

# Evolutionary dynamics of *Staphylococcus aureus* during progression from carriage to disease

Bernadette C. Young<sup>a,1</sup>, Tanya Golubchik<sup>b,1</sup>, Elizabeth M. Batty<sup>b</sup>, Rowena Fung<sup>a,c</sup>, Hanna Larner-Svensson<sup>d</sup>, Antonina A. Votintseva<sup>a</sup>, Ruth R. Miller<sup>a</sup>, Heather Godwin<sup>e</sup>, Kyle Knox<sup>f</sup>, Richard G. Everitt<sup>a</sup>, Zamin Iqbal<sup>d</sup>, Andrew J. Rimmer<sup>d</sup>, Madeleine Cule<sup>b</sup>, Camilla L. C. Ip<sup>b</sup>, Xavier Didelot<sup>b</sup>, Rosalind M. Harding<sup>g</sup>, Peter Donnelly<sup>b,d</sup>, Tim E. Peto<sup>a,c</sup>, Derrick W. Crook<sup>a,c,2</sup>, Rory Bowden<sup>b,c,d,2</sup>, and Daniel J. Wilson<sup>a,d,2,3</sup>

<sup>a</sup>Experimental Medicine Division, Nuffield Department of Clinical Medicine, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DU, United Kingdom; <sup>b</sup>Department of Statistics, University of Oxford, Oxford OX1 3TG, United Kingdom; <sup>c</sup>National Institute for Health Research Oxford Biomedical Research Centre, John Radcliffe Hospital, Oxford OX3 9DU, United Kingdom; <sup>d</sup>Wellcome Trust Centre for Human Genetics, Oxford OX3 7BN, United Kingdom; <sup>e</sup>Oxford University Hospitals National Health Service Trust, Oxford OX3 9DU, United Kingdom; <sup>f</sup>Department of Primary Care Health Sciences, University of Oxford, Oxford OX1 2ET, United Kingdom; and <sup>g</sup>Department of Zoology, University of Oxford, Oxford OX1 3PS, United Kingdom

Edited by W. Ford Doolittle, Dalhousie University, Halifax, NS, Canada, and approved February 1, 2012 (received for review August 11, 2011)

Whole-genome sequencing offers new insights into the evolution of bacterial pathogens and the etiology of bacterial disease. *Staphylococcus aureus* is a major cause of bacteria-associated mortality and invasive disease and is carried asymptotically by 27% of adults. Eighty percent of bacteremias match the carried strain. However, the role of evolutionary change in the pathogen during the progression from carriage to disease is incompletely understood. Here we use high-throughput genome sequencing to discover the genetic changes that accompany the transition from nasal carriage to fatal bloodstream infection in an individual colonized with methicillin-sensitive *S. aureus*. We found a single, cohesive population exhibiting a repertoire of 30 single-nucleotide polymorphisms and four insertion/deletion variants. Mutations accumulated at a steady rate over a 13-mo period, except for a cluster of mutations preceding the transition to disease. Although bloodstream bacteria differed by just eight mutations from the original nasally carried bacteria, half of those mutations caused truncation of proteins, including a premature stop codon in an *AraC*-family transcriptional regulator that has been implicated in pathogenicity. Comparison with evolution in two asymptomatic carriers supported the conclusion that clusters of protein-truncating mutations are highly unusual. Our results demonstrate that bacterial diversity in vivo is limited but nonetheless detectable by whole-genome sequencing, enabling the study of evolutionary dynamics within the host. Regulatory or structural changes that occur during carriage may be functionally important for pathogenesis; therefore identifying those changes is a crucial step in understanding the biological causes of invasive bacterial disease.

The past 15 years have seen great advances in understanding the within-host evolutionary dynamics of important viral pathogens, providing insights that high-throughput whole-genome sequencing now promises for the study of evolution in bacterial pathogens and their mechanisms of disease (1–3). Viral evolutionary dynamics within the host can be used to predict the stage of disease, while adaptation is known to act as a trigger for disease progression (4, 5). Bacteria have larger genomes than viruses that mutate at a slower rate owing to more faithful DNA replication and mismatch repair systems, so variation that is informative about within-host population dynamics and functional adaptation has proved much harder to capture (6). Although within-host evolution has been reported in bacterial house-keeping genes (7), and is well established in hypervariable loci encoding surface antigens or regulating phase variation (8, 9), obtaining an exhaustive catalog of change in the whole genome is a prerequisite to understanding the genetic basis of pathogenesis.

In vivo evolution of the pathogen population is an important avenue for research into the etiology of bacterial disease. *Staphylococcus aureus* is a major bacterial cause of life-threatening hospital- and community-acquired infections that has come to prominence through the rise of drug-resistant forms, notably methicillin-resistant *S. aureus* (MRSA) (10). *S. aureus*, in common with other important

bacterial pathogens, is a predominantly commensal organism and a common constituent of the nasal flora of healthy adults (11, 12), although it is undoubtedly adapted to facultative pathogenicity (13). Carriage predisposes to invasive disease; in a study of bacteremic patients, staphylococci isolated from the blood could not be distinguished by pulsed-field gel electrophoresis from concomitantly carried nasal bacteria in the majority of cases (12). However, the events leading to invasive disease are incompletely understood (14, 15). Although disease may follow ingress of commensal flora into the bloodstream through compromised epithelia, another possibility is that subtle changes in the host–pathogen interaction precipitate the onset of disease. Indeed, spontaneous mutation in the pathogen has been shown to radically alter virulence in mouse models of *S. aureus* infection (16). Regulatory protein dysfunction has been demonstrated to increase bacterial pathogenicity (17), including increased mortality in *S. aureus* bacteremia (18).

To better understand the biology of *S. aureus* carriage, we surveyed nasal carriage in more than 1,100 adults attending general practices in Oxfordshire, United Kingdom. We recruited 360 carriers for regular screening. One elderly participant (participant P) developed a *S. aureus* bloodstream infection 15 mo after joining the study. Using whole-genome sequencing, we charted the evolution of the bacterial population during carriage and disease and contrasted it to ongoing evolution in two asymptomatic carriers (Q and R). Our results reveal dynamic populations of nasally carried staphylococci that harbor genetic variation that evolves measurably over time. We found that the number of mutations separating disease-causing from asymptotically carried bacteria in participant P was very few. However, the clustering of protein-truncating mutations preceding disease progression, including a transcriptional regulator of stress response and pathogenesis, was a unique pattern absent from the asymptomatic carriers and suggests a role for loss-of-function mutations in bacterial pathogenesis.

Author contributions: R.R.M., K.K., T.E.P., D.W.C., and R.B. designed research; B.C.Y., T.G., E.M.B., R.F., H.L.-S., A.A.V., R.R.M., H.G., R.G.E., and D.J.W. performed research; Z.I., A.J.R., M.C., C.L.C.I., X.D., R.M.H., and P.D. contributed new reagents/analytic tools; B.C.Y., T.G., E.M.B., R.G.E., and D.J.W. analyzed data; and B.C.Y., T.G., T.E.P., D.W.C., R.B., and D.J.W. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

Data deposition: The sequences reported in this paper have been deposited in the European Nucleotide Archive Sequence Read Archive under study accession number ERP001185 (<http://www.ebi.ac.uk/ena/data/view/ERP001185>).

<sup>1</sup>B.C.Y. and T.G. contributed equally to this work.

<sup>2</sup>D.W.C., R.B., and D.J.W. contributed equally to this work.

<sup>3</sup>To whom correspondence should be addressed. E-mail: [daniel.wilson@ndm.ox.ac.uk](mailto:daniel.wilson@ndm.ox.ac.uk).

This article contains supporting information online at [www.pnas.org/lookup/suppl/doi:10.1073/pnas.1113219109/-DCSupplemental](http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1113219109/-DCSupplemental).



under single-dose flucloxacillin and penicillin prophylaxis. The following month the participant began a chemotherapy regimen consisting of a proteasome inhibitor and an alkylating agent, along with prophylactic antimicrobials (co-trimoxazole). Sixteen days later the participant developed fever and was admitted to hospital with features of septic shock, including neutrophilia. At this point, the late blood cultures (LBC) comprising sample B15 were taken. No source for the bacteremia was identified: there were no endovascular catheters or evidence of endocarditis or surgical-site infection from the pacemaker, but the patient developed fatal multiorgan failure.

Genomic analysis and standard molecular typing indicated a typical community-carried *S. aureus* with no obvious predisposition toward disease. All samples were methicillin-sensitive *S. aureus* (MSSA), multilocus-sequence type (19) (MLST) ST-15 and *spa* type t4714 (Table S1), a newly described allele closely related to the commonly carried, community-associated t084 (20). Alignment of genome C1285 from sample N1 to reference genomes MRSA252 and MSSA476 (21) confirmed the absence of the methicillin resistance gene *mecA* and the lack of the staphylococcal cassette chromosome that frequently encodes virulence factors (22) (Fig. S1). Sequence similarity to MSSA476 was 99.5%. No putative virulence factors were found within 13 kb of coding sequence that did not align to MSSA476 (Table S2). Two copies of a 20.7-kb plasmid that was 99.9% similar to the MSSA476 pSAS plasmid and that contained a region homologous to the staphylococcal transposon Tn552 were detected. MSSA476 prophages  $\phi$ Sa3 and  $\phi$ Sa4 were absent from C1285, and no other prophages were detected. Pathogenicity islands homologous to  $\nu$ Sa $\alpha$  and  $\nu$ Sa $\beta$  were detected with partial deletions. No virulence or toxin genes were detected beyond those that were present in MSSA476 (Dataset S1 and Fig. 1B).

**Carriage and Invasive Bacteria Formed Distinct Clades Within the Host.** Extremely limited microvariation was found among the 68 sequenced genomes (Fig. 1), well below that detectable by conventional methods and consistent with a homogeneous population arising from a single acquisition. There was no variation in MLST, in *spa* type, or in 61 minisatellite repeats (Dataset S2), suggesting that the resident bacterial population was not eradicated by several episodes of antibiotic treatment. There was no evidence for large-scale insertions/deletions (indels) or copy number variants. We discovered a total of 30 single nucleotide polymorphisms (SNPs) and four short indels in the 2.7-megabase genome comprising 5 synonymous, 16 nonsynonymous, 3 nonsense, and 6 intergenic SNPs (one of which occurred in the plasmid), 2 intergenic indels, and 2 frameshift-inducing indels (one of which led to a premature stop codon) (Table S3). There was no homoplasy or evidence for within-host recombination.

Bloodstream colonies (LBC) and nasal colonies (ENC/LNC) formed distinct clades within a population characterized by extremely limited genetic variation. Nasal colonies clustered further into those sampled before (ENC) and after (LNC) the first of a series of drug interventions beginning in month 9 (Fig. 1C). Bayesian coalescent analysis showed slow but detectable evolution of the bacterial population (Fig. 2), at a rate of 2.72 mutations per megabase per year [95% credible interval (CI): 1.64–4.42], close to other estimates of the short-term mutation rate in *S. aureus* (2, 23, 24). A similar molecular clock rate was estimated for ENC sequences alone, but between the ENC clade and the LNC/LBC clades there was greater sequence divergence than expected (Fig. S2), suggesting a departure from the neutral evolutionary model. The estimated effective population size was small, corresponding to an average life span of polymorphisms of 4 mo. The most recent common ancestor of all of the sequences was dated to 1 mo before enrollment (Table S4), but there is no evidence to rule out carriage before this time.

**Excess of Protein-Truncating Mutations Preceded Disease Progression.** Unusual patterns of molecular evolution were observed along the branches separating early nasal sequences from invasive blood-

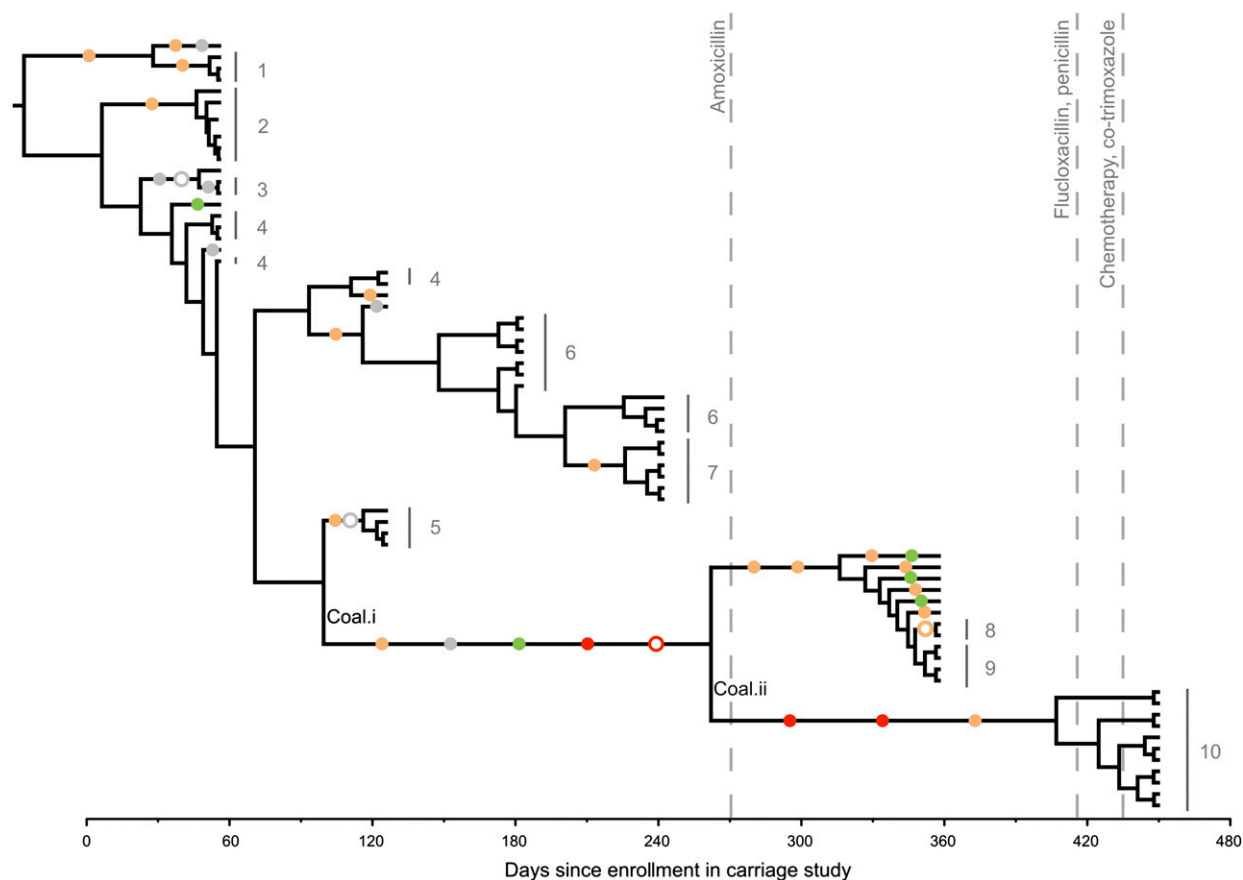
stream sequences. Tests based on neutral coalescent simulations showed that the most recent common ancestor (MRCA) of ENC and LNC/LBC (Coal.i in Fig. 2 and Table S5) was significantly older than expected ( $P = 0.005$ ). Indeed, five mutations occurred on this branch, whereas none of the derived polymorphisms from ENC sequences were retained through to LNC/LBC. This may indicate (i) cryptic populations of differentiated bacteria within the host, (ii) reseeded by a latent population of ancestral genotypes, (iii) adaptive evolution, or (iv) relaxed functional constraint associated with a population bottleneck. A similar pattern was seen for the branch leading to the LBC sequences, which coincided with periods of anti-neoplastic chemotherapy and antibiotic treatment. The MRCA of LBC and LNC (Coal.ii in Fig. 2 and Table S5) was also significantly older than expected ( $P = 0.021$ ). Although LNC sequences were diverse, no SNPs were observed within LBC. This represents significantly reduced diversity (Fig. S3) that is unlikely to be due to sampling limitations because LBC was derived from three separate blood culture bottles.

The distribution of premature stop codons among the branches of the tree was uneven, with a significant excess on the two branches leading from ENC to LBC (Fisher's exact test,  $P = 0.0015$ ; Table 1). Four SNPs and one indel separated the ENC clade from the LNC/LBC clades, including a premature stop codon in an *AraC* family transcriptional regulator (AFTR), which presents the best current candidate for functional mutation. AFTRs are regulators of carbon metabolism, stress response, and virulence that respond to changing environmental conditions such as antibiotic use and oxidative stress (17). In *Neisseria meningitidis*, a pseudogene induced by a premature stop codon in the AFTR *mpeR*, is associated with the hypervirulent ST 32 complex (25). The mutation that we observed maps to MSSA476 SAS2271, radically truncating the sequence from 702 to 77 amino acids. A premature stop codon induced by a frameshift on the same branch occurred in a protein of unknown function, SAS1429. We observed two further premature stop codons predicting significantly truncated proteins on the branch leading to the LBC clade: SAS0973, an iron-compound binding protein/transporter, and SAS1361, a GNAT family acetyltransferase.

**Clusters of Protein-Truncating Mutations Were Not Observed in Noninvasive Carriage Populations.** To investigate the evolution of *S. aureus* during asymptomatic nasal carriage, we used the Illumina GAIIX and HiSeq 2000 platforms to sequence the whole genomes of 101 colonies isolated from two other participants (Table S1). Twenty-two colonies isolated from two swabs taken 2 mo apart were sequenced from participant Q, who had no history of staphylococcal disease or recent antibiotic use. Seventy-nine colonies isolated from eight swabs taken over an 18-mo period were sequenced from participant R, who similarly had no history of staphylococcal disease. However, participant R had completed a treatment of flucloxacillin shortly before enrollment in the carriage study and took a course of amoxicillin in month 20. No bacterial growth was detected in any of six nasal swabs taken from month 22 to month 32, suggesting that carriage was cleared. The bacterial populations in both carriers were MSSA, and both exhibited a single *spa* type (t164 and t012, respectively) and multilocus-sequence type (ST-20 and ST-30, respectively), consistent with a single founding colonization in each case. The repertoire of virulence and toxin genes was indistinguishable among genomes sequenced within a single participant, and it was more similar between the genomes sequenced from participants P and Q than those from R (Dataset S1).

Limited microvariation was detected in both participant Q and participant R. We discovered a total of 42 SNPs and 4 short indels in participant Q, comprising 10 synonymous, 20 nonsynonymous, 1 nonsense, and 11 intergenic SNPs, 2 intergenic indels, and 2 frameshift-inducing indels, both of which led to premature stop codons (Table S3). Two large deletions were also detected in one of the genomes: an 8-kb deletion partially matching *S. aureus* pathogenicity island SaPI4 and a 1.6-kb deletion of an integrase. In participant R, we discovered a total of 39 SNPs and 9 short indels





**Fig. 2.** Bayesian coalescent tree. The maximum clade credibility tree representing the genealogy of sequences in the study, reconstructed from SNPs using BEAST. Genotypes are enumerated as in Fig. 1C. SNPs (filled circles) and indels (open circles) are superimposed on the tree and color-coded by type: synonymous (green), nonsynonymous (orange), premature stop codon (red), and intergenic (gray). The ordering of mutations within a branch is arbitrary.

comprising 14 synonymous, 15 nonsynonymous, 1 nonsense, and 9 intergenic SNPs, 6 intergenic indels, and three frameshift-inducing indels, all of which led to premature stop codons. There was no significant difference in the overall pattern of mutation types across participants P, Q, and R (Fisher's exact test,  $P = 0.457$ ). As in participant P, there was no homoplasy or evidence for within-host recombination in participants Q and R.

Rather than forming distinct clusters, the colonies isolated 2 mo apart in participant Q were genetically overlapping, with the descendants from multiple lineages detected in the earlier nasal swab present in the latter (Fig. S4). There was a clearer temporal trend in participant R, such that the diversity sampled at one time was usually descended from a single one of the lineages present in the previous sample, leading to a steady accumulation of mutations over time. Bayesian coalescent analysis revealed a molecular clock rate of 1.87 mutations per megabase per year in participant R (95% CI: 1.08–3.06), consistent with the rate

estimated in P. There was insufficient power to independently estimate the rate of evolution in participant Q. Assuming the same clock rate as in R implies a large effective population size in Q, corresponding to an average life span of polymorphisms of 17 mo. The effective population size estimated for participant R was intermediate between P and Q, with an average life span of polymorphisms of 5 mo (Table S4).

The evidence from participants Q and R provided additional support for the view that a significant excess of protein-truncating mutations occurred on the two branches separating the genomes sampled early during asymptomatic nasal carriage (ENC) from those sampled from the invasive bloodstream infection (LBC) in participant P. Three of 48 mutations detected in participant Q, and 4 of 48 mutations detected in participant R, were protein-truncating. Treating the mutations in Q and R as control groups confirmed that the number of premature stop codons occurring on the ENC-LBC branches in participant P was statistically

**Table 1.** Evidence for an excess of protein-truncating mutations on the two branches leading from ENC to LBC

Branches	No. of mutations		<i>P</i> value (Fisher's exact test)
	Protein-truncating	Other	
From ENC to LBC	4	4	—
All others in participant P	0	26	0.0015
All others in participant Q	3	45	0.0055
All others in participant R	4	44	0.0103
All others in P, Q, and R	7	115	0.0017

significant (Table 1). To maximize statistical power, we combined information across participants P, Q, and R, yielding a highly significant  $P$  value of 0.0017. To further investigate the unusual clustering of premature stop codons in participant P, we constructed an empirical distribution for this  $P$  value by considering every possible pair of branches occurring in participants P, Q, or R. None of the 589 possible permutations yielded a  $P$  value as significant as 0.0017 (Fig. S5), demonstrating that the cluster of protein-truncating mutations on the two branches leading from ENC to LBC was indeed highly unusual.

## Discussion

Just eight mutations accompanied the transition of an asymptotically carried MSSA population to a fatal bloodstream infection. Half of those mutations were premature stop codons, one of which truncated a putative transcriptional regulator of virulence (17). Two further premature stop codons were detected only among invasive bloodstream bacteria. Loss-of-function mutations that truncate the amino acid sequence may play an important role in pathogenesis because point mutations of this sort can quickly effect radical functional change (18). However, the patient's general health was also likely to have been important, with the interaction between genome evolution and clinical context likely to have been critical.

Using whole-genome sequencing of 169 bacterial colonies isolated from three nasal MSSA carriers, we have detected limited but measurable cross-sectional diversity and ongoing evolution within singly colonized carriers that would be undetectable by traditional means and have charted the evolutionary changes associated with the progression to invasive disease in one individual. High-throughput sequencing offers opportunities for understanding bacterial molecular evolution within the host and promises to shed light on the in vivo dynamics of bacterial carriage and infection. The role of chance, circumstance, and genetics in invasive bacterial disease is yet to be determined, but the exhaustive characterization of bacterial genetic variation within the host is an important step.

## Materials and Methods

**Isolate Collection and Preliminary Analysis.** Ethical approval for the carriage study was obtained from the Oxfordshire B Oxfordshire Research Ethics Committee (reference no. 08/H0605/102). Each nasal swab culture was prepared and stored in glycerol. We incubated an inoculum of the glycerol stock on SAselct agar (BioRad) overnight at 37 °C, then picked 12 colonies, streaked each onto Columbia blood agar, and incubated the colonies overnight at 37 °C. Methicillin sensitivity was determined by the disk diffusion method. Blood cultures were prepared using the BD Bactec system; blood was drawn from the patient at two times 6 h apart, and each sample was inoculated separately into two bottles. Both bottles from the first sample and one of two from the second sample flagged positive for bacterial growth. Blood extracted from the bottles was cultured on SAselct (BioRad) agar, and four colonies were picked from each bottle for sequencing. DNA was extracted using a commercial kit (FastDNA by MP Biomedicals) employing mechanical disruption of bacteria and column-based purification of DNA. Staphylococcal protein A (*spa*) type was determined by Sanger sequencing of the variable X region of the 3' end of the *spa* gene, using commercially designed primers (*spa*F: 5'-AGACGATCCTTCGGTGAGC-3'; *spa*R: 5'-GCTTTTGCAATGTCATTTACTG-3'). The software Ridom StaphType (26) was used for *spa* sequence analysis.

**Sequencing and Assembly.** For samples Q2, Q4, R2, and R4, we used the Illumina GAIIx platform with 12-fold multiplexing, read lengths of 51 bp, insert sizes of 200 bp, and mean depth of 62.9 reads. For the remaining samples, we used the Illumina HiSeq 2000 platform with 96-fold multiplexing, read lengths of 99 bp, insert sizes of 200 bp, and mean depth of 214 reads. In 68 of 84 colonies (participant P), 22 of 24 colonies (participant Q), and 79 of 96 colonies (participant R), we successfully performed DNA extraction, library preparation, and sequencing to a standard that passed stringent quality control measures. We used Velvet (27) to assemble reads into contigs de novo for each genome. We used Stampy (28) with no BWA premapping and an expected substitution rate of 0.01 to map each genome against a host-specific internal reference genome comprising the contigs assembled for genomes C1285 (2.68 Mb), C0965 (2.59 Mb), or C0764 (2.76 Mb). These

represent the earliest sequenced sample groups in participants P, Q, and R, respectively, comprising 137, 948, and 167 contigs with an N50 of 71,927, 22,136, and 148,695 bp, respectively. Altogether, 99.5, 96.9, and 96.6% of reads in C1285, C0965, and C0764 mapped to the respective de novo assemblies. Participant P genomes were also mapped to the MRSA252 (2.90 Mb) and MSSA476 (2.80 Mb) references (21). Repetitive regions, defined by BLASTing the reference genome against itself, were masked before variant calling. This masked 4.0, 2.2, and 2.7% of the host-specific reference genomes, respectively, and 5.9% of MRSA252 and 4.5% of MSSA476. The average proportions of the reference genome that we called by mapping were 92, 92, 93, 85, and 81%, respectively.

**Variant Calling.** We used SAMtools (29) and Picard (<http://picard.sourceforge.net>) to call variants from mapping, which we then filtered using criteria including base quality, mapping quality, and depth. We used Cortex (30) to detect SNPs and short indels. Visual inspection of every filtered and unfiltered variant call in participant P was used to manually validate the approach. To detect large deletions relative to the host-specific references, the mapped read depth was scanned for regions of at least 1 kb in which 500 bp or more exhibited zero coverage. To detect large insertions relative to the host-specific references, unmapped reads were assembled by Velvet with a hash length 31 bp. We validated our ability to identify large indels by comparing our sequences with MSSA476. We used Tandem Repeats Finder (31) to identify minisatellite-like repeats in the MRSA252 reference and searched for their flanking sequences in each genome using BLAST. Indeterminate results were obtained when just one of the flanking sequences was found, or when the two were located on different contigs. We likewise used BLAST to search for the presence of known or putative virulence factors, including toxins, adhesins, and regulators (32–34).

**Experimental Validation.** We chose the four protein-truncating mutations detected in participant P for validation using PCR and capillary sequencing. The variants detected at positions 1043150 (C→A), 1458121 (G→A), 1555915 (deletion of A), and 2430183 (C→T) relative to the MSSA476 genome were successfully amplified and sequenced in each of two single-colony isolates from N1 and two single-colony isolates from B15 using the following primer pairs: F1043150 5'-GATTTAGCCACTGACGGGA-3' and R1043150 5'-ATGTAACGATGCGCCAATTC-3'; F1458121 5'-ATACGTGTCCAACCTGTCCC-3' and R1458121 5'-GGCGCCTTTGTTATTCATCG-3'; F1555915 5'-GCAATCGAATCTCCTGTCCA-3' and R1555915 5'-ACATTAGTGATGGTGTGCC-3'; and F2430183 5'-TGGTGAACCAAGACGTAAG-3' and R2430183 5'-GTCTATGAACACCGGATTGCT-3'. For every variant, both the N1 and both the B15 isolates showed the expected sequence, confirming the existence of the variant in our samples.

**Mobile Elements.** We used BLAST to search for short flanking sequences of six staphylococcal cassette chromosome (SCC)-associated loci (22) in C1285 using a word size of 16. As a control, we repeated the searches in MRSA252 (21) and MSSA476. We used ClustalX (35) to align each pair of SCC direct repeats in MRSA252 and MSSA476 to C1285. We used xbase (36) to align C1285 against MRSA252 and MSSA476 using MUMMER (37) and to annotate the genome. The alignment in the SCC region was inspected using the Artemis Comparison Tool (38). We used BLAST to search for *S. aureus* transposons Tn552 and Tn554 in C1285. We used Stampy (28), MUMMER (37), and Mauve (39) to search for MSSA476 phages  $\phi$ Sa3 and  $\phi$ Sa4 and pathogenicity islands  $\nu$ Sa $\alpha$  and  $\nu$ Sa $\beta$ . We searched for novel prophages using Prophage Finder (40).

**Population Genetics Analysis.** We used a permutation test for recombination that detects any correlation between physical distance and linkage disequilibrium (41). We inferred tree topology and branch lengths using maximum likelihood (ML) under the assumption of no repeat mutation and homogeneous mutation rates. We used the ML tree to reconstruct haplotypes. We performed Bayesian coalescent inference to estimate evolutionary parameters, including the molecular rate using BEAST (42), assuming constant population size and the Hasegawa, Kishino, and Yano mutation model (43). All validated SNPs were included, together with 1% of invariant nucleotides. Separate analyses of participant P, Q, and R genomes were undertaken, along with separate analyses of ENC alone and LNC and LBC together. Further analyses of each sample (N1, N2, N4, N6, N8, N12, and B15) within participant P were undertaken to estimate diversity ( $\theta = 2N_e\mu$ ) for each group separately by fixing  $\mu$ . For the analysis of participant Q sequences, there was insufficient power to estimate  $\mu$ ; instead,  $\mu$  was fixed at the rate estimated for participant R. In all cases, we assumed an improper uniform prior on  $N_e\mu$  (the product of effective population size and generation length), an improper uniform prior on  $\mu$  (mutation rate per day, unless fixed), a uniform prior on nucleotide frequencies, and a log-normal prior on

$\kappa$  (transition:transversion ratio) with mean 1 and SD 1.25 on the logarithmic scale. Pairs of chains of 10 million iterations each were run, which were sampled every 1,000 iterations with a burn-in of 100,000 iterations removed before merging the chains to obtain final results. We quote the posterior median and (2.5%, 97.5%) quantiles as point estimates and credible intervals, respectively. To obtain the maximum clade credibility tree with BEAST, we used an outgroup constructed with 1% of the fixed differences between MSSA476 and the host-specific internal reference, which allowed us to infer the direction of mutation. To remedy the strong leverage that the outgroup sequence has on estimates of the molecular rate, we assumed an uninformative improper uniform prior on the sampling date of the outgroup sequence. A pair of chains of 400 million iterations were run, which were sampled every 10,000 iterations with a burn-in of 100,000 iterations removed before merging the chains to obtain final results.

**Evolution Associated with Progression to Invasive Disease.** To test whether the number of premature stop mutations occurring on the two branches leading from ENC to LBC was unusual, we used Fisher's exact test, cross-tabulating the number of protein-truncating premature stop mutations versus all other mutations against the branch on which they occurred: those leading from ENC to LBC versus (i) all others in participant P, (ii) all others in participant Q, (iii) all others in participant R, and (iv) all others combined. To test empirically whether the clustering of protein-truncating mutations on the two

branches of the tree leading from ENC to LBC was unusual, we considered all pairs of branches within each participant and calculated a *P* value using Fisher's exact test based on the total number of premature stop codons seen in those two branches versus all other branches in all participants. We then compared *P* value (*iv*) to this empirically generated distribution. To test whether the coalescence times for the branches leading from ENC to LBC were unusually ancient, we used coalescent simulations based on the output from BEAST to calculate a predictive *P* value under the standard neutral model of evolution. For each branch independently, we calculated the prior probability of observing a coalescent time as long or longer, which was conditional on the rest of the inferred tree. The *P* value was taken as a mean over the iterations of the Markov chain Monte Carlo.

**ACKNOWLEDGMENTS.** We thank L. O'Connor, A. S. Walker, P. Piazza, and the Oxford Medical Research Council High Throughput Sequencing Hub Team. This study was supported by the National Institute for Health Research Oxford Biomedical Research Centre and the Modernising Medical Microbiology Consortium, the latter funded under the UK Clinical Research Collaboration Translational Infection Research Initiative supported by the Medical Research Council; Biotechnology and Biological Sciences Research Council; National Institute for Health Research on behalf of the UK Department of Health Grant G0800778; and Wellcome Trust Grant 087646Z/08/Z. We acknowledge the support of Wellcome Trust core funding through Grant 090532/Z/09/Z. T.E.P. and D.W.C. are National Institute for Health Research Oxford Biomedical Research Centre senior investigators.

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