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### Authors

Bosi, Emanuele  
Monk, Jonathan M  
Aziz, Ramy K  
et al.

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# Comparative genome-scale modelling of *Staphylococcus aureus* strains identifies strain-specific metabolic capabilities linked to pathogenicity

Emanuele Bosi<sup>a,1</sup>, Jonathan M. Monk<sup>b,1</sup>, Ramy K. Aziz<sup>c</sup>, Marco Fondi<sup>a</sup>, Victor Nizet<sup>d,e</sup>, and Bernhard Ø. Palsson<sup>b,d,2</sup>

<sup>a</sup>Department of Biology, University of Florence, I-50019 Sesto Fiorentino, Italy; <sup>b</sup>Department of Bioengineering, University of California, San Diego, La Jolla, CA 92093-0412; <sup>c</sup>Department of Microbiology and Immunology, Faculty of Pharmacy, Cairo University, 11562 Cairo, Egypt; <sup>d</sup>Department of Pediatrics, University of California, San Diego, La Jolla, CA 92093-0760; and <sup>e</sup>Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California, San Diego, La Jolla, CA 92093-0760

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*Staphylococcus aureus* is a preeminent bacterial pathogen capable of colonizing diverse ecological niches within its human host. We describe here the pangenome of *S. aureus* based on analysis of genome sequences from 64 strains of *S. aureus* spanning a range of ecological niches, host types, and antibiotic resistance profiles. Based on this set, *S. aureus* is expected to have an open pangenome composed of 7,411 genes and a core genome composed of 1,441 genes. Metabolism was highly conserved in this core genome; however, differences were identified in amino acid and nucleotide biosynthesis pathways between the strains. Genome-scale models (GEMs) of metabolism were constructed for the 64 strains of *S. aureus*. These GEMs enabled a systems approach to characterizing the core metabolic and panmetabolic capabilities of the *S. aureus* species. All models were predicted to be auxotrophic for the vitamins niacin (vitamin B3) and thiamin (vitamin B1), whereas strain-specific auxotrophies were predicted for riboflavin (vitamin B2), guanosine, leucine, methionine, and cysteine, among others. GEMs were used to systematically analyze growth capabilities in more than 300 different growth-supporting environments. The results identified metabolic capabilities linked to pathogenic traits and virulence acquisitions. Such traits can be used to differentiate strains responsible for mild vs. severe infections and preference for hosts (e.g., animals vs. humans). Genome-scale analysis of multiple strains of a species can thus be used to identify metabolic determinants of virulence and increase our understanding of why certain strains of this deadly pathogen have spread rapidly throughout the world.

systems biology | mathematical modeling | pathogenicity | core genome | pangenome

The preeminent gram-positive bacterial pathogen, *Staphylococcus aureus*, is capable of colonizing diverse ecological niches within its human host, including the respiratory tract, skin, and nasal passages. As a species it possesses several immune resistance and evasion factors, toxins, and invasiveness mechanisms. As a result, *S. aureus* is a leading cause of skin and soft tissue infections, pneumonia, sepsis, and endocarditis. In recent years, clinical management of this leading pathogen has been complicated by its continuous acquisition of resistance to front-line antibiotics (1). Despite this deadly status, broad diversity exists among strains within this species. Some strains are present as asymptomatic colonizers of the human nose (2), others cause skin and soft tissue infections, and some can cause life-threatening and severe disease (3). One strain, known as USA300, has replaced other strains of *S. aureus* to become the predominant cause of methicillin-resistant *S. aureus* (MRSA) infections in the United States (4), and its prevalence is increasing rapidly worldwide (5). Thus, it is important to understand the genetic factors that allow some strains to spread aggressively, whereas others exist asymptotically.

The epidemiology of *S. aureus* and phenotypic diversity present in different strains is reflected in their genotypes. Therefore, it may be

possible to analyze multiple *S. aureus* genomes to discover factors that could be predictors of disease phenotypes and virulence capabilities. Unfortunately, classical genotyping methodologies used today in the clinic such as pulsed-field gel electrophoresis (PFGE) and multilocus sequence tags (MLST) rely on the evaluation of highly conserved housekeeping genes representative of the vertical gene pool that do not provide sufficient resolution for prediction of disease phenotypes (6). Due to the revolution in DNA sequencing technologies, hundreds of full *S. aureus* genome sequences are now available and can be analyzed using new methods. One such method is the analysis of shared and unique genes, within a species, termed the “pangenome.” A bacterial species can be effectively described by its pangenome (7), which can be divided into the core genome (genes shared by genomes of all strains in the species and that are likely to encode functions related to basic cellular biology) and the dispensable genome [genes present in some, but not all, of the representatives of a species (8)]. The dispensable genome includes functions that confer specific advantages under particular environmental conditions, such as adaptation to distinct niches, antibiotic resistance, and the ability to colonize new hosts.

Despite many studies focusing on *S. aureus* genomics, molecular epidemiology, and mechanisms of drug resistance and cytotoxicity, there are relatively few studies that examine *S. aureus* basic biochemistry and metabolic function on a genome scale. Metabolic

## Significance

Comparative analysis of multiple strains within a species is a powerful way to uncover pathoadaptive genetic acquisitions. Hundreds of genome sequences are now available for the human pathogen *Staphylococcus aureus*, mostly known for its antibiotic-resistant variants that threaten the emergence of panresistant superbugs. In this study, genome-scale models of metabolism are used to analyze the shared and unique metabolic capabilities of this pathogen and its strain-specific variants. The models are used to distinguish *S. aureus* strains responsible for severe infections based solely on growth capabilities and presence of different virulence factors. The results identify metabolic similarities and differences between *S. aureus* strains that provide insights into the epidemiology of *S. aureus* and may help to combat its spread.

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<sup>1</sup>E.B. and J.M.M. contributed equally to this work.

<sup>2</sup>To whom correspondence should be addressed. Email: palsson@ucsd.edu.

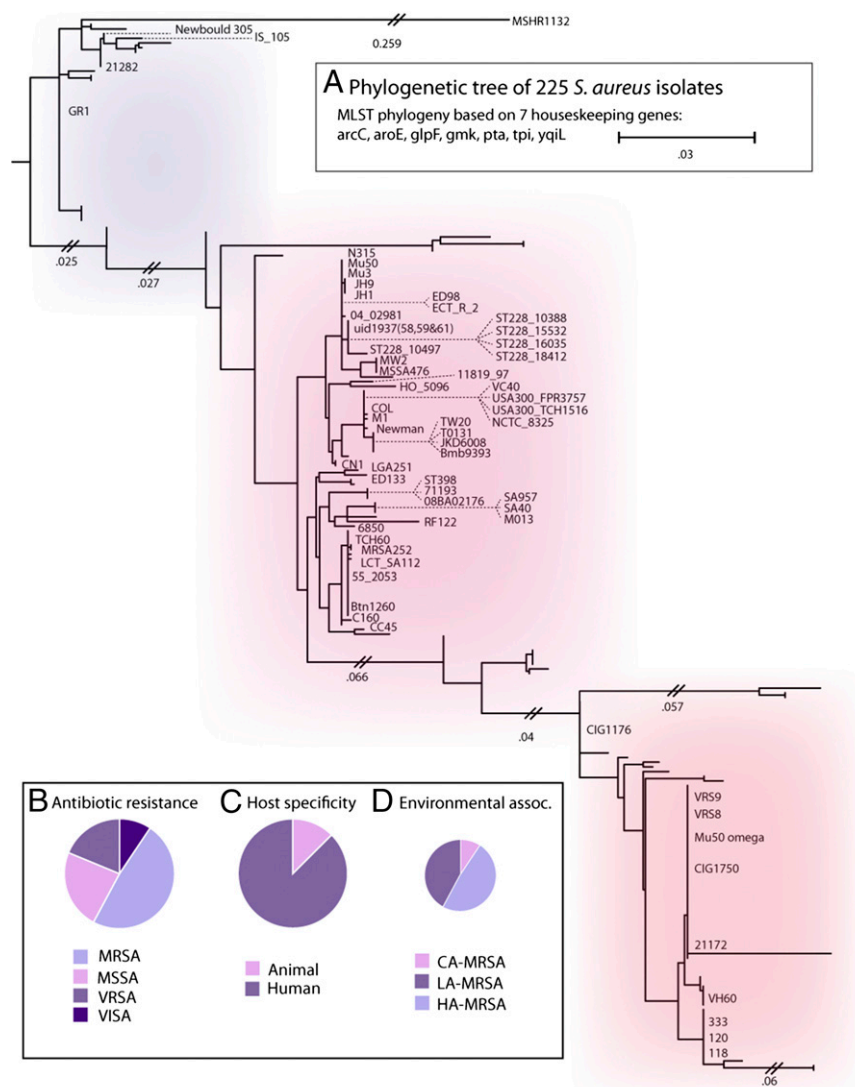
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network reconstructions have proven to be powerful tools to probe the genetic diversity of metabolism between organisms (9) and among strains within a species (10, 11). As useful as genome annotation is, it does not provide an understanding of the integrated function of gene products to produce phenotypic states. Becker et al. provided a well-curated genome-scale model of *S. aureus* N315 (named iSB619) that represented the first biochemically, genomically, and genetically structured knowledge base for *S. aureus* metabolism, and several updates of the model for this strain have followed (12–14). However, knowledge from one strain is never sufficient to represent an entire species. Currently, there are 48 complete genome sequences available for *S. aureus* strains in the National Center for Biotechnology Information (NCBI) Genome database (15) with an additional 450 draft sequences for comparison, which provide comprehensive insight into the pangenome of the *S. aureus* species. In this study we set out to construct the pangenome of the *S. aureus* species and to build GEMs of strains that represent the breadth of genetic, phenotypic, and pathogenic characteristics within this

species. Together, the genomic sequences and the GEMs provide two different and complementary ways to explore diversity within the *S. aureus* species and to compare core genomic vs. pangenomic functionality and metabolic capabilities. Our integration of genomic and biochemical data illustrates the dynamic evolution of *S. aureus* strains, and the results provide insights into the metabolic determinants of pathogenicity.

## Results

**Characteristics of the *S. aureus* Core Genome and Pangenome.** A set of publicly available *S. aureus* genome sequences was downloaded from the NCBI including 48 completely assembled genomes (16). From this set, a phylogenetic tree was constructed using the concatenated sequence of seven conserved housekeeping genes (*arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yqiL*) commonly used to define clonal complexes in clinical studies of *S. aureus* using the MLST approach (Fig. 1A). The most distantly related strain was the Australian *S. aureus* isolate MSHR1132 belonging to the clonal complex 75 lineage (17). Next, a representative set of 64 *S. aureus*



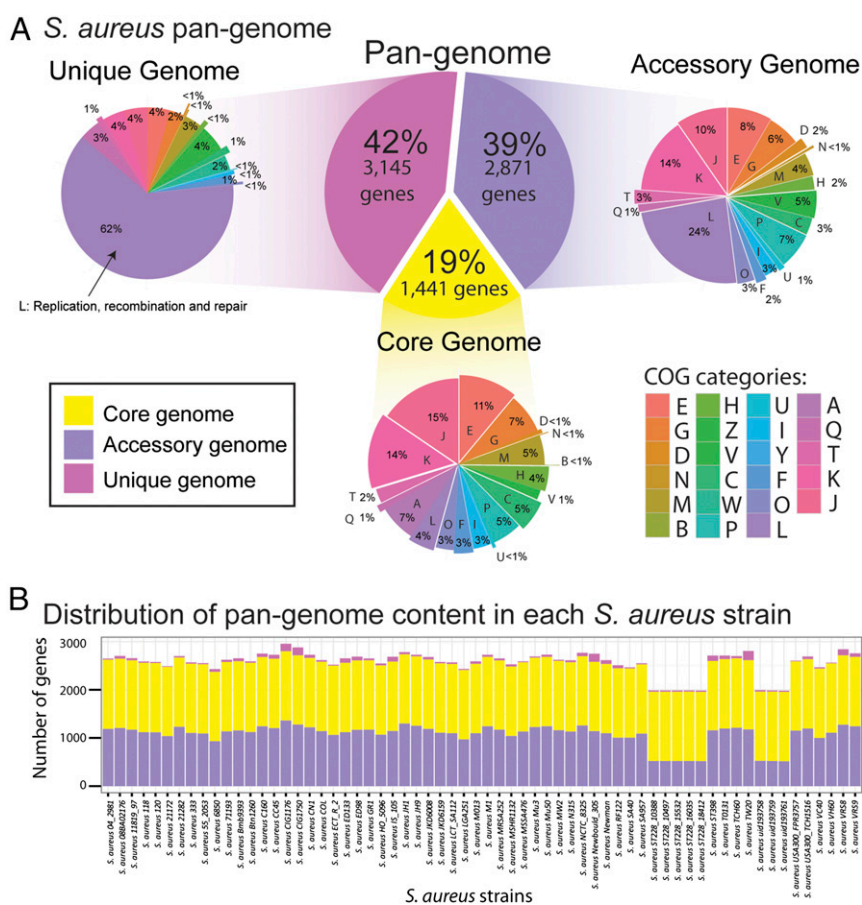
**Fig. 1.** *S. aureus* dataset construction. (A) Phylogenetic tree of 225 *S. aureus* genomes based on seven housekeeping genes (*arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yqiL*). A set of 64 strains (labeled) were selected from this set to create a heterogeneous dataset of *S. aureus* strains based on the evolutionary distance (tree topology), as well as (B) drug resistance (MRSA, MSSA, VRSA, and VISA), (C) host specificity (human vs. animal), and (D) virulence/environmental association (CA-MRSA, community-associated MRSA; HA-MRSA, healthcare-acquired MRSA; LA-MRSA, livestock-associated MRSA). Evolutionary distance is based on tree topology.

strains (Dataset S1) were filtered from this larger dataset based on four criteria: (i) drug resistance [MRSA, methicillin-sensitive *S. aureus* (MSSA), vancomycin-resistant *S. aureus* (VRSA), and vancomycin-intermediate *S. aureus* (VISA)] (Fig. 1B), (ii) host specificity (human vs. animal) (Fig. 1C), and (iii) epidemiological source [community-associated MRSA (CA-MRSA), healthcare-acquired MRSA (HA-MRSA), and livestock-associated MRSA (LA-MRSA)] (Fig. 1D). Thus, the topology of the tree coupled with clinical and epidemiological phenotypic information allowed for development of a heterogeneous dataset spanning representative strains of the *S. aureus* species.

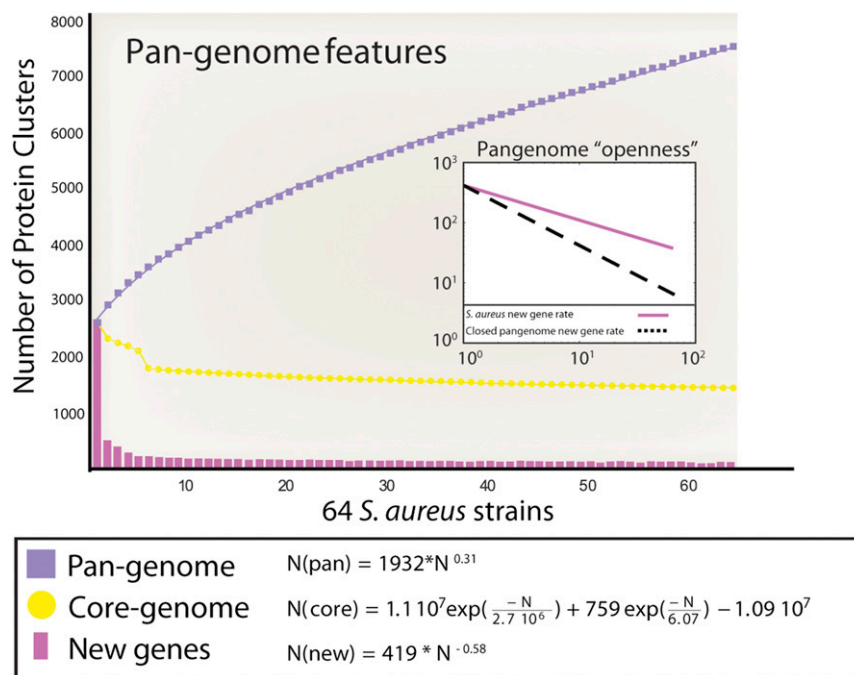
We used these 64 strains to construct the *S. aureus* pangenome that can be used to describe this bacterial species. The pangenome is divided into three sections: (i) the core genome (the set of genes shared by virtually all strains in a species), (ii) the accessory genome (the set of genes present in some, but not all, representatives of a species), and (iii) the unique genome (genes unique to individual members of the species). Because the pangenome is made up of thousands of genes, it offers a much higher resolution for strain typing compared with genotyping techniques such as MLST (18).

The 64 *S. aureus* strains examined yielded a pangenome size of 7,457 genes. Based on this dataset, the core genome is composed of 1,441 genes, the accessory genome is composed of 2,871 genes, and the unique genome is composed of 3,145 genes (Fig. 2A). Functional annotation of genes in the pangenome performed using the Clusters of Orthologous Groups (COG) database (19) revealed a unique distribution of functional categories among the three different pangenome sets. When genes were classified into metabolic and nonmetabolic, the largest fraction of the core genome consisted of genes with metabolic functions (58%). These included those involved in central metabolism including glycolysis/gluconeogenesis, the pentose phosphate pathway, and TCA cycle. A much lower fraction of metabolic gene content is observed in the accessory and unique genome (33% and 18%, respectively).

The size of the pangenome and its increase in size upon addition of new strains can be used to predict the future rate of discovery of novel genes in a species. We used a sampling approach to obtain a set of randomly permuted *S. aureus* pangenomes (with specific breakdowns of total genes, shared genes, and new genes). By fitting a double exponential decay function to



**Fig. 2.** *S. aureus* pangenome statistics. (A) The *S. aureus* pangenome can be subdivided into three categories: (i) the core genome (the set of genes shared by all genomes), (ii) the accessory genome (the set of genes present in some but not all genomes), and (iii) the unique genome (genes that are unique to a single genome). The function of each gene in a group is classified using COGs. COG categories are as follows: For cellular processes and signaling, D is cell cycle control, cell division, and chromosome partitioning; M is cell wall/membrane/envelope biogenesis; N is cell motility; O is posttranslational modification, protein turnover, and chaperones; T is signal transduction mechanisms; U is intracellular trafficking, secretion, and vesicular transport; V is defense mechanisms; W is extracellular structures; Y is nuclear structure; and Z is cytoskeleton. For information storage and processing, A is RNA processing and modification; B is chromatin structure and dynamics; J is translation, ribosomal structure, and biogenesis; K is transcription; and L is replication, recombination, and repair. For metabolism, C is energy production and conversion; E is amino acid transport and metabolism; F is nucleotide transport and metabolism; G is carbohydrate transport and metabolism; H is coenzyme transport and metabolism; I is lipid transport and metabolism; P is inorganic ion transport and metabolism; and Q is secondary metabolites biosynthesis, transport, and catabolism. (B) Distribution of the genes in the pangenome for each examined *S. aureus* strain.



**Fig. 3.** Pan-genome, core, and novel genes of the 64 analyzed *S. aureus* strains. Pan-genome features are as follows: The purple squares denote the number of novel genes discovered with the sequential addition of new genomes. The yellow dots denote the values of the core genes as genomes are added to the pan-genome. The purple bars indicate the number of new genes added to the total pan-genome size as new genomes are added. Each of the values represents the median from a distribution of randomly selected genomes at each genome addition. The purple line represents the number of new genes found for each genome addition. For comparison, the same trend for a closed genome is reported as a dashed line. The equations below the graph show parameters for fits to Heap's law. Positive exponents indicate an open state and that the category is boundless so new genes are likely to be discovered continually as new genomes are sequenced.

the number of shared genes, we estimated the *S. aureus* core genome to have 1,425 genes (Fig. 3). Discovery of new genes and count of total genes were used to obtain fits to the Heap's law function, resulting in  $\gamma$  values of  $-0.58$  and  $0.31$ , respectively. The  $\gamma$  parameter determines the behavior of the curve. For  $\gamma$  values  $>0$ , the function has no asymptote, indicating that the *S. aureus* pan-genome repertoire is likely to grow indefinitely as more strains are sequenced.

#### Size and Content of the Core Genome Provide Insight into Essential *S. aureus* Capabilities.

The average *S. aureus* genome encodes 2,800 genes; therefore, the size of the core genome represents a high portion (56%, on average) of each *S. aureus* genome (Fig. 2B). This portion is particularly high compared with other organisms including *Clostridium difficile* (core represents 25% of the average genome) (20) and *Escherichia coli* (core represents 40% of the average genome) (21). The fact that each *S. aureus* strain has such a high portion of shared genes can be interpreted as a direct consequence of the proposed clonal structure for the species (22). The majority of the 1,441 core genes (shared by all 64 *S. aureus* strains examined) are involved in housekeeping processes. These include nonmetabolic functions such as transcription (15%), translation, ribosomal structure and biogenesis (14%), and RNA processing and modification (7%). A core set of 239 genes functionally annotated to be involved in transcription and translation was found in all *S. aureus* strains examined (Fig. S1). A detailed discussion of these genes is provided in *SI Text*. Of these 239 conserved genes, 94 and 87 are known to be experimentally essential on LB media in *E. coli* (23) and *Bacillus subtilis* (24), respectively. Therefore, inhibitors of these proteins may be good targets for antibiotic therapies against *S. aureus*. Genes involved in metabolic functions were also highly conserved. Such functions included amino acid transport and metabolism (11%),

carbohydrate transport and metabolism (7%), coenzyme transport and metabolism (4%), cell wall and membrane biosynthesis (5%), and energy production and conversion (12%). Genome-scale network reconstructions for each of the 64 strains were built to perform an integrated analysis of the function of these metabolic genes (see below). Together, genes involved in metabolic, transcription, and translation processes make up 75% of the *S. aureus* core genome.

Relative to the core genome, widely different functional assignments are present for those genes in the accessory and unique genomes. The accessory genome has a large portion of genes associated with mobile genetic elements such as transposons and bacteriophages [replication, recombination, and repair (24%)] or those with nonmetabolic functions including defense mechanisms (5%). Metabolic functions were also included in the accessory genome, including those related to amino acid metabolism (8%), inorganic ion transport (7%), and carbohydrate metabolism (6%). The unique genome is heavily enriched in genes related to mobile elements (62%), with the other categories being poorly represented. This high proportion of mobile elements in the unique genome is similar to other organisms, including *E. coli*, and indicates that horizontal gene transfer (HGT) has had a large effect on *S. aureus* evolution (25–27). Indeed, the archaic MRSA clone, thought to be the ancestor strain of MRSA in Europe, obtained its methicillin resistance because of the horizontal transfer of the *mecA* gene from an unknown source (1). Even strains in the same household have been shown to engage in HGT with other *Staphylococcus* species (28). Thus, analysis of all HGT events in different strains of *S. aureus* and their putative source can shed light on the evolution of this species (see *SI Text* for analysis of atypical genes and their putative transfer source).

**Ancestral Events of Gene Loss and Acquisition Affect the Pathogenicity of *S. aureus* Strains.** We used the identified 1,441 core genes to produce an *S. aureus* phylogeny based on a concatenated sequence of the aligned genes. The patterns of gene presence were used with this phylogeny based on the core genome to infer events of ancestral gene gains and losses using a parsimony approach (29). This analysis allowed, for each internal node of the phylogenetic tree, us to compute (i) the number of gene gains and losses and (ii) the corresponding genetic repertoire. In particular, we focused on the evolutionary events related to the virulence factors in each strain because they have profound implications on pathogenesis of different *S. aureus* clones. The mapping of the events of gene gains and losses revealed that *S. aureus* strains have undergone extensive rearrangement of their genetic repertoire. A large number of evolutionary events occurred both ancestrally and recently (Fig. S2). For example, the ancestor of the ST228 lineage is characterized by a massive genome reduction (522 genes lost), whereas all of the LA-MRSA strains have recently acquired a large number of genes. Considering that the *S. aureus* last common ancestor (Sa-LCA) comprised 2,673 ortholog groups, the species has acquired a large number of new genes (4,784) during its evolutionary history.

**Known *S. aureus* Virulence Factors Are Unequally Distributed Between Core and Accessory Genomes.** To gain insight into the conservation of virulence factors across the *S. aureus* species we curated a set of known virulence factors (VFs) present in different strains based on literature and database searches (30). We identified a total of 90 different VFs that were present in at least one of the 64 different *S. aureus* strains. Of the 90 VFs, 35 were shared by all of the strains, forming a core set of VFs. Nine of the conserved VFs are cap8 genes (B, C, E, F, L, M, N, O, and P), which are involved in the synthesis of the polysaccharide capsule (PC). Other conserved VFs included five involved in the production of two different cytotoxins, namely, the Pantone–Valentine leukocidin (PVL), encoded by the genes *lukS* and *lukF*, and the gamma–hemolysin, encoded by *hlgA*, *hlgB*, and *hlgC*. Four other conserved VFs encode iron-regulated proteins (*isdA*, *isdC*, *isdE*, and *isdF*) that bind to extracellular matrix components such as fibrinogen and fibronectin to promote cell adherence. Among these, the *isdA* gene plays a role in the *S. aureus* iron acquisition system, important for *S. aureus* in vivo replication and disease pathogenesis (31). Other canonical *S. aureus* virulence factors were highly conserved but were not found across all 64 strains. Protein A (binds immunoglobulin G to disrupt phagocytosis, encoded for by *spa*) was found in 90% of strains. Alpha toxin (disrupts the membrane and enhances invasiveness, encoded for by *hla*) was found in 96% of strains. Biosynthesis genes for the staphyloxanthin pigment (encoded for by *crtMN PQ*) were found in all but one of the strains. This strain (MSHR1132) was specifically reserved because of its lack of the yellow pigment (17).

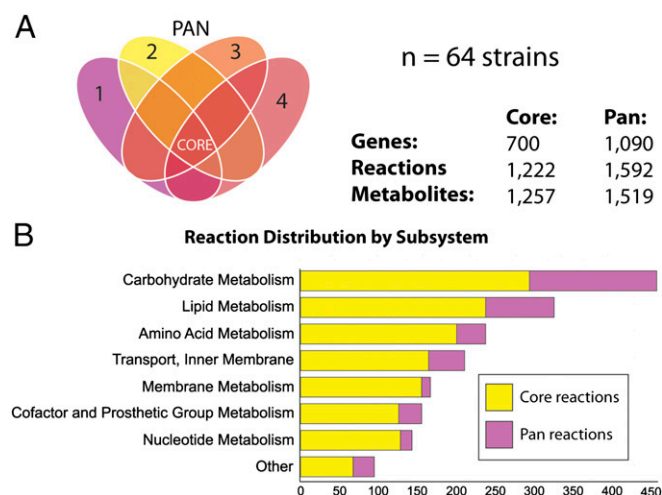
Several other VFs were strain-specific. For example, the Staphylococcal complement inhibitor (SCIN) protein (encoded by *scn*) was present in 48 of the 64 strains, and Chemotaxis inhibitory protein of staphylococci (CHIPS, encoded by *chp*) was present in 27 of the 64 strains. The AGR quorum sensing system (regulates biofilm development, encoded for by *agrABCD*) was found in 25% of strains. Meanwhile, the exfoliative toxin B was found only in a single strain (*S. aureus* 11819-97). *S. aureus* MW2 was found to have the highest count of established virulence factors (79 VFs) followed by MSSA476 (74 VFs), Mu3, and Mu50 (70 VFs). In contrast, the ST288 strains had the fewest number of VFs (53 total).

**Metabolic Models Facilitate Investigation into Genetic Basis of Strain-Specific Growth Capabilities.** Because so much of the *S. aureus* core genome is dedicated to metabolic functions, the 64 *S. aureus* genome sequences were used to construct strain-specific genome-scale metabolic reconstructions (Dataset S2) that were used to compare gene, reaction, and metabolite content between the strains. Each reconstruction serves as a comprehensive representation of the

metabolic capabilities of an *S. aureus* strain. Content shared among all reconstructions defines the core metabolic capabilities of the *S. aureus* species. Similarly, the metabolic capabilities of all strains were combined to define the full potential of metabolic capabilities for the *S. aureus* species, or its panmetabolic network (Fig. 4A) (10).

The core metabolic network contained 700 metabolic genes that catalyze 1,222 reactions involving 1,257 metabolites. Highly conserved metabolic subsystems across all *S. aureus* models include lipid metabolism, energy metabolism, glycan biosynthesis, and metabolism of polyketides and terpenoids. Reactions involved in these metabolic subsystems were highly represented (>95% conserved) in the core metabolic network. By contrast, only 80% of amino acid biosynthesis reactions were part of the core metabolic network. Conserved amino acid biosynthesis pathways included those for valine, alanine, and serine biosynthesis. The core metabolic network also contained known metabolic virulence determinants including catalase, an enzyme that hydrolyzes hydrogen peroxide into water and oxygen and that is used in the clinical laboratory to distinguish staphylococci from enterococci and streptococci. Catalase production and oxidant resistance have been shown to be predisposing factors for nasal colonization and subsequent infection (32).

The panmetabolic reactions are composed of the union of different metabolic reactions found in all strains and thus indicate the pool of metabolic capabilities within a species. The *S. aureus* panmetabolic network contains 1,090 metabolic genes, 1,592 reactions, and 1,519 metabolites. About 45% of reactions in carbohydrate metabolism were not present in the core metabolic network. Thus, these reactions were not shared by all strains of *S. aureus* examined. These formed the largest group of reactions present in the set difference between the core metabolic and panmetabolic networks (Fig. 4B). A majority of these reactions are involved in alternate carbon metabolism, including catabolic pathways for unique niche-specific nutrients. Amino acid metabolism was also disproportionately present in the panmetabolic network including biosynthesis pathways for leucine, arginine, and histidine metabolism, indicating that these capabilities may have been lost by several strains of *S. aureus*.



**Fig. 4.** Core metabolic and panmetabolic capabilities of the *S. aureus* species. The core metabolic and panmetabolic content was determined for genome-scale metabolic models (GEMs) of 64 unique *S. aureus* strains. (A) The core content, illustrated by the intersection of the Venn diagram, is shared with all strains. The pancontent consists of all content in any model and includes the core content. Note that the Venn diagram is not to scale and is simplified to only include the first 4 out of  $n = 64$  strains. (B) Classification of reactions in the core reactomes and panreactomes by metabolic subsystem.

The conversion of metabolic network reconstructions into computable mathematical models enables computation of phenotypes in diverse nutrient environments based on the content of each reconstruction. Thus, the 64 *S. aureus* strain-specific reconstructed networks were converted into genome-scale metabolic models (GEMs) that were used to compute phenotypes in more than 300 unique, growth-supporting environmental conditions. A detailed biomass composition was defined (Table S1) and used to identify the distribution of metabolic fluxes leading to optimal growth. Reactions belonging to the amino acid metabolism subsystem made up the majority of reactions in set difference between the core reactomes and panreactomes (Fig. 4B). Thus, we hypothesized that functional differences in amino acid biosynthesis capabilities of different strains of *S. aureus* may allow different strains to adapt to different nutritional environments. To test this hypothesis, we simulated growth in silico for all 64 *S. aureus* GEMs on a variety of minimal media growth conditions, including a minimal growth media reported for *S. aureus* N315 (12). The in silico growth analysis revealed that all 64 models growing in glucose minimal media require at least vitamins B1 (thiamin) and B3 (niacin) to be added to the media. Some of the strains required more components to be added to the minimal media beyond just these (Table 1). The thiamin auxotrophy is due to a lack of the pathway that converts tyrosine to thiamin via tyrosine lyase and thiazole phosphate synthase. The niacin auxotrophy is due to a lack of nicotinate-nucleotide diphosphorylase (EC 2.4.2.19). These auxotrophies have been experimentally documented for *S. aureus* in the past (33).

There were also several additional strain-specific auxotrophies with 90% of the models (55/64) unable to grow in the in silico glucose minimal media containing thiamin and niacin (Table S2). These models lacked the ability to synthesize additional compounds including the nucleotide guanine and the vitamin riboflavin as well as the amino acids leucine, arginine, histidine, tryptophan, phenylalanine, methionine, proline, and tyrosine (Table 1). Previous work has demonstrated that some strains of *S. aureus* are able to grow in minimal media devoid of amino acids after a long period of training or weaning away from amino acid addition (33, 34). We experimentally confirmed that four strains (USA300, N315, 8325, and Mu50) could grow in minimal media supplemented with proline, serine, leucine, threonine, thiamin, and niacin (Fig. S3). *S. aureus* is known to have a complicated gene regulatory structure that may inhibit its growth on minimal media, despite the presence of biosynthetic pathways for these metabolites (12, 14). Longer-term growth or training in the laboratory for these strains may allow for eventual growth in the minimal medium defined here (35).

**Table 1. Percentage of the models with a predicted strain-specific auxotrophy**

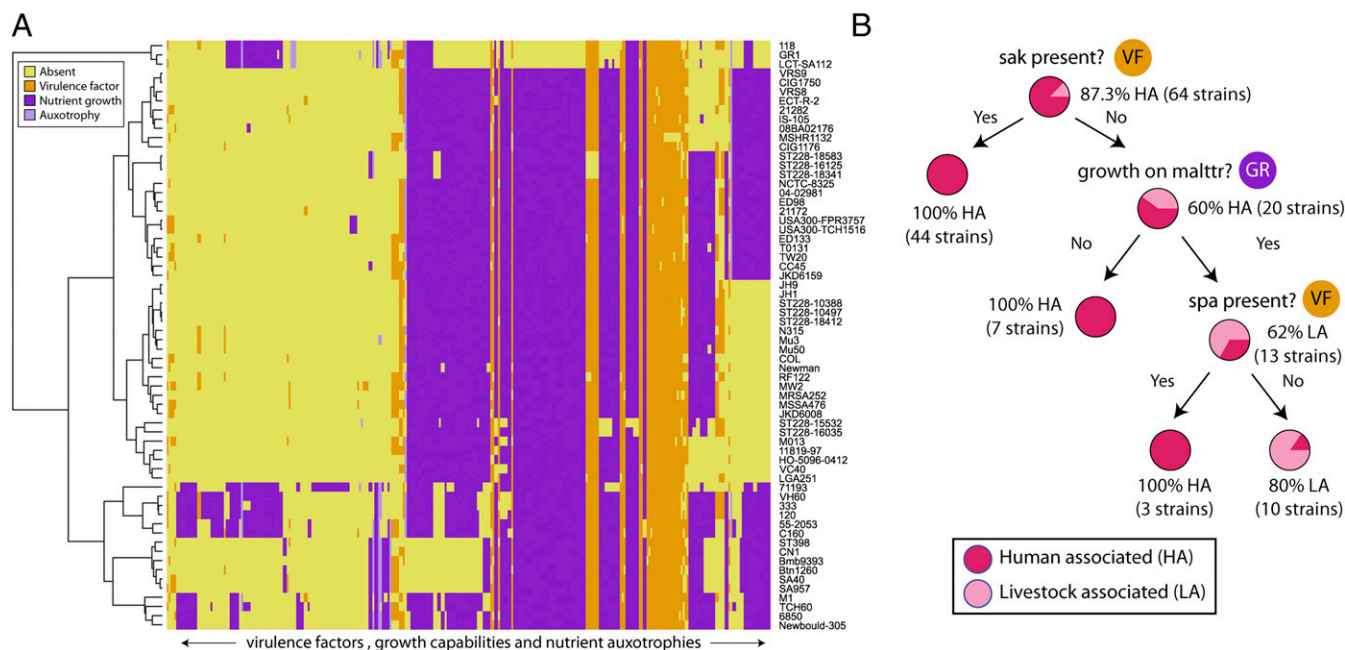
Compound	Percent auxotrophic
Thiamin	64/64 (100%)
Niacin	64/64 (100%)
L-leucine	38/64 (59%)
Guanine	31/64 (48%)
L-methionine	20/64 (31%)
L-cysteine	16/64 (25%)
Riboflavin	16/64 (25%)
L-proline	12/64 (19%)
L-asparagine	11/64 (17%)
L-histidine	3/64 (5%)
L-phenylalanine	3/64 (5%)
L-tyrosine	3/64 (5%)
L-arginine	2/64 (3%)
L-tryptophan	1/64 (2%)

***S. aureus* Strains Can Be Differentiated Using GEM-Predicted Metabolic Capabilities and Presence of Virulence Factors.** The 64 *S. aureus* GEMs were used to predict growth capabilities on alternative carbon, nitrogen, phosphorous, and sulfur sources by removing glucose, ammonia, sulfate, and phosphate from the in silico growth media and adding alternative sources one at a time. Over 300 alternative nutrient sources were tested in aerobic and anaerobic conditions using flux balance analysis (FBA) (36) to assess whether each *S. aureus* strain grew in silico (Fig. 5A). Overall, 238 nutrients were universally catabolized by all strains including glucose and glycerol as carbon sources and arginine as a nitrogen source. Other nutrients were strain-specific, including dulcose (42 strains) and inosine (13 strains) as carbon sources and uracil and thymidine as nitrogen sources (42 strains each). We specifically looked to see if the models were predicted to be able to use arginine for growth. The arginine mobile catabolic element (ACME) is present in MRSA USA300 strains and has been shown to be a key element for its successful colonization (37). We, however, found that the arginine catabolic capability was conserved across all *S. aureus* models due to the presence of a separate *arc* operon (encoded for by *arcA-arcD*) present in all strains. This finding is consistent with previous work (38). Despite this similarity, several other growth capabilities could be used to distinguish groups of strains (Fig. 5). For example, simulation results showed that the two *S. aureus* USA300 isolates were the only strains capable of using spermidine as a sole source of carbon and nitrogen due to the presence of spermidine acetyltransferase (encoded for by *speG*). Spermidine is produced at high levels in areas of keratinocyte proliferation, inflammation, and wound healing (39), conditions under which *S. aureus* invades and causes skin infection. We experimentally confirmed that only *S. aureus* USA300 could grow in TSB media supplemented with 6 mM of spermidine, whereas *S. aureus* strains Newman and NCTC8325 could not (Fig. S4).

Another example of using model predictions to distinguish strains was made possible by combining the simulated growth results with presence/absence of virulence factors in each strain. This combination allowed the formation of a classification schema that could be used to differentiate strains with a lifestyle that is livestock associated (LA) compared with those that were human associated (HA). The classifier was capable of distinguishing strains with these two host preferences based on evaluation of only three factors: presence of staphylokinase precursor (*sak*), the ability to catabolize maltotriose (*mlttr*), and presence of IgG binding protein A precursor (*sak*) (Fig. 5B). Interestingly, 100% of the *sak* positive strains were human-associated. We also observed that although many of the strains from the same clonal complexes clustered near each other, some important differences arose. For example, strains from the ST228 lineage appear to have lost several metabolic capabilities relative to others, causing two separate groups from this clonal complex to cluster apart from each other. Therefore, GEM-predicted metabolic capabilities and presence of virulence factors can be used to classify strains of *S. aureus* based on lifestyle and preferred niche. Thus, the tools presented here are capable of offering more precise classification schemes than those used routinely in the clinic to type *S. aureus* strains today.

## Discussion

The pangenome of *S. aureus* was assembled and applied to highlight the genetic, metabolic, and pathogenic diversity of the species. A particular emphasis was placed on the analysis of strain-specific metabolic capabilities and the distribution of virulence factors. Based on the content of the pangenome, GEMs of metabolism were reconstructed and deployed for 64 *S. aureus* strains to determine their functional differences by computing minimal media compositions and growth capabilities across more than 300 nutrient sources both aerobically and anaerobically. All strains were predicted to be auxotrophic for niacin and thiamin, whereas strain-specific auxotrophies were predicted for riboflavin, guanine, and



**Fig. 5.** *S. aureus* virulence factors and predicted metabolic capabilities. (A) Presence and absence of virulence factors and predicted metabolic capabilities across the 64 *S. aureus* strains examined in this study. Metabolic capabilities were predicted using the strain-specific metabolic models (dark purple, growth capability; light purple, nutrient required; yellow, no growth or nutrient is not required). The virulome consists of curated virulence factors known to be present in different strains of *S. aureus*. Orange indicates a factor is present, and yellow indicates a factor is absent. Full matrix with strains, predicted growth capabilities, and virulence factor is available in [Dataset S1](#). Virulence factor and growth profiles can be used to classify strains. For example in B, a classification is constructed that separates human-associated *S. aureus* strains from livestock-associated strains using only the presence of two virulence factors and the ability to catabolize maltotriose. Abbreviations are as follows: staphylokinase precursor (sak), maltotriose (malttr), and IgG binding protein A precursor (spa).

leucine among others (Table 1). Model-predicted growth capabilities and identification of virulence factors allowed for the creation of classification schemas that could distinguish groups of strains based on relatively few traits. The results presented here demonstrate that comparisons of GEM-predicted metabolic capabilities among different strains in a species can be used to identify strain-specific biomarkers and to uncover and elucidate metabolic determinants of virulence.

Identification and characterization of the makeup of a species' pangenome is a powerful tool to analyze genomic diversity within a taxon. The *S. aureus* pangenome described here is composed of 7,457 unique genes. Of these, 1,441 genes are shared among all strains in the species, forming a core genome. Functional assignment of the core genes revealed that they are mostly associated with housekeeping functions (i.e., control of gene expression machinery and basic biochemistry). On average, 56% of genes in the average *S. aureus* genome are part of the core genome. This confirms previous observations that *S. aureus* is a clonal species (22). Recent studies have shown that even mutations in the core genome of closely related *S. aureus* isolates can have significant effects on virulence, proliferation, and persistence of *S. aureus* strains (6). Therefore, a deeper analysis of strain-specific genetic variants in this set could offer further insights into *S. aureus* pathogenicity.

The 1,441 core genes were aligned to produce a reliable phylogeny compared with those derived from a limited number of genes (Fig. S2). In general, the phylogeny is consistent with information from literature including agreement between strains' ST and clade. However, some exceptions were observed: (i) the ST5 group is included within a paraphyletic clade corresponding to CC5 and (ii) *S. aureus* COL (ST250) is placed in the ST8 clade. Although these exceptions are consistent with the evolutionary history of *S. aureus* [i.e., the paraphyletic clade includes members of the same clonal complex and ST8 is the predicted ancestor of ST250 (40)], the differences between MLST geno-

typing and the presented phylogeny underline the limitations of molecular typing techniques to differentiate between closely related strains. Furthermore, in regards to host specificity, the distribution of the LA-MRSA strains along the tree indicates that they have originated through different independent events. In particular, we identified a monophyletic clade of ST398 strains, a clade of strains from different lineages (ST425, ST133, and ST151), and two strains included in different human-associated clades (corresponding to ST5 and ST1).

In contrast to the core genome, the size of the pangenome was used to predict, via extrapolation, the number of genome sequences required for bounding the gene repertoire of a clade. Our regression analysis shows that the *S. aureus* pangenome is open, indicating that the gene repertoire of this species is theoretically boundless. This result is in agreement with a previous DNA microarray experiment involving 36 *S. aureus* strains (41), in which extensive genetic variability was reported. A high portion of unique genes were found to be related to mobile genetic elements (i.e., transposon, phages, and plasmids), which may drive acquisition of novel functional modules via HGT, including drug resistance and virulence (42). Beyond these evolutionary insights, the pangenome has important practical implications. The presence/absence of genes from the dispensable genome (i.e., genes unique to some strains) represents a high-resolution alternative to MLST that we have used to diagnose distinct groups of pathogenic bacteria based on specific biomarkers.

We specifically examined the presence of virulence factors in the core genomes and pangenomes. Overall, most of the *S. aureus* virulome is conserved across the 64 strains examined. There is only one VF unique to a single strain (i.e., the *etb* gene, encoding the exfoliative toxin B), whereas 54 VFs were shared by a small number of strains but were not present in all strains. Some virulence factors are redundantly present across the species to overcome herd immunity. For example, several enterotoxins are



found as unique variants in different strains to avoid detection by a dynamic host immune system. The vast majority of the virulome is composed of core and pseudocore (shared by most of the strains) genes. The presence of these genes is interesting from an evolutionary point of view because it implies that *S. aureus* has evolved a highly conserved system to carry out its infectious cycle. From an applied viewpoint, these genes may represent targets for virulence inhibitor or antibody-based therapeutic strategies (43).

To gain insights into the metabolic diversity between *S. aureus* strains, we produced 64 strain-specific GEMs and used them to simulate growth capabilities in different nutrient sources. Experimental verification of minimal growth-supporting media was partially confirmed. It is conceivable that certain infectious niches may activate latent biosynthetic pathways under the appropriate conditions, and the fact that genes in these pathways have not developed into pseudogenes indicates that they may have relevance in niche adaptation (44).

A clustering analysis based on computed metabolic phenotypes distinguished groups of strains from each other. One distinction that arose was the identification of USA300 isolate as the only strain with a capability to catabolize spermidine due to the presence of *speG* encoded spermidine acetyltransferase. In humans, spermidine is known to potentiate keratinocyte killing of *S. aureus* in conjunction with antimicrobial peptides (45) and several classes of antibiotics, especially  $\beta$ -lactams (46). Recently, studies have found regions of ACME (including the arginine catabolic *arc* region) in many different, non-USA300 strains (47, 48). However, the association of the *speG* locus with ACME is uncommon outside of USA300. Therefore, acquisition of *speG* has likely been helpful in the rapid spread of USA300 strains. The *speG* gene was horizontally transferred from *S. epidermis*, a commensal colonizer of human skin (49). Therefore, a future comparison of shared metabolic capabilities between *S. aureus* strains and *S. epidermis* might provide further insights. Furthermore, design of antibiotics that specifically interfere with strain-specific capabilities such as those identified here offer new ways to inhibit epidemiological spread of particularly infectious strains.

The GEMs presented here open the *S. aureus* species to a wide array of systems biology methods and techniques (50). For example, using these models, it is straightforward to predict essential genes in different conditions (we found an average of 190 essential genes for the 64 strains in LB media; [Dataset S1](#)). Furthermore, single gene essentiality screens have been extended to the study of synthetic lethality and discovery of synergistic antibiotics (51). In conclusion, the multiscale comparative approach used in this work provides insights into the diversity of the *S. aureus* species. Historical methods for classification of *S. aureus* strains have developed considerably from phage-typing to PFGE and MLST approaches (52). New high-throughput DNA sequencing technologies and analysis techniques will continue to improve our ability to track and distinguish pathogens. Computational analysis of GEMs built for multiple strains in a species provides a powerful tool that can be deployed to provide insights into metabolic determinants of virulence, to identify novel biomarkers capable of distinguishing certain strains from one another, and to discover new pharmacological targets.

## Materials and Methods

Tools and methods used to identify and construct the core genome and pangenome of *S. aureus* as well as the analysis of atypical genes are presented in [SI Materials and Methods](#). Briefly, InParanoid (53) was used to identify orthologous genes to construct a pangenome. The strain-specific model reconstruction procedure was performed with Simpheny (Genomatica, Inc.), and gap-filling algorithms and in silico growth simulation conditions were implemented in Constraints-Based Reconstruction and Analysis Toolbox for Python (COBRAPy) (54) and are described in [SI Materials and Methods](#). Heat map, phylogenetic tree, and decision tree construction are described in [SI Materials and Methods](#). *S. aureus* strains NCTC8325, Newman, N315, Mu50, and USA300 were used for carbon source and growth testing. All experimental protocols are described in [SI Materials and Methods](#).

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