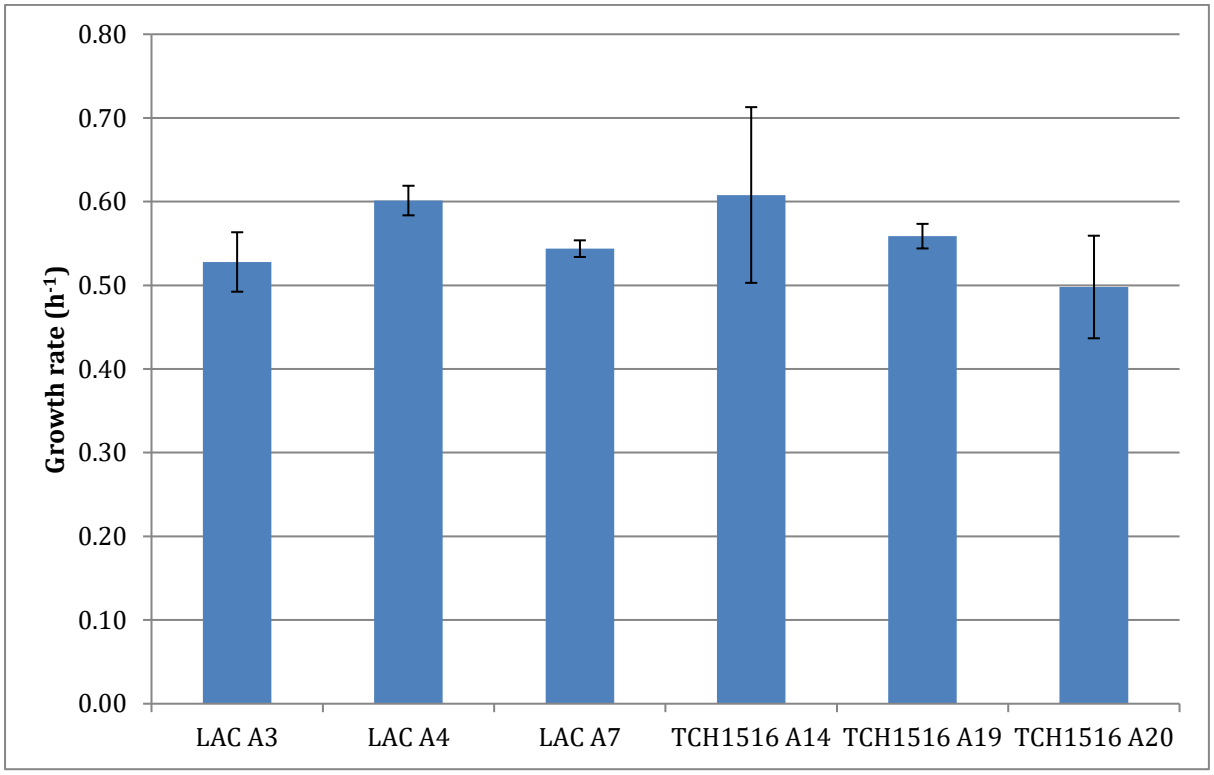
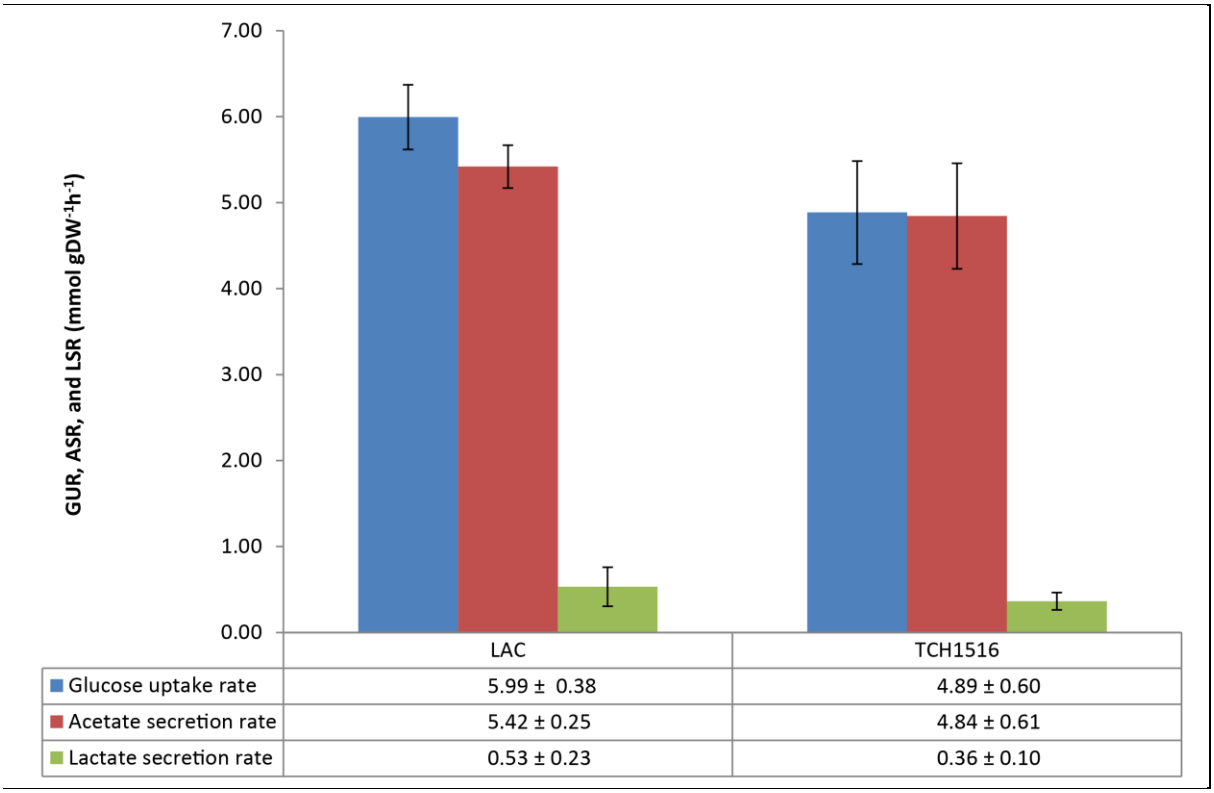


Figure 2. Growth rates of the representative evolved clones of USA300 LAC (A3, 4, 7) and TCH1516 (A14, 19, 20) strains. All of the selected clones possess a comparable growth rate with the endpoint growth rates calculated from populations at the end of the ALE experiments.



	LAC	TCH1516
Glucose uptake rate	5.99 ± 0.38	4.89 ± 0.60
Acetate secretion rate	5.42 ± 0.25	4.84 ± 0.61
Lactate secretion rate	0.53 ± 0.23	0.36 ± 0.10

Figure 3. Phenotypic characteristics of the evolved LAC (A4) and TCH1516 (A19) clones. The glucose uptake rates (GURs) and the acetate secretion rates (ASRs) are roughly equivalent across both strains. The lactate secretion rates (LSRs) are relatively low compared, but detectable.

Evolved clones from each of the USA300 strains were selected for MIC testing using various antibiotics in SMM and another relevant antibiotic susceptibility testing media, RPMI+10%LB [21]. RPMI+10% LB has previously been demonstrated to be a more relevant physiological medium due to it more closely mimicking the nutritional environment of the human host [22]. The drugs selected to assess each strains antibiotic sensitivity included Ampicillin (AMP), Azithromycin (AZM), Linezolid (LNZ) and Nafcillin (NAF) (**Table 2**). Overall, there was no significant differences in antibiotic susceptibility when comparing wild-type strains with the SMM adapted strains in RPMI+10%LB (**Table 2**). This similarly implies that the mutations present in the evolved strains do not impact susceptibility on the physiological RPMI+10%LB media. However, when cultivated in SMM, there is a significant shift in the

susceptibility to AZM, with an approximately 32-fold MIC increase compared to RPMI+10%LB for both the evolved A7 and A19 clones. The strain independent differences in antibiotic susceptibility implies that the media conditions impact susceptibility more than the genetic differences between the strains.

Table 2. Comparison of Minimal inhibitory concentrations (MICs) between the wild-type strains in rich media (RPMI+10% LB) and one of each adapted strain in both the rich media and SMM. The antibiotics tested here include ampicillin (AMP), azithromycin (AZI), linezolid (LINE) and nafcillin (NAF) The table at the top shows the MICs of the LAC strains (wild-type and A7). The table at the bottom shows the MICs of the TCH1516 strains (wild-type and A19).

AMP (ug/mL)	**0.000**	**0.031**	**0.063**	**0.125**	**0.250**	**0.500**	**1.000**	**2.000**	**4.000**	**8.000**	**16.000**	**32.000**
LAC RPMILB	0.72	0.49	0.41	0.34	0.21	0.05	0.05	0.05	0.05	0.05	0.05	0.05
A7 RPMILB	0.76	0.53	0.44	0.40	0.05	0.04	0.04	0.04	0.04	0.04	0.04	0.05
A7 SMM	0.38	0.28	0.32	0.28	0.15	0.07	0.05	0.04	0.04	0.04	0.04	0.04
AZM (ug/mL)	**0.000**	**0.004**	**0.008**	**0.016**	**0.031**	**0.063**	**0.125**	**0.250**	**0.500**	**1.000**	**2.000**	**4.000**
LAC RPMILB	0.64	0.40	0.06	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
A7 RPMILB	0.65	0.22	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
A7 SMM	0.28	0.37	0.41	0.40	0.36	0.34	0.24	0.07	0.05	0.04	0.04	0.04
LNZ (ug/mL)	**0.000**	**0.125**	**0.250**	**0.500**	**1.000**	**2.000**	**4.000**	**8.000**	**16.000**	**32.000**	**64.000**	**128.000**
LAC RPMILB	0.71	0.64	0.61	0.45	0.20	0.05	0.05	0.05	0.05	0.05	0.04	0.04
A7 RPMILB	0.97	0.63	0.58	0.49	0.21	0.05	0.05	0.05	0.05	0.05	0.05	0.04
A7 SMM	0.27	0.29	0.24	0.13	0.07	0.04	0.04	0.04	0.04	0.04	0.04	0.04
NAF (ug/mL)	**0.000**	**0.016**	**0.031**	**0.063**	**0.125**	**0.250**	**0.500**	**1.000**	**2.000**	**4.000**	**8.000**	**16.000**
LAC RPMILB	0.95	0.94	0.88	0.57	0.45	0.12	0.05	0.05	0.05	0.05	0.05	0.05
A7 RPMILB	0.82	0.68	0.62	0.59	0.53	0.12	0.05	0.05	0.05	0.05	0.04	0.05
A7 SMM	0.76	0.65	0.57	0.26	0.38	0.05	0.04	0.04	0.04	0.04	0.04	0.04
AMP (ug/mL)	**0.000**	**0.500**	**1.000**	**2.000**	**4.000**	**8.000**	**16.000**	**32.000**	**64.000**	**128.000**	**256.000**	**512.000**
TCH RPMI LB	0.86	0.61	0.57	0.56	0.54	0.55	0.51	0.04	0.04	0.04	0.04	0.05
A19 RPMI LB	0.81	0.43	0.43	0.41	0.43	0.04	0.04	0.04	0.04	0.04	0.04	0.04
A19 SMM	0.66	0.27	0.33	0.35	0.33	0.33	0.29	0.14	0.04	0.04	0.04	0.04
AZM (ug/mL)	**0.000**	**0.031**	**0.063**	**0.125**	**0.250**	**0.500**	**1.000**	**2.000**	**4.000**	**8.000**	**16.000**	**32.000**
TCH RPMI LB	0.99	0.61	0.55	0.50	0.24	0.04	0.04	0.04	0.04	0.04	0.04	0.05
A19 RPMI LB	0.67	0.41	0.40	0.39	0.09	0.04	0.04	0.04	0.04	0.04	0.04	0.04
A19 SMM	0.49	0.35	0.31	0.28	0.24	0.45	0.52	0.41	0.31	0.25	0.07	0.04
LNZ (ug/mL)	**0.000**	**0.125**	**0.250**	**0.500**	**1.000**	**2.000**	**4.000**	**8.000**	**16.000**	**32.000**	**64.000**	**128.000**
TCH RPMI LB	1.03	0.63	0.54	0.47	0.32	0.07	0.05	0.04	0.04	0.04	0.04	0.04
A19 RPMI LB	0.79	0.36	0.30	0.30	0.09	0.05	0.04	0.04	0.04	0.04	0.04	0.04
A19 SMM	0.66	0.27	0.31	0.22	0.09	0.04	0.04	0.04	0.04	0.04	0.04	0.04
NAF (ug/mL)	**0.000**	**0.016**	**0.031**	**0.063**	**0.125**	**0.250**	**0.500**	**1.000**	**2.000**	**4.000**	**8.000**	**16.000**
TCH RPMI LB	0.96	0.61	0.53	0.51	0.50	0.61	0.51	0.05	0.05	0.04	0.04	0.04
A19 RPMI LB	0.78	0.43	0.39	0.38	0.45	0.05	0.05	0.04	0.04	0.04	0.04	0.04
A19 SMM	0.57	0.30	0.29	0.27	0.17	0.04	0.04	0.04	0.04	0.04	0.04	0.04

2.4 Genetic Analysis of the Mutations Presented in the Evolved Strains

Whole genome sequencing of the evolved *S. aureus* strains revealed a high level of parallel evolution between replicates of the same strain, and between the two different evolved USA300 strains. Overall, sequencing revealed a total of 101 observed mutations across all strains, of which 84 are unique (**Table S1**). Of the 84 unique mutations, 21 out of 29 mutations (72%) found in the evolved TCH1516 strains were seen in both population and clonal samples across 7 replicates and 31 out of 46 mutations (67%) found in the evolved LAC strains were observed in both populations and clones across 8 replicates. Mutations were also analyzed for the evolved D592 strains by mapping the reads to the wild-type reference genome to study the required growth-enabling mutations. Interestingly, 13 out of 13 mutations (100%) found in the evolved D592 strains were seen in both populations and clones across 3 replicates.

The 85 unique mutations mapped to 23 key genes/genetic regions (**Table 3**). A key gene is defined as one that mutated across multiple experiments. As shown in **Figure 4**, within the 23 key mutated genes/genetic regions, 3 occurred in D592, 9 in TCH1516 and 14 in LAC. Highly-occurring ($n \geqslant 4$) mutated genes/genetic regions include LAC_H_02272 (closely related to TM_0288), bioD1 (intergenic), yhdG_2, polA_2 in LAC, and USA300HOU_RS13140 (hypothetical protein), MarR, USA300HOU_RS03930 (peptide ABC transporter permease), USA300HOU_RS14110 (amino acid permease) in TCH1516. Mutations in bioW, rsbU, and spoVG appeared in the two evolved USA300 strains, encoding for 6-Carboxyhexonate-CoA ligase, phosphoserine phosphatase, and putative septation protein, respectively. There were no mutated genes shared between all three strains or any between the two USA300 (LAC and TCH1516) and one USA100 (D592)

starting strains. However, there was a high degree of parallelism seen in the USA100 D592 strain as mutations in three genes, tetA_1, codY, and gltA_2 appeared across all evolved replicates.

Table 3. Key mutated genes identified in all three strains and their occurrences. Three shared mutated genes are identified in USA300 strains.

Strain	Gene or Genetic Region	Product and Function	Occurrence
LAC (n = 7)	LAC_H_02272 (TM_0288), bioD1	putative ABC transporter ATP-binding protein/ATP-dependent dethiobiotin synthetase BioD 1	6
	yhdG_2	putative amino acid permease	5
	polA_2	DNA polymerase I, thermostable	4
	spoVG*	Putative septation protein	4
	bioW*	6-carboxyhexanoate--CoA ligase	4
	slyA_1, LAC_H_01002	Transcriptional regulator SlyA/hypothetical protein	3
	rsbU*	Phosphoserine phosphatase	3
	dtpT	Di-/tripeptide transporter	3
	ebpS, LAC_H_00573	Elastin-binding protein EbpS/Glucosaminate ammonia-lyase	3
	ftsH	ATP-dependent zinc metalloprotease FtsH	2
	rpoC	DNA-directed RNA polymerase subunit beta'	2
	rhaS	HTH-type transcriptional activator RhaS	2
	ecfA1	Energy-coupling factor transporter ATP-binding protein	2
	ebh_2	Extracellular matrix-binding protein	1
	speA, recR	Arginine decarboxylase/Recombination protein RecR	1
TCH1516 (n = 8)	spoVG*	Putative septation protein	7
	USA300HOU_RS13140	Hypothetical protein	6
	MarR	MarR family transcriptional regulator. DNA-binding transcription factor activity	6
	bioW*	6-carboxyhexanoate--CoA ligase	5
	USA300HOU_RS03930	peptide ABC transporter permease	5
	rsbU*	Phosphoserine phosphatase	4
	USA300HOU_RS14110	Amino acid permease	4
	SigB	RNA polymerase sigma factor	3
	cbiO	energy-coupling factor transporter ATPase	2
	rrf	5S ribosomal RNA	1
D592 (n = 3)	tetA_1	Tetracycline resistance protein. Response to antibiotic	3
	codY	GTP-Sensing transcriptional pleiotropic repressor	3
	gltA_2	Glutamate synthase [NADPH] large chain	3

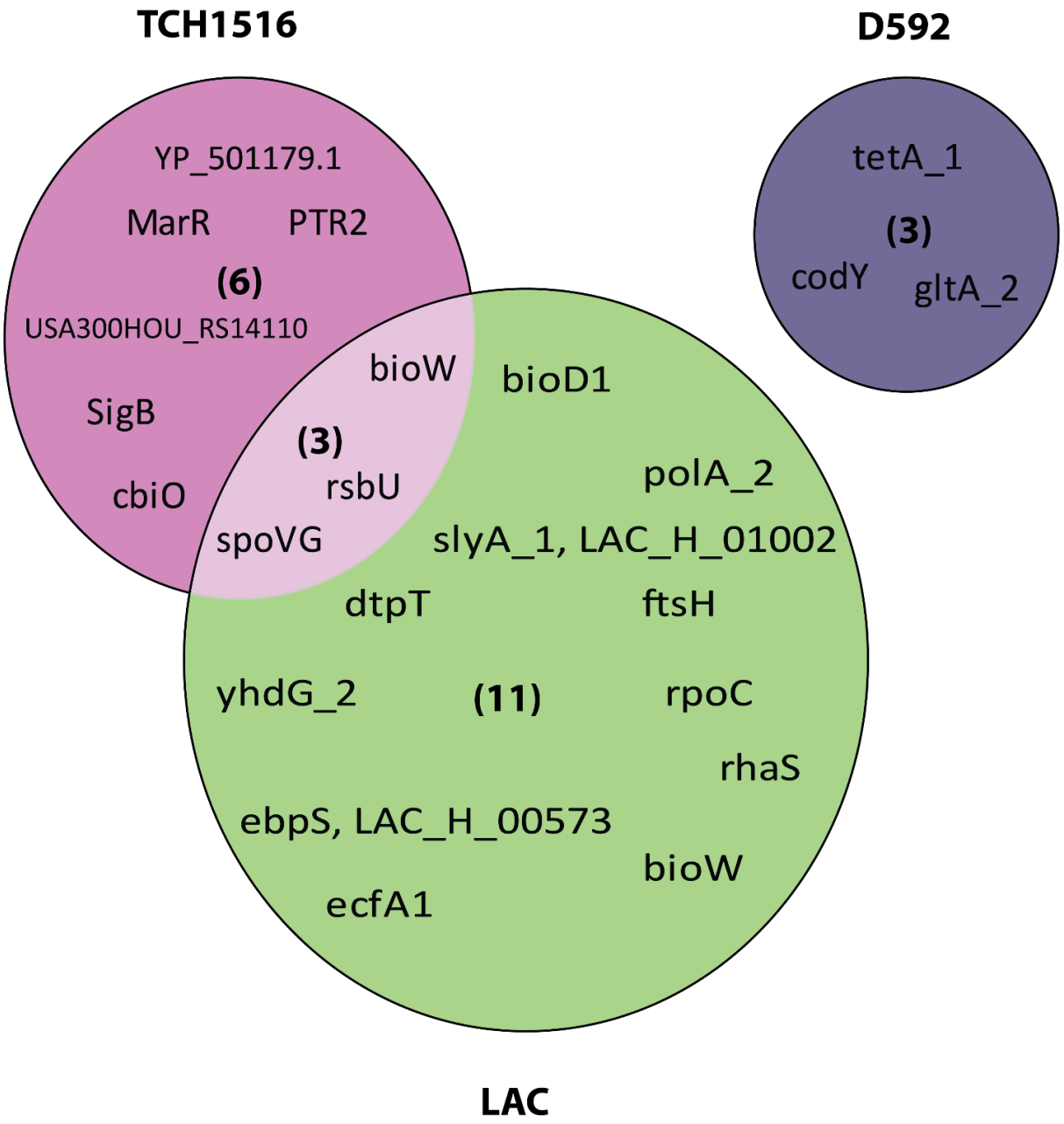


Figure 4. Venn diagram of mutated genes that are present in multiple ALE adapted strains. USA300 LAC and TCH1516 acquired mutations in 6 and 11 unique genes, respectively, and share three key mutated genes. In addition, USA100 D592 acquired mutations in 3 unique genes.

Chapter 3

Materials and Methods

3.1 Preparation of SMM

The components of SMM were prepared in bulk as stock solutions and were split into three categories base, salt mixture, and supplements, and were dissolved in Mili-Q water. The salt mixture includes $FeSO_4 \cdot 7H_2O$ and $MnSO_4 \cdot H_2O$, and was sterilized by vacuum filtration. The base contains KCl, NaCl, $MgSO_4 \cdot 7H_2O$, $(NH_4)_2SO_4$ and Tris, and was autoclaved after adjusting its pH to 7.4 with 4M HCl. The remaining components are the supplements and were prepared and vacuum filtered individually except for L-Arginine and L-Proline, which were autoclaved. All the sterilized solutions were mixed, and vacuum filtered again to form SMM.

3.2 Cell Culture and ALE

The parental *S. aureus* strains were grown on LB agar plates and individual colonies were isolated and inoculated into RPMI + 10% LB. The cultures were cultivated overnight, and 1 mL of each overnight culture were transferred into 15 mL tubes containing SMM at 37 ℃. Cultures were constantly stirred at high speed (1000 rpm) for sufficient aeration and serially transferred once the Optical Density reached between 0.20 and 0.25 (Tecan Sunrise plate reader, equivalent to an OD_{600} between

0.67 and 0.83 with 1 cm light path length), when the cultures were proliferating exponentially. The average duration of the ALE experiments was approximately 34 days and frozen stocks of the endpoint strains were created.

3.3 Physiological Characterizations

The endpoint strains, A3, 4, 7 (LAC) and A14, 19, 20 (TCH1516), were first plated on solid SMM agar plates. After being incubated at 37 ℃ until decent sizes of colonies were observed (~2 days), isolates of each ALE strains were selected and cultured overnight in SMM in duplicate for physiological characterizations. The overnight cultures were diluted to an OD_{600} of 0.1 and the optical density were sampled throughout the cultivation periods. The growth rates (h^{-1}) were calculated by performing linear regression on the log-linear region and the average of the two replicates was determined. At every sampling point, a small portion of the growing cultures were collected and filtered for determinations of metabolites including glucose, acetate and lactate, and their production/secretion rates using high-performance liquid chromatography (HPLC). The filtrates were injected through HPLC column (Aminex HPX-87H Column #125-0140) and analyzed for the concentrations of the metabolites mentioned above by comparing to the concentrations of the standards. The production rates and the secretion rates were then computed by linear regression and were converted into yield (mmol gDW^{-1} h^{-1}).

3.4 Susceptibility Testing

Azithromycin (Fresenius Kabi), and Linezolid (Pfizer) were purchased from a clinical pharmacy. Ampicillin, and nafcillin was purchased from Sigma-Aldrich. All drugs were resuspended in 1x Dubelcco's phosphate-buffered saline (DPBS) (Corning). The bacterial strains to be used in antibiotic susceptibility testing were first streaked on Luria agar plates from stocks stored at -80 °C (in 20% glycerol / 80% MHB) and grown stationary at 37 °C overnight. Isolated colonies were picked from the plate and inoculated into 5 mL of either CA-MHB (MHB (Difco) amended with 20 mg/L Ca^{2+} and 10 mg/L Mg^{2+}) or RPMI+ (phenol free RPMI (Gibco 1640) amended with 10% LB (Criterion)) media in a 14 mL Falcon polypropylene round-bottom snap cap tube (Corning #352059) and grown shaking at 100 rpm at 37 °C overnight. The following day the overnight cultures were sub-cultured 1:50 in the desired medium and volume in either the 14 mL snap cap tubes and grown shaking at 100 rpm at 37 °C until they reached mid-logarithmic phase (~OD_{600}= 0.4). Unless otherwise noted experiments were conducted in Costar flat-bottom 96 well plates (Corning #3370) with a final volume of 200 µl / well. For the MIC experiments the bacteria were cultured in the same media throughout (CA-MHB or RPMI+) prior to the addition of antibiotics. The mid-logarithmic phase cultures were diluted to approximately $5*10^5$ cfu (~OD_{600}= 0.002) and 180 µl was added to each experimental well of the 96 well flat bottom plate (Costar #3370). Either 20 µl of 1x DPBS or 20 µl of the desired 10x drug stock were added into each culture containing well. Plates were then incubated shaking at 100 rpm at 37 °C overnight. Bacterial growth, as determined by measuring the OD_{600} of each well, was determined by utilizing an Enspire Alpha multimode plate reader (PerkinElmer). To determine the MIC_{90}, defined as the amount of drug required to inhibit ≥90% of the growth of the untreated controls, the density of each drug treated well was compared to the untreated control (Chapter 3.4, in full, was written by Dr.

Nicholas Dillon. The thesis author was the primary investigator and author of this paper).

3.5 Next-generation DNA Sequencing and Mutational Analysis

Genomic DNA was isolated using the Zymo Quick-DNA Fungal/Bacterial Miniprep kit. DNA was quantified and quality-controlled using the Qubit dsDNA high-sensitivity assay. Paired-end sequencing libraries were prepared using the KAPA HyperPlus Kit and sequenced using an Illumina NextSeq 500. Reads were QCed and mutations were identified by a bioinformatics pipeline [26]. Reads generated from the USA300 TCH1516 were aligned to a reference TCH1516 genome (NCBI accession NC_010079) while the other two strains (USA300 LAC and USA100 D592) were aligned to *de novo* reference genomes generated in the lab. The output mutations across all three strains were first filtered by a mutation frequency threshold set at 20% followed by manual inspections and filtrations of the mismapped results. All samples were confirmed for an averaged mapped coverage of at least 31X.

Chapter 4

Discussion

In this study, a chemically defined minimal medium (SMM) was explored, redefined, and verified for cultivation of MDR USA300 *S. aureus* strains LAC, TCH1516, and the USA100 strain D592. Initially, slow growth rates and stochasticity in growths were observed for all strains in SMM. The strains were then introduced to an approximately month-long ALE experiment to improve growth rates and adaptabilities in SMM. Starting with 10 and 15 replicates for USA300 strains and USA100 strain respectively, 8 USA300 TCH1516 strains, 7 USA300 LAC strains, and 3 USA100 D592 strains were able to grow consistently throughout the cultivation period. The endpoint evolved strains were stored and phenotypically characterized to determine their growth rate, GUR, ASR, and LSR in SMM. In addition, susceptibility testing (MICs) was performed to compare the impacts of nutritional environments between SMM and RPMI + 10%LB for a variety of antibiotics such as AMP, AZM, LNZ, and NAF. Next-generation sequencing was utilized to reveal the genotypic changes occurring during the adaptation process and key mutations were identified, highlighted, and compared across strains.

A number of interesting findings were revealed from this study. The initial screening of growth in the SMM recipe demonstrated a surprising stochastic growth capability of *S. aureus,* as inconsistent results were observed among all strains examined. This finding hints at a complex process inherent to *S. aureus* in adaptation

to a new environment, requiring *S. aureus* to mutate early in the growth experiments in order to proliferate. Further, the finding that more replicates of the USA300 strains were able to grow than the USA100 strains suggests that there exists a strain-specific behavior which may further provide insights on this stochastic behavior. Phenotypic profiling results also provided specifics on the functional growth models for the evolved strains which would be used to calculate maintenance energies and provide a baseline for nutritional requirements in the defined SMM. The SMM media could also be used to look at more specific relationships between media additives and responses given its minimal components. MIC testing of the evolved strains also provided an interesting differential media-specific behavior. The observed media-dependent differences in AZM susceptibility between strains cultivated in SMM and in RPMI + 10%LB suggests one possibility that the cellular responses activated during cultivation in SMM/RPMI interacted and impaired the efficacy of the antibiotic. Further investigations of the mechanism will be useful in interpreting this phenomenon.

The mutational analysis provides mechanisms required for enhanced growth of these strains in a minimal nutrition environment. Most of the mutated genes are involved in energy transport and nutrient acquisition and some are related to bacterial pathogenesis. This can be highlighted by three key mutated genes that overlap between the USA300 strains, bioW, rsbU, and spoVG. BioW encodes for 6-Carboxyhexonate-CoA ligase [23], which is involved in the biosynthesis of biotin [24]. Biotin is a critical nutrient as it is involved in a number of metabolic bioprocesses such as carboxylation, and more importantly, it is highly demanded by the bacteria during infection [25]. The other two genes in this set are involved in transcriptional regulation. The phosphatase-encoding gene rsbU is known for its role in activation of SigB, an important transcription factor [26,27]. Moreover, it has been observed that spoVG, a

SigB-controlled protein, is a critical factor that impacts on cell wall synthesis and is involved in antibiotic resistance and virulence factor [28,29]. The transcription factor sigB has found to be related to expression of high methicillin resistance and regulated in response to growth phase and environmental stress [30,31].

In summary, a chemically defined minimal medium has been defined and validated. ALE was used to produce robust and faster-growing *S. aureus* strains for phenotypic profiling and MIC testing and to understand mechanisms at work to enable optimal growth. Further investigation of the strains and specific results presented here are warranted, but the strains and media compositions provide valuable tools for the study of this important pathogen.

(This paper is also co-authored by Dr. Adam M. Feist. The thesis author was the primary investigator and author of this paper).

Appendix A

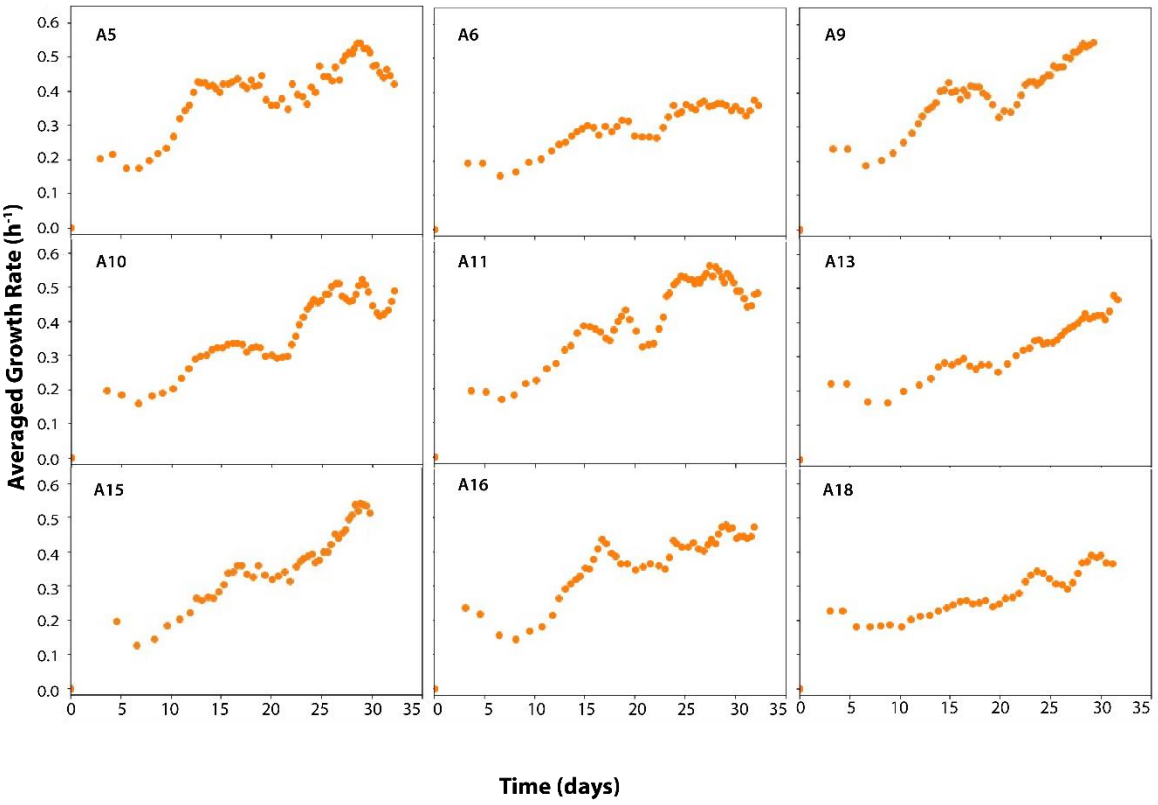


Figure S1. Fitness trajectories of additional replicates for the ALE experiment on SMM. A5, A6, A9, and A10 are three biological replicates of *S. aureus* USA300 LAC. A11, A13, A15, A16, and A18 are five biological replicates of USA300 TCH1516. A25, A30, A35 are three biological replicates of USA100 D592. All strains demonstrated fitness increase over the course of the experiment. The endpoint clones were used for physiological characterizations and both clones and populations were sequenced to understand the genetic basis for increased growth.

Table S1. All key mutations identified in all three *S.A.* strains.

Gene/Protein	Mutation	n (Population)/n (Clone)	Strain/Exprt
bioW	*231K (TAG→AAG)	5/1	LAC A10, TCH1516 A16, A11, A15, A19x2
bioW	*231W (TAG→TGG)	2/1	LAC A7, TCH1516 A16X2
bioW	coding (693/693 nt)	1/1	TCH1516 A18x2
bioW	coding (689-691/693 nt)Δ3 bp	0/1	LAC A3
bioW, LAC_H_02278	intergenic (+3/-8)	1/0	LAC A5
spoVG	coding (75/303 nt)	2/1	TCH1516 A11, LAC A9x2
spoVG	P63T (CCT→ACT)	1/2	LAC A10, TCH1516 A20x2
spoVG	S66* (TCA→TAA)	1/1	TCH1516 A13x2
spoVG	R7* (AGA→TGA)	1/1	TCH1516 A15x2
spoVG	P48T (CCA→ACA)	1/1	LAC A5x2
spoVG	P48L (CCA→CTA)	1/1	LAC A4x2
spoVG	L8H (CTT→CAT)	1/1	TCH1516 A14x2
spoVG	K50I (AAA→ATA)	1/1	TCH1516 A19x2
spoVG	A61P (GCG→CCG)	1/1	TCH1516 A16x2
rsbU	Y135* (TAT→TAA)	1/1	TCH1516 A14x2
rsbU	Q92* (CAA→TAA)	1/1	LAC A6
rsbU	Q115* (CAA→TAA)	1/1	TCH1516 A19x2
rsbU	coding (963/1002 nt)	1/1	LAC A7
rsbU	A220T (GCT→ACT)	1/1	LAC A3
rsbU	coding (703/1002 nt)	1/0	TCH1516 A11
rsbU	A220G (GCT→GGT)	1/0	TCH1516 A13
cbiO	E2V (GAG→GTG)	0/1	TCH1516 A14
ecfA1/cbiO	E2E (GAG→GAA)	1/1	LAC A7, TCH1516 A15
ecfA1/cbiO	F23L (TTC→TTG)	1/0	LAC A5
nrdI, ribN	intergenic (-511/-289)	7/7	All 7 LAC strains (Population + clone)
leuA_1	N20K (AAT→AAA)	7/7	All 7 LAC strains (Population + clone)
gltA_2	*675Q (TAA→CAA)	2/2	D592 A30x2, A35x2
gltA_2	*675K (TAA→AAA)	1/1	D592 A25x2
patA_2	I334F (ATC→TTC)	3/3	All 3 D592 strain (Population + clone)
pflB	E341G (GAA→GGA)	3/3	All 3 D592 strain (Population + clone)
codY	T96I (ACA→ATA)	1/1	D592 A25x2
codY	G239S (GGT→AGT)	1/1	D592 A35x2
codY	coding (487-546/774 nt)	1/1	D592 A30x2

Table S1. (Continued)

SAV1028	L22V (CTT→GTT)	3/3	All 3 D592 strain (Population + clone)
tetA_1	coding (839-898/1167 nt)	1/1	D592 A25x2
tetA_1	coding (44/1167 nt)	1/1	D35 A35x2
tetA_1	coding (170-298/1167 nt)	1/1	D592 A30x2
KJJPFECL_01783	coding (4/183 nt)	3/3	All 3 D592 strain (Population + clone)
yfmJ	D282A (GAT→GCT)	3/3	All 3 D592 strain (Population + clone)
yhdG_2	R410C (CGT→TGT)	1/0	LAC A7
yhdG_2	L369P (CTT→CCT)	1/0	LAC A7
yhdG_2	W303* (TGG→TGA)	0/1	LAC A9
yhdG_2	S174* (TCA→TAA)	0/1	LAC A3
yhdG_2	L402F (TTA→TTT)	0/1	LAC A6
yhdG_2	A73V (GCT→GTT)	0/1	LAC A4
yhdG_2	A365T (GCA→ACA)	0/1	LAC A6
LAC_H_02272, bioD1	intergenic (+383/-69)	3/3	LAC A4x2, A7x2, A9x2
LAC_H_02272, bioD1	intergenic (+404/-48)	2/1	LAC A5x2, A10
LAC_H_02272, bioD1	intergenic (+397/-55)	0/1	LAC A10
LAC_H_02272, bioD1	intergenic (+390/-62)	0/1	LAC A10
slyA_1, LAC_H_01002	intergenic (-15/-138)	4/3	LAC A4x2, A5x2, A9x2
polA_2	R616I (AGA→ATA)	3/2	LAC A10, A4x2, A9x2
polA_2	coding (1574/1962 nt)	1/1	LAC A5x2
ftsH	P323L (CCA→CTA)	1/1	LAC A6x2
ftsH	K687N (AAA→AAT)	1/1	LAC A3x2
ftsH	coding (2062/2094 nt)	1/1	LAC A3
ftsH	T36S (ACA→TCA)	1/0	LAC A6
ebpS, LAC_H_00573	intergenic (+77/-325)	3/2	LAC A4x2, A9x2, A10
dtpT	I351F (ATT→TTT)	1/3	LAC A10x2, A5, A9
dtpT	S6S (TCC→TCG)	1/1	LAC A5x2
rpoC	R259G (CGT→GGT)	1/1	LAC A5x2
rpoC	A1177V (GCT→GTT)	1/1	LAC A5x2
rpoC	G247E (GGA→GAA)	0/1	LAC A10
rhaS	R377I (AGA→ATA)	2/2	LAC A5x2, A7x2
lspA, LAC_H_00861	intergenic (-306/-205)Δ59 bp	2/0	LAC A5, A9
SigB	W19G (TGG→GGG)	1/1	TCH1516 A16x2
SigB	R241L (CGA→CTA)	1/1	TCH1516 A18x2

Table S1. (Continued)

SigB	coding (438-440/771 nt)	1/1	TCH1516 A15x2
SigB	R241G (CGA→GGA) ‡	1/0	TCH1516 A18
MarR	V43F (GTC→TTC)	1/1	TCH1516 A15x2
MarR	R86P (CGT→CCT)	1/1	TCH1516 A19x2
MarR	L44* (TTA→TAA)	1/1	TCH1516 A16x2
MarR	K74I (AAA→ATA)	1/1	TCH1516 A14x2
MarR	R92L (CGT→CTT)	1/0	TCH1516 A13
MarR	D65Y (GAT→TAT)	1/0	TCH1516 A11
MarR	coding (79/444 nt)	1/0	TCH1516 A11
USA300HOU_RS14110	G91D (GGT→GAT)	1/1	TCH1516 A19
USA300HOU_RS14110	A335T (GCA→ACA)	1/1	TCH1516 A15
USA300HOU_RS14110	W111R (TGG→AGG)	0/1	TCH1516 A14
USA300HOU_RS14110	S351* (TCA→TAA)	0/1	TCH1516 A16
YP_501179.1	*56L (TAA→TTA)	3/3	TCH1516 A15x2, A16x2, A20x2
YP_501179.1	E51D (GAA→GAC)	2/2	TCH1516 A13x2, A14x2
YP_501179.1	coding (166-167/168 nt)	1/1	TCH1516 A19x2
YP_501179.1	A54S (GCG→TCG)	1/1	TCH1516 A20x2
USA300HOU_RS03930	I351F (ATT→TTT)	5/2	TCH1516 A11, A14, A16, A19x2, A20x2

‡: Initiation codon

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