The Energy-Coupling Factor Transporter Module EcfAA’T, a Novel Candidate for the Genetic Basis of Fatty Acid-Auxotrophic Small-Colony Variants of Staphylococcus aureus

Nina Schleimer1, Ursula Kaspar1, Mike Drescher1, Jochen Seggewiß2, Christof von Eiff1*, Richard A. Proctor3, Georg Peters1, André Kriegeskorte1† and Karsten Becker1*

1 Institute of Medical Microbiology, University Hospital Münster, Münster, Germany, 2 Institute of Human Genetics, University Hospital Münster, Münster, Germany, 3 Departments of Medical Microbiology/Immunology and Medicine, University of Wisconsin School of Medicine and Public Health, Madison, WI, United States

Staphylococcal small-colony variants (SCVs) are invasive and persistent due to their ability to thrive intracellularly and to evade the host immune response. Thus, the course of infections due to this phenotype is often chronic, relapsing, and therapy-refractory. In order to improve treatment of patients suffering from SCV-associated infections, it is of major interest to understand triggers for the development of this phenotype, in particular for strains naturally occurring in clinical settings. Within this study, we comprehensively characterized two different Staphylococcus aureus triplets each consisting of isogenic strains comprising (i) clinically derived SCV phenotypes with auxotrophy for unsaturated fatty acids, (ii) the corresponding wild-types (WTs), and (iii) spontaneous in vitro revertants displaying the normal phenotype (REVs). Comparison of whole genomes revealed that clinical SCV isolates were closely related to their corresponding WTs and REVs showing only seven to eight alterations per genome triplet. However, both SCVs carried a mutation within the energy-coupling factor (ECF) transporter-encoding ecf module (EcfAA’T) resulting in truncated genes. In both cases, these mutations were shown to be naturally restored in the respective REVs. Since ECF transporters are supposed to be essential for optimal bacterial growth, their dysfunction might constitute another mechanism for the formation of naturally occurring SCVs. Another three triplets analyzed revealed neither mutations in the EcfAA’T nor in other FASII-related genes underlining the high diversity of mechanisms leading to the fatty acid-dependent phenotype. This is the first report on the ECF transporter as genetic basis of fatty acid-auxotrophic staphylococcal SCVs.

Keywords: Staphylococcus aureus, small-colony variants (SCVs), fatty acid-auxotrophy, whole-genome sequencing, energy-coupling factor transporter (EcfAA’T), phenotype switch
INTRODUCTION

As an integral part of the normal bacterial life cycle and the infection process, the formation of the small-colony variant (SCV) phenotype enables staphylococcal cells to adapt to an intracellular lifestyle protecting them against the host defense system and antimicrobial therapy resulting in persistent, relapsing, and often therapy-refractory chronic infections (Tuchscherr et al., 2010, 2011; Kriegeskorte et al., 2011, 2014b; Edwards, 2012; Kahl et al., 2016).

Two major types of SCVs have been most frequently associated with clinical cases (Proctor et al., 2006, 2014): (i) electron transport-deficient SCVs, which are typically recovered from patients suffering from chronic osteomyelitis and/or treated with aminoglycosides and found to exhibit auxotrophies for hemin, menadione, or thiamine, respectively (Proctor et al., 1995; von Eiff et al., 1997b; Kohler et al., 2008) and (ii) thymidine-auxotrophic SCVs with thymidylate biosynthesis defects recovered from cystic fibrosis patients especially after long-term trimethoprim–sulfamethoxazole treatment (Kahl et al., 1998; Kriegeskorte et al., 2014a). Based on in vitro generation of deletion mutants and sequencing, the genetic basis of only a small fraction of these SCV phenotypes could be clarified so far discovering defects in genes including aroB, aroD, hemA-D, hemG, hemH, menA-F, and thyA, respectively (von Eiff et al., 1997b; Bates et al., 2003; Schaaff et al., 2003; Chatterjee et al., 2008; Lannerberg et al., 2008; Köser et al., 2012; Hammer et al., 2013; Dean et al., 2014; Painter et al., 2015; Cao et al., 2017; Zhang et al., 2017). However, for clinically derived SCVs, only mutations of the hemG, menB, menC, menE, menF, and thyA genes were identified. Besides these intensively studied mechanisms of SCV formation, less is known on SCVs dependent on unsaturated fatty acids or other compounds (Sherris, 1952; Sličkin et al., 1971; Kaplan and Dye, 1976; Gómez-González et al., 2010; Lin et al., 2016). Hitherto, the fatty acid metabolism-linked genes accC, accD, fabF, fabl (eventually combined with fabD), and plsX have been associated with the phenotype switch of fatty acid-auxotrophic SCVs, with fabF mutation being the only one found in a clinical isolate (Parsons et al., 2011, 2013, 2014; Lin et al., 2016; Bazaid et al., 2018). Moreover, the underlying auxotrophy could be elucidated only for a part of the SCVs (Garcia et al., 2013), which further complicates the identification of potential genes. As an example, a mutation in relA was identified as potential trigger for the phenotype switch (Gao et al., 2010).

Here, we comprehensively analyzed two different triplets of isogenic S. aureus isolates, each comprising a clinically derived, fatty acid–auxotrophic SCV phenotype, its corresponding wild-type (WT) strain, both sampled in parallel, and a spontaneous in vitro revertant (REV) displaying the normal phenotype. In particular, the unsaturated fatty acid-based auxotrophism of SCVs was characterized and the phenotype switch was assessed by a whole-genome sequencing (WGS) approach. WGS revealed SCV formation-associated mutations within the energy-coupling factor (ECF) transporter-encoding ecf module (EcfAA'T) for both triplets and, in one triplet, an alteration within the Agr system most likely responsible for the decreased hemolytic activity displayed by the SCV and REV.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Clinical S. aureus WT isolates and their corresponding SCVs were recovered in parallel from patients of the University Hospital Münster in Germany suffering from different infections (Table 1 and Supplementary Table 1 in the Supplementary Material). Strains were cultivated and grown on Columbia blood agar (BBL™ Columbia agar with 5% sheep blood, Becton Dickinson, Franklin Lakes, NJ, United States) at 37°C, then frozen and stored at −80°C until testing. Isolates exhibiting the following characteristics were identified as SCVs: (i) pinpoint colonies on Columbia blood agar (Becton Dickinson, Franklin Lakes, NJ, United States) after 24–72 h of incubation, (ii) reduced hemolytic activity, and (iii) decreased pigmentation. Corresponding REVs exhibiting the normal phenotype (Becker et al., 2006; Proctor et al., 2006) emerged spontaneously from subcultured SCV isolates after several in vitro passages on Columbia blood agar (Becton Dickinson, Franklin Lakes, NJ, United States).

To monitor possible spontaneous reversions of the SCVs into the WT, every step of the cultivation procedures and the inoculum preparations were performed exclusively on solid media. SCVs were tested for reversion by subculturing of individual colonies onto Columbia blood agar (Becton Dickinson, Franklin Lakes, NJ, United States) under non-stress conditions (37°C, 24–48 h). The ability of the SCV colonies to revert to normal-sized colonies was judged visually regarding change in size and hemolysis behavior. Colonies that matched the SCV criteria underwent several (at least 10) passages of subculturing on solid media.

Differences in colony sizes were assessed on solid media by measuring the size of 50 single colonies on Columbia blood agar after 24 h of incubation. To analyze the results of colony sizes, statistical analyses were performed applying non-parametric tests using the Kruskal–Wallis test, with $p = 0.05$ set for statistical significance.

DNA Manipulations

Unless otherwise stated, all DNA manipulations were carried out following standard procedures and manufacturer’s recommendations. Chromosomal DNA from S. aureus cells was extracted after lysostaphin treatment (20 μg/mL, 1 h, 37°C) (Wak-Chemie Medical, Steinbach, Germany) using the PrestoSpin D kit (Molzym, Bremen, Germany). Plasmid DNA was isolated with the Qiagen Plasmid Mini kit (Qiagen, Venlo, Netherlands). PCRs were performed using oligonucleotides listed in Table 2 and Taq DNA Polymerase (Segenetic, Borken, Germany). Standard-PCR conditions consist of 5-min initial denaturation step at 95°C followed by 35 cycles of (i) denaturation at 95°C for 30 s, (ii) annealing at 65°C for 30 s, and (iii) extension at 72°C for 3 min. The final extension was performed at 72°C for 7 min. PCR products were analyzed by agarose gel electrophoresis and purified using the QIAquick
PCRs were performed by standard PCR with amplification of genes by standard PCR with annealing at 55°C and extension for 1 min for genes accB, fabG, fabI, and fabZ and extension for 1.5 min for genes accC, accD, fabD, fabF, fabH, and plsX followed by Sanger sequencing (Eurofins Genomics, oligonucleotides listed in Table 2).

Genotyping by PFGE
Clonal relationship within the strain triplets for the corresponding WT, SCV, and REV strains was confirmed by Smal macrorestriction analyses of total bacterial DNA followed by resolving the digests using pulsed-field gel electrophoresis (PFGE) as previously described (Goering and Winters, 1992; von Eiff et al., 1997a). Instead of tryptic soy broth (TSB), BHI broth (Merck, Darmstadt, Germany) was used in order to optimize growth conditions for SCVs. Strains were considered clonally identical if less than two bands varied on the gel according to the published guidelines (Tenover et al., 1995).

MLST, Spa Typing, and Microarray Analysis
MLST was done based on the WGS data applying the PubMLST database and software available on PubMLST.org/SAUREUS/ (Jolley and Maiden, 2010). Typing of the spa gene was performed with spaTyp 1.0 available from the Center for Genomic Epidemiology homepage (Bartels et al., 2014) also using whole-genome data. Verification of MLST and spa typing and determination of regulatory and hemolysin genes were performed at the genetic level using DNA microarray analyses (IdentibAC Microarray, Alere, Jena, Germany).

Auxotrophism Studies
Auxotrophism testing was performed in triplicate on surface-dried, in particular condensation water-free MHA (Merck, Darmstadt, Germany). For further evaluation of growth, samples were adjusted to McFarland 0.5 (in 0.9% NaCl), diluted (1:1,000 for WTs and 1:100 for SCVs), and an amount of 100 µL was streaked on unsupplemented MHA (Merck, Darmstadt,

**Table 1 | Characteristics of the strain triplets analyzed in this study.**

| Strain | Phenotype | Source | Expression of catalase | Hemolysis | Reference |
|--------|-----------|--------|------------------------|-----------|----------|
| Triplet No. 24117² | | | | | |
| 24117-WT | Wild-type | Wound swab (metatarsal bone V) | + | ++ | ++ | This study |
| 24117-SCV | SCV | Wound swab (metatarsal bone V) | + | – | – | This study |
| 24117-REV | Wild-type | In vitro culture | + | – | –/+(+) | This study |
| Triplet No. 1549³ | | | | | |
| 1549-WT | Wild-type | Gall bladder content | + | ++ | ++ | (Kriegeskorte et al., 2014b) |
| 1549-SCV | SCV | Gall bladder content | + | – | ++/+ | (Kriegeskorte et al., 2014b) |
| 1549-REV | Wild-type | In vitro culture | + | +/++ | ++ | This study |

¹Hemolytic behavior on blood agar: ++, strongly positive with clear zone of β-hemolysis; +, positive with small zone of β-hemolysis; +, weak positive with weak and very small zone of β-hemolysis; –, without hemolysis. ²From a diabetic patient with neuropathic osteoarthropathy-associated osteomyelitis. ³From a patient with a gall bladder empyema suffering from hepatitis C-induced liver cirrhosis.

**S. aureus Knockout Mutants**
The ΔthyA mutant was constructed as previously published using the vector pB79-thyA:ermB that was transformed by electroporation into clinical 1549-WT applying the standard protocol (Kriegeskorte et al., 2014a). Further cultivation and integration (first recombination) of pBT9-thyA:ermB into the genome of 1549-WT were performed as described (Kriegeskorte et al., 2014a) with the exceptions of using brain heart infusion broth (BHI, Merck, Darmstadt, Germany) supplemented with erythromycin (2.5 µg/mL), chloramphenicol (10 µg/mL), and thymidine (100 µg/mL). Integration was verified by PCR of selected colonies (Supplementary Table 2). For resolution (second recombination), an overnight culture of verified 1549-thyA:ermB were then grown in BHI (Merck, Darmstadt, Germany) containing erythromycin and thymidine at 25°C. Further procedure and selection of the mutants was performed as described elsewhere (Kriegeskorte et al., 2014a) with the exceptions for erythromycin at a concentration of 2.5 µg/mL and susceptibility disks (Oxoid, Hampshire, United Kingdom) impregnated with 10 µL of thymidine (10 mg/mL) for supplementation of Mueller-Hinton agar (MHA, Merck, Darmstadt, Germany). Deletion of thyA was verified via PCR amplification followed by sequencing (Eurofins Genomics). Furthermore, a ΔermB mutant of the clinical S. aureus A3878-WT and a ΔmenD mutant derived from the laboratory strain COL, both constructed as previously published (von Eiff et al., 2006; Kriegeskorte et al., 2011), were also included in the experiments (Table 3).

**Screening for Alterations Within the ecf Module and the FASII Pathway Genes**
In order to screen further strain triplets (listed in Supplementary Table 1) for alterations within the ecf module, amplification of the module was performed by standard PCR with annealing at 55°C and oligonucleotides ecF-F and ecF-R. For sequencing (Eurofins Genomics), oligonucleotides listed in Table 2 were used. Alterations within the FASII pathway genes were screened by amplification of genes by standard PCR with annealing at 55°C and extension for 1 min for genes accB, fabG, fabI, and fabZ and extension for 1.5 min for genes accC, accD, fabD, fabF, fabH, and plsX followed by Sanger sequencing (Eurofins Genomics, oligonucleotides listed in Table 2).

**Table 2 | Characteristics of the strain triplets analyzed in this study.**

| Strain | Phenotype | Source | Expression of catalase | Hemolysis | Reference |
|--------|-----------|--------|------------------------|-----------|----------|
| Triplet No. 24117² | | | | | |
| 24117-WT | Wild-type | Wound swab (metatarsal bone V) | + | ++ | ++ | This study |
| 24117-SCV | SCV | Wound swab (metatarsal bone V) | + | – | – | This study |
| 24117-REV | Wild-type | In vitro culture | + | – | –/+(+) | This study |
| Triplet No. 1549³ | | | | | |
| 1549-WT | Wild-type | Gall bladder content | + | ++ | ++ | (Kriegeskorte et al., 2014b) |
| 1549-SCV | SCV | Gall bladder content | + | – | ++/+ | (Kriegeskorte et al., 2014b) |
| 1549-REV | Wild-type | In vitro culture | + | +/++ | ++ | This study |

¹Hemolytic behavior on blood agar: ++, strongly positive with clear zone of β-hemolysis; +, positive with small zone of β-hemolysis; +, weak positive with weak and very small zone of β-hemolysis; –, without hemolysis. ²From a diabetic patient with neuropathic osteoarthropathy-associated osteomyelitis. ³From a patient with a gall bladder empyema suffering from hepatitis C-induced liver cirrhosis.

**MLST, Spa Typing, and Microarray Analysis**
MLST was done based on the WGS data applying the PubMLST database and software available on PubMLST.org/SAUREUS/ (Jolley and Maiden, 2010). Typing of the spa gene was performed with spaTyp 1.0 available from the Center for Genomic Epidemiology homepage (Bartels et al., 2014) also using whole-genome data. Verification of MLST and spa typing and determination of regulatory and hemolysin genes were performed at the genetic level using DNA microarray analyses (IdentibAC Microarray, Alere, Jena, Germany).

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Auxotrophism testing was performed in triplicate on surface-dried, in particular condensation water-free MHA (Merck, Darmstadt, Germany). For further evaluation of growth, samples were adjusted to McFarland 0.5 (in 0.9% NaCl), diluted (1:1,000 for WTs and 1:100 for SCVs), and an amount of 100 µL was streaked on unsupplemented MHA (Merck, Darmstadt,
| Oligonucleotide primer | Sequence (5′ → 3′) | Purpose | Reference |
|------------------------|---------------------|---------|-----------|
| F1 (thyA) fwd          | ATA TGA GCT CGA CAT TGC GGA CTT AAA GGA TG | Forward primer of upstream flanking region of thyA; binds within pBT9-thyA::ermB and chromosomal DNA | (Kriegeskorte et al., 2014a) |
| F2 (thyA) rev          | GCG CGT CGA CTA GTT GGT AAA TATCTT CAA TA | Reverse primer of downstream flanking region of thyA; binds within pBT9-thyA::ermB and chromosomal DNA | (Kriegeskorte et al., 2014a) |
| GP1 (thyA)             | GCT TTA TTC AAA GGT CAA GAT TTA GTT TAT TTT ATG CCT AGA QA | Forward primer of upstream region of thyA; binds only within chromosomal DNA | This study |
| GP2 (thyA)             | TAC ATG TCG TCC ACT TTA TCA ATC ATT TCT TCA AAT ATT GTT TGC | Reverse primer of downstream region of thyA; binds only within chromosomal DNA | This study |
| ecf-F                  | CCC AGT CAA TGA TCT CAT ATA CA | Forward primer for amplification of the ecf module and Sanger sequencing of ecfA | This study |
| ecf-R                  | TGC GTT GTA ATA GCT TTT CA | Reverse primer for amplification of the ecf module and Sanger sequencing of ecfT | This study |
| ecfA1-R                | AAT AGC TTG ATG CTG GTA TG | Sequencing primer for Sanger sequencing of ecfA | This study |
| ecfA2-F                | ATA AAT CAA ATG CTG GGA CA | Sequencing primer for Sanger sequencing of ecfA' | This study |
| ecfA2-R                | CTT GGA TCA AGA TGA TGA AC | Sequencing primer for Sanger sequencing of ecfA' | This study |
| ecfT-F                 | CAT ATT GGT TTG CCT AGA AT | Sequencing primer for Sanger sequencing of ecfT | This study |
| accA-N315-F            | TCT AAA AAT CCA TCA AGA GG | Forward primer for Sanger sequencing of accA (FASII biosynthesis/FA metabolism) | This study |
| accA-N315-R            | AAA CCC AGT AAC GAT TTA AC | Reverse primer for Sanger sequencing of accA (FASII biosynthesis/FA metabolism) | This study |
| accB-N315-F            | TGG GAT AGA CCT ATA ATG TC | Forward primer for Sanger sequencing of accB (FASII biosynthesis/FA metabolism) | This study |
| accB-N315-R            | AGA TTG CAA CAG TTT GGA TG | Reverse primer for Sanger sequencing of accB (FASII biosynthesis/FA metabolism) | This study |
| accC-N315-F            | TAG AGT ATG GCC AAC CTT TA | Forward primer for Sanger sequencing of accC (FASII biosynthesis/FA metabolism) | This study |
| accC-N315-R            | ATC AGT TAC TTT GAC CAT GG | Reverse primer for Sanger sequencing of accC (FASII biosynthesis/FA metabolism) | This study |
| accD-N315-F            | GAT AAA CAT TCA ACA GTC AA | Forward primer for Sanger sequencing of accD (FASII biosynthesis/FA metabolism) | This study |
| accD-N315-R            | TCA AGC ATG TCA ATT TCT TC | Reverse primer for Sanger sequencing of accD (FASII biosynthesis/FA metabolism) | This study |
| fabD-F                 | AGC AAA AAT AGC AGG AGA GC | Forward primer for Sanger sequencing of fabD (FASII biosynthesis/FA metabolism) | This study |
| fabD-R                 | GTC CAA TTC TCT TTG GTG CA | Reverse primer for Sanger sequencing of fabD (FASII biosynthesis/FA metabolism) | This study |
| fabF-F-3               | ATT ATG AGC ATT GTG CTG TC | Forward primer for Sanger sequencing of fabF (FASII biosynthesis/FA metabolism) | This study |
| fabF-R-2               | ATT GTT CTT GTC GGA TTC GG | Reverse primer for Sanger sequencing of fabF (FASII biosynthesis/FA metabolism) | This study |
| fabG-F                 | TAG AAG ATG TGA AAG GAT GG | Forward primer for Sanger sequencing of fabG (FASII biosynthesis/FA metabolism) | This study |
| fabG-R                 | TCC ATT GGA TGA CCA GTC AA | Reverse primer for Sanger sequencing of fabG (FASII biosynthesis/FA metabolism) | This study |
| fabH-F                 | TTA TTA AGA AGG TGT TCA AC | Forward primer for Sanger sequencing of fabH (FASII biosynthesis/FA metabolism) | This study |
| fabH-R                 | CTA CTC TTA TAT TTT GAC TC | Reverse primer for Sanger sequencing of fabH (FASII biosynthesis/FA metabolism) | This study |
| fabI-F                 | GCT TTG CTC ACA TAT ATA AT | Forward primer for Sanger sequencing of fabI (FASII biosynthesis/FA metabolism) | This study |

(Continued)
TABLE 2 | Continued

| Oligonucleotide primer | Sequence (5’ → 3’) | Purpose | Reference |
|------------------------|--------------------|---------|-----------|
| fabI-R                 | CTG GGA TTA GAT ATT CTA TC | Reverse primer for Sanger sequencing of fabI (FASII biosynthesis/FA metabolism) | This study |
| fabZ-F                 | GGT GCA GAC ATT GAA CGT AT | Forward primer for Sanger sequencing of fabZ (FASII biosynthesis/FA metabolism) | This study |
| fabZ-R                 | TTC AAA GAT TAT GGC AAC AC | Reverse primer for Sanger sequencing of fabZ (FASII biosynthesis/FA metabolism) | This study |
| plsX-F                 | CGT CGA AGT AAA GTC ATA TG | Forward primer for Sanger sequencing of plsX (FA metabolism) | This study |
| plsX-R                 | TTT CAG TTG CCT GAT CTT TG | Reverse primer for Sanger sequencing of plsX (FA metabolism) | This study |

*thyA, thymidylate synthase; ecfA, ECF transporter ATPase; ecfT, ECF transporter transmembrane protein; accA, acetyl-CoA carboxylase, carboxyltransferase, alpha-subunit; accB, acetyl-CoA carboxylase, biotin carboxyl carrier protein-subunit; accC, acetyl-CoA carboxylase, biotin carboxylase-subunit; accD, acetyl-CoA carboxylase, carboxyltransferase beta-subunit; fabD, malonyl-CoA-acyl-carrier-protein (ACP) transacylase; fabF, β-hydroxyacyl-ACP dehydratase; plsX, acyl-ACP-phosphate acyltransferase; FASII, fatty acid biosynthesis type II; and FA, fatty acid.

TABLE 3 | Genetically defined SCVs used as positive controls in auxotrophism studies.

| Strain          | Phenotype | Description                          | Reference |
|-----------------|-----------|--------------------------------------|-----------|
| A3878::hemB     | SCV       | ΔhemB mutant of clinical A3878-WT (hemB::ermB knockout) | (Kriegeskorte et al., 2011) |
| DB-24-COL       | SCV       | ΔmenD mutant of COL (menD::ermC knockout) | (von Eiff et al., 2006) |
| 1549::thyA      | SCV       | ΔthyA mutant of clinical 1549-WT (thyA::ermB knockout) | This study |

*hemB, delta-aminolevulinic acid dehydratase; ermB, erythromycin resistance methylase; ermC, tRNA adenine N-6-methyltransferase; menD, 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate synthase; and thyA, thymidylate synthase.

Germany) and incubated for 24–48 h at 37°C. For evaluation of an underlying auxotrophism, samples were adjusted and streaked on MHA (Merck, Darmstadt, Germany) as described and a maximum of three blank antimicrobial susceptibility disks was laid on top of the MHA (Merck, Darmstadt, Germany) surface. Disks were impregnated with 10 µL of each of the solutions listed in Supplementary Table 3. Auxotrophism of the SCVs was presumed if a growth-promoting effect became exclusively visible only surrounding the impregnated disks after incubation for 24–48 h at 37°C. As positive controls for hemin-, menadione-, and thymidine-auxotrophy, ΔhemB, ΔmenD, and ΔthyA mutants, respectively, were included in all experiments (Table 3). For mutants, MHA (Merck, Darmstadt, Germany) supplemented with erythromycin at 2.5 µg/mL was used.

Further Characterization of Strains

Hemolysis activity was examined by culturing the strains on Columbia blood agar (Becton Dickinson, Franklin Lakes, NJ, United States) for 24 and 48 h. Hemolysis was considered as strongly positive (+++) if showing a clear zone of β-hemolysis, positive (+) if showing a weak and small zone of β-hemolysis, and negative (−) when no hemolysis could be detected. The presence of catalase was confirmed using 3% hydrogen peroxide (Merck, Darmstadt, Germany).

Whole-Genome Sequencing of Triplets

For the PacBio RS II sequencing platform (Pacific Biosciences, Menlo Park, CA, United States), genomic DNAs of WTs, SCVs, and REVs were extracted after lysostaphin treatment (20 µg/mL, 1 h, 37°C) (Wak-Chemie Medical, Steinbach, Germany) using the Genomic-tip 20/G kit (Qiagen, Venlo, Netherlands). This was followed by sequencing on the Pacific Biosciences RS II instrumentation with use of P6 DNA polymerase with C4 chemistry (P6-C4), 110 pM of complexed 20 kb-SMRTbell library, and 240 min continuous movie collection. Initial de novo assembly of reads was performed using the HGAP3 (Chin et al., 2013) v 2.3.0 assembler. Assembly coverages were ranging between 96.77× and 607.93×, with 126,769–153,216 mapped reads and a mean read length from 11,158 to 12,746 bp (N50, 16,228–19,044 bp). Assembled genomes were annotated via the GenDB pipeline (Meyer et al., 2003) and BLAST+. The Staphylococcal regulatory RNAs Database (SRD) (Sassi et al., 2015) was used for detection of small non-protein-coding RNAs (npcRNAs; often referred to as “non-coding RNA”) within the 23S ribosomal RNA module.

Availability of Supporting Data

The genome sequences of the sequenced strains were deposited in the European Nucleotide Archive ENA (Accession No. LT992434–LT992436 for triplet 1549 and LT996889–LT996891 for triplet 24117, respectively).
RESULTS

Phenotypic, Biochemical, and Molecular Characterization

WTs and REVs exhibited a normal phenotype after 24 h (Figure 1) and SCVs demonstrated a significantly reduced colony size on Columbia blood agar (Becton Dickinson, Franklin Lakes, NJ, United States) after 24 h of incubation (Figures 1A,B). Their phenotypes were also stable after 48 h of incubation (Figure 2). WTs and REVs displayed hemolysis after 48 h with 24117-REV showing only a very weak hemolytic activity after 48 h of incubation. 1549-SCV exhibited hemolysis only after 48 h of incubation, whereas 24117-SCV was not hemolytic (Table 1 and Supplementary Figure 1). When cultivated on MHA (not supplemented), 1549-SCV was not able to grow after 48 h of incubation, whereas 24117-SCV exhibited no visible growth after 24 h but grew in micro-colonies after 48 h of incubation (Figure 2). Both SCVs were catalase-positive as their corresponding WTs and REVs (Table 1).

Pulsed-field gel electrophoresis fragment patterns of each strain triplet were identical or varied in only one band (data not shown). MLST and spa typing revealed that all phenotypes of triplet 1549 belonged to a single locus variant of ST45 and SCV exhibited a frameshift mutation in the gene ecfT mutations, Table 4). When cultivated on MHA (not supplemented), 1549-SCV was not able to grow after 48 h of incubation, whereas 24117-SCV exhibited no visible growth after 24 h but grew in micro-colonies after 48 h of incubation (Figure 2). Both SCVs were catalase-positive as their corresponding WTs and REVs (Table 1).

AUXOTROPHISM

Supplementation with hemin, menadione, or thymidine had no growth-supporting effect on the SCVs (data not shown), as shown as an example for the respective knockout mutant SCVs (Supplementary Figure 2). Supplementation with oleic acid solved in Tween 80, a synthetic ester from polyethoxylated sorbitan and oleic acid, restored the normal growth phenotype of both SCVs (Figure 2). SCVs were further tested for auxotrophy for oleic acid and/or polyethylene oxide (PEG) and sorbitan. Auxotrophy was detected for oleic acid solved in NaOH (Figure 2), but not for PEG or sorbitan.

Whole-Genome Sequencing of Strain Triplets

Genome comparison of the three phenotypes of triplet 24117 revealed a total of seven alterations (point and frameshift mutations, Table 4): Due to a deletion of two nucleotides, 24117-SCV exhibited a frameshift mutation in the gene ecfT, which is part of the ecf module and encodes the ECF transporter transmembrane protein EcfT. This mutation caused a premature termination of the gene 19 amino acids downstream of the mutation locus resulting in a truncated protein with only 133 instead of 268 amino acids. While this mutation was still present in the 24117-REV, this strain showed an additional suppressor mutation (one nucleotide deletion) directly upstream of the first mutation locus resulting in the original WT open-reading frame with only one missing amino acid and a conservative amino acid exchange (Y→F). The genomic arrangement of the genes belonging to the ecf module in the genome of triplet 24117 is shown in Figure 3.

Besides these alterations, four other mutations occurring between 24117-WT and 24117-SCV were detected that were still present in 24117-REV without any additional suppressor mutation. All of them constituted point mutations with two of them being silent. Two other point mutations were functional missense mutations in the genes coding for a Na+/phosphate symporter (I358M) and the accessory gene regulator protein A (agrA) (G82R), a part of global virulence regulator agr. Another missense mutation could only be detected in 24117-REV, namely in a gene coding for the aminobenzoyl-glutamate utilization protein B (E385K).

WGS analysis of the triplet 1549 revealed eight alterations with seven of them occurring between 1549-WT and 1549-SCV (Table 4). Similar to the other strain triplet, 1549-SCV exhibited a nucleotide deletion in the ecf module. Here, the gene ecfA encoding for the ECF transporter ATP-binding protein EcFA was affected by a nonsense mutation resulting in a truncated EcFA protein in 1549-SCV with 156 instead of 269 amino acids. Again, similar to 24117-REV, 1549-REV exhibited this mutation as well as a suppressor mutation (one nucleotide insertion) directly upstream of the mutation locus resulting in a restored ecfA gene of 269 nucleotides, but with three amino acid exchanges. The genomic arrangement of the genes belonging to the ecf module was identical to strain triplet 1549 (Figure 3). In each case, the ecf module consisted of two adjacent genes encoding ecf transporter ATP-binding proteins EcFA and EcFB directly upstream of the ecfT gene. A sequence alignment of the ecf modules of 1549-WT and 24117-WT revealed 137 mismatches, one insertion (one nucleotide) and one deletion (one nucleotide). Within the ecfA gene, the sequence of the npcRNA Sau-31 (Abu-Qataouseh et al., 2010) could be identified exhibiting one and two nucleotide exchanges in triplets 1549 and 24117, respectively, when compared to NCTC 8325. Furthermore, for both triplets, 348 and 380 nucleotides upstream of ecfA, the npcRNA sequences Sau-30 (Abu-Qataouseh et al., 2010) and sRNA347 (Howden et al., 2013) could be found (Figure 3). Compared to NCTC 8325, there were three nucleotide exchanges in Sau-30 in triplet 1549 and one in triplet 24117, whereas sequences encoding sRNA347 were identical to NCTC 8325.

All other nucleotide alterations between 1549-WT and 1549-SCV could also be identified in 1549-REV without any suppressor mutation and comprise a nucleotide exchange that resulted in a conservative missense mutation in a phosphodiesterase (G290V). Furthermore, a total of five loci showed insertions of a number of 1–12 nucleotides in the genome of 1549-SCV resulting in (i) a frameshift within a hypothetical protein, (ii) an insertion of two amino acids followed by a frameshift within a histidinol-phosphate aminotransferase, and (iii) three different inframe insertions in the DNA topoisomerase IV subunit A, within a
FIGURE 1 | (A) Clinical S. aureus strain triplets 1549 and 24117 each comprising isogenic wild-type (WT), small-colony variant (SCV), and revertant (REV) phenotypes after 24 h of incubation at 37°C on Columbia blood agar with 5% sheep blood; scale bar indicates 5 mm. (B) Colony sizes of WTs, SCVs, and REVs after 24 h incubation on Columbia blood agar at 37°C; ***P ≤ 0.001.

FIGURE 2 | Phenotype of clinical S. aureus strain pairs after 48 h of incubation at 37°C on MHA with and without supplementation with oleic acid and on Columbia blood agar with 5% sheep blood; scale bar indicates 5 mm; diameter of the discs is 6.4 mm.
permease domain-containing protein and another hypothetical protein, respectively (Table 4).

Screening for Alterations Within the ecf Module and the FASII Pathway Genes

Another three isogenic strain triplets comprising SCVs with dependency on oleic acid were screened for alterations within genes of the ecf module and genes linked to the FASII pathway. For all genes analyzed, mutations between WT and SCV phenotypes could not be detected.

DISCUSSION

For clinical SCVs, only few underlying genetic alterations for distinct auxotrophies were hitherto identified, whereas the genetic basis for the phenotypic switch remains unclear for the majority of clinical SCVs (von Eiff et al., 1997b; Schaaff et al., 2003; Chatterjee et al., 2008; Lannergård et al., 2008; Abu-Qatouseh et al., 2010; Köser et al., 2012; Lin et al., 2016).

The SCVs investigated here did not show any of the well-characterized auxotrophies for hemin, menadione, and/or thymidine, but dependency on monounsaturated oleic acid. 1549-SCV was originally described as heme auxotroph (Kriegeskorte et al., 2014b), which becomes explainable due to the use of Tween 80 as solvating reagent for hemin in earlier studies (Kriegeskorte et al., 2014b), which becomes explainable due to the use of Tween 80 as solvating reagent for hemin in earlier studies. For clinical SCVs, only few underlying genetic alterations for distinct auxotrophies were hitherto identified, whereas the genetic basis for the phenotypic switch remains unclear for the majority of clinical SCVs (von Eiff et al., 1997b; Schaaff et al., 2003; Chatterjee et al., 2008; Lannergård et al., 2008; Abu-Qatouseh et al., 2010; Köser et al., 2012; Lin et al., 2016).

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### TABLE 4 | Genetic alterations between the three phenotypes of two *S. aureus* strain triplets detected with a whole-genome sequencing approach.

| Triplet No. 24117 | DNA profile/mutation (5′ → 3′) | Function | Locus tag (identities in %) | Effect of mutation3 in: SCV compared to the WT | REV compared to the SCV |
|------------------|--------------------------------|----------|----------------------------|-----------------------------------------------|--------------------------|
| T (900)          | A                              | A H      | Hypothetical protein       | SAOUHSC_00179 (99)                           | Silent                   |
| A (1,074)        | G                              | G        | Na+/phosphate symporter    | SAOUHSC_00060 (99)                           | Missense mutation (G358M) |
| GGCTTTATATAC      | G-CGGTTATAT-C                  | G-C      | ECF transporter transmembrane protein EcfT (ecf module) | SAOUHSC_02481 (99) | Frameshift with stop codon after 19 AAs (S114fsX134) |
|                  | (529–541)                      | C-C      |                             |                                               |                         |
|                  |                                |          |                             |                                               | Suppressor mutation, frameshift, inframte with WT (L111fsX26824) |
| G (1,153)        | G                              | A H      | Aminobenzoyl-glutamate utilization protein B | SAOUHSC_02374 (99) | Missense mutation (E385K) |
| G (244)          | C                              | C H      | Accessory gene regulator protein A (agrA) | SAOUHSC_02265 (99) | Missense mutation (G82R) |
| C (1,137)        | G                              | G        | SLT orf 527-like protein    | SAOUHSC_01523 (97)                           | Silent                   |

| Triplet No. 1549 | DNA profile/mutation (5′ → 3′) | Function | Locus tag (identities in %) | Effect of mutation3 in: SCV compared to the WT | REV compared to the SCV |
|------------------|--------------------------------|----------|----------------------------|-----------------------------------------------|--------------------------|
| T-----A (1,704–1,705) | TGCAGATA  | TGCAGATA | DNA topoisomerase IV subunit A | SAOUHSC_01352 (99) | Insertion, infame (D568Ins66 insAD) |
| C-AAGTTGAT (461–470) | C-AAGTTGAT-A | C-AAGTTGAT-A | ECF transporter ATPase EcfA (ecf module) | SAOUHSC_02483 (98) | Nonsense mutation (L157X) |
|                  |                                |          |                             |                                               | Suppressor mutation, frameshift, inframte with WT (S155fsX27037) |
|                  |                                |          |                             |                                               |                         |
|                  |                                |          |                             |                                               |                         |
|                  |                                |          |                             |                                               |                         |
|                  |                                |          |                             |                                               |                         |

1. Changed, inserted, or deleted nucleotides with respect to the WT were given in bold and underlined. Nucleotide numbers of + strand of respective gene of the WT were given in parentheses. 2. Gene identification number in *S. aureus* NCTC 8325 (NC_007795.1) (Zhang et al., 2000; Gillapsy et al., 2006). Nucleotide identities between the locus of the respective WT and NCTC 8325 were given in %. 3. New protein profile of the respective strain is given in parentheses.

## Notes:
- **REV** exhibited the same reading frame like WT with the exception of one exchanged and one missing amino acids.
- **REV** exhibited the same reading frame like WT with three exchanged amino acids.
- Here, nucleotide triplet encoding the amino acids upstream of the insertion site was interrupted leading to a change of the respective amino acids. **∅**, no difference in protein profile; AA, amino acid; ins, insertion (one or more amino acids were inserted between the two amino acids mentioned); fs, frameshift (first amino acid affected is mentioned first and the new open-reading frame of the mutated protein is open until stop codon X at position mentioned).
identical to an ecf module type described in *Bacillus subtilis* (Rodionov et al., 2009).

To date, 21 different EcFS have been identified, among these pantothenic acid, also called vitamin B₅ (Rodionov et al., 2009; Slotboom, 2014). This vitamin is necessary for the biosynthesis of the ubiquitous coenzyme A (CoA). CoA is essential for biosynthesis of fatty acids (Begley et al., 2001) and a lack in CoA levels will thus inevitably lead to complications in fatty acid synthesis. Moreover, CoA plays also a key role in the energy production of the cell. Once in the form of acetyl-CoA, it enters the TCA cycle and the electrons obtained are used during oxidative phosphorylation for the generation of ATP. For 1549-SCV and other clinical and genetically defined SCVs, a down-regulation of TCA cycle activity could already be shown (Kohler et al., 2003; Chatterjee et al., 2005, 2007; Gaupp et al., 2010; Kriegeskorte et al., 2014b). Therefore, for both SCVs, one can speculate that the mutations within the ecf module might be also the cause for a reduced uptake of vitamin B₅, resulting in insufficient amounts of intracellular CoA. Thus, mutations within the ecf module may lead to both the impaired FASII metabolism and the downregulated TCA cycle as explanation for the slow growth of the analyzed SCVs.

While being essential in fatty acid synthesis, CoA is not needed for the incorporation of fatty acids into the membrane in the case of *S. aureus* (Parsons et al., 2011, 2014). Uptake of supplemented oleic acid may therefore restore membrane synthesis in oleic acid-auxotrophic SCVs. Accordingly, higher amounts of CoA are available for entering the TCA cycle and contributing to the reversion of the phenotype. Furthermore, bacilli deficient for pantothenate were found to be limited in growth (Baigori et al., 1991) and their defective uptake of glutamic acid was shown to be reversible by supplementation of unsaturated fatty acids (Holden and Bunch, 1972).

Since for type-2 ECF transporters multiple EcFS can use the same EcFαAT module to form an active transporter complex (Rodionov et al., 2009), there might be also a lack of other substrates being responsible for the SCV phenotype such as riboflavin, niacin, and biotin. Riboflavin (vitamin B₂), an essential component of the basic metabolism, represents a precursor of coenzyme flavin adenine dinucleotide (FAD) (Vitreschak et al., 2002; LeBlanc et al., 2017). Niacin (vitamin B₃), a component of NAD, as well as biotin (vitamin B₇) are also known to be required or to constitute stimulatory factors for growth of *S. aureus* (Peterson and Peterson, 1945). Besides CoA, NAD and FAD are also important cofactors needed in the TCA cycle and, thus, maybe also implicated in growth characteristics of the tested SCVs.

Staphylococcal SCVs not defective for fatty acids may also exhibit alterations in ecf modules mediating the uptake of other B-group vitamins. Auxotrophy for thiamine (vitamin B₁) was already identified being responsible for SCVs (Ziv and Sompolinsky, 1976; Acar et al., 1978). The same is probably the

### TABLE 5 | Genetically defined and FabI inhibitor selected strains with defects in fatty acid incorporation or biosynthesis (FASII).

| Strain | Description/mutation¹ | Phenotype | Reference |
|--------|-----------------------|-----------|-----------|
| PS01 (ΔaccD) | *S. aureus* with 900 bp intron insert, at 164 bp (knockout mutant) | Fatty acid and lipoic acid auxotroph | (Parsons et al., 2011) |
| JP102 (ΔaccDAfabl) | *S. aureus* with 900 bp intron inserts, at 164 and 167 bp, respectively (knockout mutant) | Fatty acid auxotroph | (Parsons et al., 2011) |
| MWF23 (ΔaccD) | *S. aureus* with g232t STOP at residue 77 (AFN-1252-selected) | Fatty acid auxotroph | (Parsons et al., 2011) |
| MWF26 (accC<sup>G65E</sup>) | *S. aureus* with a257t (AFN-1252-selected) | Fatty acid auxotroph | (Parsons et al., 2011) |
| MWF28 (ΔaccC) | *S. aureus* with 301 bp deletion at residue 77 (AFN-1252-selected) | Fatty acid auxotroph | (Parsons et al., 2011) |
| JP103 (RN6930ΔaccD) | RN6930 with 900 bp insert, at 164 bp (knockout mutant) | Fatty acid and lipoic acid auxotroph | (Parsons et al., 2013) |
| PCD39 (SA178R1 ΔpIsX) | SA178R1 with 366 bp intron insert (knockout mutant) | Fatty acid auxotroph | (Parsons et al., 2014) |
| ATCC43300_P10 (fabD<sup>G113C</sup>) | ATCC43300 with SNP in both the fabl and the fabD gene (triclosan-selected) | Fatty acid auxotroph | (Bazaid et al., 2018) |
| Newman_P10 (fabD<sup>G59V</sup>) | Newman with SNP in both the fabl and the fabD gene (triclosan-selected) | Fatty acid auxotroph | (Bazaid et al., 2018) |
| NCTC13277_P10 (fabD<sup>G59V</sup>) | NCTC13277 with SNP in the fabl gene (triclosan-selected) | Fatty acid auxotroph | (Bazaid et al., 2018) |
| SAR17_P10 (fabD<sup>G59V</sup>) | SAR17 with SNP in the fabl gene (triclosan-selected) | Fatty acid auxotroph | (Bazaid et al., 2018) |

¹The method of generation of the SCVs is mentioned in parentheses; AFN-1252 and triclosan represent FabI inhibitors. accC, acetyl-CoA carboxylase, biotin carboxylase-subunit; accD, acetyl-CoA carboxylase, carboxyl transferase beta-subunit; fabl, enoyl-acyl-carrier-protein (ACP) reductase; fabD, malonyl-CoA-ACP transacylase; pIsX, acyl-ACP-phosphate acyltransferase; and SNP, single-nucleotide polymorphism. 
case for the ECF substrate folate (vitamin B\textsubscript{9}). In a previous study, we could show a down-regulation of a protein cluster involved in the folate metabolism for a clinically derived SCV (Kriegeskorte et al., 2011).

By contrast, other SCVs dependent on oleic acid screened in this study showed neither mutations within the ecf module nor alterations in genes linked to the FASII pathway. This was also the case for genes acc\textsubscript{C}, acc\textsubscript{D}, and pls\textsubscript{X} for which genetically defined knockout mutants were already proven to exhibit auxotrophy for fatty acids (Table 5; Parsons et al., 2011, 2013, 2014). This underlines the high diversity of potential mutation loci leading to the phenotype switch. Furthermore, high reversion rates of clinical SCVs may indicate phenotypical heterogeneity based on genetic alterations being not yet detectable (Avery, 2006). Besides the genomic background, regulation processes, e.g., via differentially expressednpcRNAs are also involved in SCV formation (Abu-Qatouseh et al., 2010). ECF transporter can be coupled with npcRNAs (riboswitches) (Rodionov et al., 2009). It was previously demonstrated that npcRNA Sau-31 is developmentally regulated and not expressed in a clinical SCV during stationary phase (Abu-Qatouseh et al., 2010) indicating potential impairment of ECF substrate uptake in the according SCV. This might be a hint for the involvement of these npcRNAs in the SCV phenotype switch.

By WGS, we were furthermore able to find probable genetic drivers for the variable hemolysis behavior between the REV phenotypes of the two strain triplets. Whereas 1549-REV showed a normally restored \(\beta\)-hemolysis after 48 h of incubation at 37\(^\circ\)C, 24117-REV only exhibited weak hemolysis under the same conditions. Sequence analysis of the strain triplet 24117 revealed no alterations in the genes hla, hlb, hld, and hlg (encoding for \(\alpha\), \(\beta\), \(\gamma\), and \(\delta\)-hemolysin), but a mutation in the gene agr\textsubscript{A} occurring in 24117-SCV and 24117-REV. This mutation leads to a non-conservative amino acid exchange (G82R) in Agr\textsubscript{A}, part of the global virulence regulator agr (Table 4). Agr\textsubscript{A} is responsible for the activation of agr promotor P3 and therefore for the transcription of RNAIII (Novick et al., 1993), which also encodes the agr-regulated \(\delta\)-hemolysin hld (Janzon et al., 1989). RNAIII stimulates the translational regulation of proteolytic enzymes and several exotoxins like hla encoded \(\alpha\)-hemolysin (Morfeldt et al., 1995) and its downregulation is shown to be a characteristic trait in SCVs (Proctor et al., 2014). The accompanying loss of hemolytic activity was recently shown to be caused by a point mutation within the agr\textsubscript{C} gene of S. aureus resulting in an amino acid substitution that leads to a destabilization of the AgrC–Agr\textsubscript{A} interaction (Maipady Shambat et al., 2016). Moreover, it was already shown that another mutation in agr\textsubscript{A} is responsible for the non-hemolytic phenotype of laboratory strain RN4220 due to defective translation of hla and hld (Traber and Novick, 2006). Accordingly, in 24117-REV, the mutated agr\textsubscript{A} may most likely contributed to the poor hemolysis.

Comparison of whole genomes of the included strains revealed the close relationship between the isogenic, but phenotypically different strains showing only seven to eight alterations per strain triplet. The number of the genetic events was found to be similar to other WGS approaches tracking the mutability of isogenic S. aureus strains. However, in many cases, isogenic strains were isolated before and after extensive chemotherapy resulting in approx. 2–30 mutations most likely driven by adaptation to the antibiotics and by pathogenesis mechanisms (Mwangi et al., 2007; Lannergård et al., 2011; Peleg et al., 2012; Lin et al., 2016).

Our study emphasized that WGS-based identification of all mutations leading to a phenotype switch may prove workable, while generation of knockout mutants can be expedient only for analyzing one or a few genes of interest. Furthermore, if the gene affected is not directly linked to the respective auxotrophism, knockout mutant generation is ineffectual. This should be considered especially for undefined auxotrophies.

**CONCLUSION**

This study demonstrated that the genetic background of SCVs is highly diverse and that the detection of underlying genes inducing the phenotypic switch is mandatory for a better understanding of this phenotype. Furthermore, the study reveals a novel dynamic process of phenotype switching between naturally occurring SCVs and REVs displaying the normal phenotype. WGS seems to be the most reliable and efficient tool for detecting the underlying genetic mechanisms responsible for the SCV formation and the way back to the WT phenotype. Alterations within ECF transporters as detected here display further drivers for the phenotype switch from WT to SCV by limitation of cellular nutrient uptake, thus leading to a downregulation of the TCA cycle activity. Moreover, this study underlines the importance of including clinically derived strains when studying the genetic background of phenotypic variation.

**AUTHOR CONTRIBUTIONS**

KB designed the study concept. AK performed initial studies. NS designed the experiments, performed laboratory work, evaluated the data, drafted, and wrote the manuscript. UK contributed in data evaluation and writing the manuscript. MD performed cloning experiments. JS provided scientific support regarding whole-genome sequencing. RP, GP, and CE provided scientific support regarding SCVs and data interpretation. All authors have read and approved the final draft of the article.

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SUPPLEMENTARY MATERIAL
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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