Livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) of clonal complex CC398 typically carry various antimicrobial resistance genes, many of them located on plasmids. In the bovine LA-MRSA isolate Rd11, we previously identified plasmid pAFS11 in which resistance genes are co-localized with a novel ica-like gene cluster, harboring genes required for polysaccharide intercellular adhesin (PIA)-mediated biofilm formation. The ica genes on pAFS11 were acquired in addition to a pre-existing ica locus on the *S. aureus* Rd11 chromosomal DNA. Both loci consist of an icaADBC operon and icaR, encoding a corresponding icaADBC repressor. Despite being different on the amino acid sequence level, the two IcaR repressor proteins are mutually replaceable and are able to interact with the icaA promoter region of the other copy. We speculate that this regulatory crosstalk causes the biofilm-negative phenotype in *S. aureus* Rd11. The data shed light on an unexpected regulatory interplay between pre-existing and newly acquired DNA traits in *S. aureus*. This also raises interesting general questions regarding functional consequences of gene transfer events and their putative implications for the adaptation and evolution of bacterial pathogens.

**Keywords:** *Staphylococcus aureus*, biofilm regulation, PIA/ica, IcaR, horizontal gene transfer, plasmid-chromosome crosstalk
INTRODUCTION

*Staphylococcus aureus* is a common human and animal pathogen, causing a wide range of clinical manifestations (Tong et al., 2015; Ballhausen et al., 2017). Due to the capability to readily acquire many different resistance genes, *S. aureus* and other staphylococcal species are regarded as pathogens of concern for public health (Foster, 2017; Lakhundi and Zhang, 2018). Thus, methicillin-resistant *S. aureus* (MRSA) and coagulase-negative staphylococci (MR-CoNS) are among the most common causes of healthcare-associated infections (Lee et al., 2018a; Becker et al., 2020). In this respect, the ability to form biofilms on the inert surfaces of medical devices is considered as important pathomechanism that contributed to the establishment of staphylococci as notorious nosocomial pathogens (Heilmann et al., 2019; Becker et al., 2020; Schilcher and Horswill, 2020). Biofilms are understood as bacterial communities that adhere to surfaces by encasing into a self-produced extracellular polymeric matrix (Costerton et al., 1999). The staphylococcal biofilm matrix may contain exopolysaccharides (Heilmann et al., 1996) and proteins (Rohde et al., 2005) as well as extracellular (e)DNA (Qin et al., 2007) [for a recent review see reference (Schilcher and Horswill, 2020)]. The key exopolysaccharide component of staphylococcal biofilms is PIA (polysaccharide intercellular adhesin), a beta-1,6 linked N-acetyl glucosaminoglycan, whose synthesis enzymes are encoded by the *ica* (intercellular adhesin) locus [recently reviewed in (Nguyen et al., 2020)]. PIA/ica was originally discovered in *Staphylococcus epidermidis* and was later also detected in *S. aureus* and other staphylococcal species (Heilmann et al., 1996; Mack et al., 1996; Cramton et al., 1999). Interestingly, *ica* locus homologs also exist in phylogenetically unrelated bacteria such as *Escherichia coli* (Wang et al., 2004), suggesting an eminent role of the factor in the evolution of bacterial biofilm functions. In these organisms, PIA is also often referred to as PNAG (poly-1,6-N-acetylglicosamine). The staphylococcal *ica* locus consists of two divergently oriented transcription units, one comprising the *icaABCD* operon (encoding the enzymes required for PIA synthesis) and the other harboring *icaR* which codes for a transcription factor of the TetR family (Figure 1A). IcaR, for which the crystal structure was solved, binds to a region upstream of *icaA* and represents a potent repressor of *icaABCD* operon transcription (Conlon et al., 2002a; Jefferson et al., 2004; Jeng et al., 2008). Regulation of the *ica* locus is highly complex and a plethora of environmental cues are known to influence PIA production many of which either directly or indirectly influencing *icaR* transcription (Conlon et al., 2002b; Cerca et al., 2008; Fey and Olson, 2010; Cue et al., 2012; Hoang et al., 2019; Nguyen et al., 2020). Expression of *icaR* is further controlled post-translationally through RNA-mediated mechanisms that influence stability and translation of the *icaR* mRNA, with direct consequences for PIA production and biofilm formation (Ruiz de los Mozos et al., 2013; Rochat et al., 2018; Bronsky et al., 2019; Lerch et al., 2019; Schoenfelder et al., 2019). While nearly all *S. aureus* genomes carry the *ica* locus, distribution of the gene cluster among *S. epidermidis* and other CoNS species is more diverse and often associated with distinct clonal lineages (Kozitskaya et al., 2005; Conlan et al., 2012; Thomas et al., 2014; Méric et al., 2015; Méric et al., 2018; Lee et al., 2018b; Espadinha et al., 2019). The *ica* locus is usually located in the bacterial chromosomal DNA in all staphylococcal species. Previously, however, we detected an *ica* gene cluster of unknown genetic origin on plasmid pAFS11 in the bovine MRSA isolate *S. aureus* Rd11 (Feßler et al., 2017). *S. aureus* Rd11 is a livestock-associated (LA)-MRSA strain of sequence type ST398, a clonal lineage known for its potential to carry a broad range of both common and novel antibiotic resistance genes, many of which located on plasmids (Kadlec et al., 2012; Feßler et al., 2018). On pAFS11, antimicrobial and heavy metal resistance genes were found to be co-localized with a novel *ica* gene cluster. The *ica* locus on pAFS11 differed both on nucleotide and protein levels from the copy in the *S. aureus* Rd11 chromosome, and initial analyses (i.e. BLAST searches against the entire non-redundant sequence collections at NCBI) suggested that the plasmid-borne *ica* locus might have its origin in the CoNS species *Staphylococcus sciuri* [recently re-classified as *Mammmalianicus sciuri* (Madhaiyan et al., 2020)] (Feßler et al., 2017). The mosaic structure of pAFS11 further suggests that the plasmid arose by a series of recombination events and was acquired by *S. aureus* Rd11 through horizontal gene transfer (HGT). As a result, *S. aureus* Rd11 carries two *ica* loci. Surprisingly, however, the strain did not produce biofilm when tested in standard tissue culture plate assays. Also, transformation of the pAFS11 plasmid into another *S. aureus* strain did not prompt biofilm formation, but even slightly reduced it (Feßler et al., 2017). In this study, we address the molecular mechanism underlying the biofilm-negative phenotype of pAFS11-bearing *S. aureus*. We identified an unexpected IcaR-mediated regulatory crosstalk between the plasmid-borne and chromosomally encoded *ica* loci, resulting in downregulation of biofilm formation. We discuss these findings in the context of co-evolution of virulence and resistance traits and raise the question of how genes newly acquired by HGT might become integrated into the regulatory network of host bacteria.

MATERIALS AND METHODS

Sequence Alignments and Data Base Searches

Alignments of the nucleotide sequences of *ica* loci as well as that of amino acid sequences of Ica proteins from different species were performed with CLUSTAL Omega multiple sequence alignments (https://www.ebi.ac.uk/Tools/msa/clustalo) (Madeira et al., 2019). Average distance between the *ica* loci and pairwise alignments were calculated with the aid of Jalview (Waterhouse et al., 2009). Strains and sequences included into the analyses comprised *S. epidermidis* RP62A (accession no. NC_002976), *S. epidermidis* O-47 (accession no.
CP040883), S. aureus ST398 (accession no. AM990992.1),
S. aureus RN4220 (accession no. AFGU01000118.1), S. sciuri
NS1 (accession no. LDTK01000031.1) and plasmid pAFS11
(accession no. FN806789.3). Data base queries with nucleotide
and protein sequences were performed using the Basic Local
Alignment Search Tool available at the National Center for
Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.
gov/Blast.cgi).

**Plasmid and Strain Construction**

Strains, plasmids and oligonucleotides used for this work are
listed in **Tables 1** and **2**, respectively.
### TABLE 1 | Strains and plasmids.

| Strains | Description | Reference |
|---------|-------------|-----------|
| **E. coli** | *E. coli* for plasmid transformation into staphylococci | (Monk et al., 2012) |
| **S. aureus** | Restriction-deficient *S. aureus* strain | (Nair et al., 2011) |

#### Rd11
RN4220
LA-MRSA carrying pAFS11 plasmid
RN4220 transformed with pAFS11
RN4220 transformed with pGM10 (picaR)
RN4220 transformed with pGM11 (picaR, ΔicaR)
RN4220 transformed with pGM14 (picaR, ΔicaR, icaR)
GM20
RN4220 transformed with pGM10 (picaR)
GM28
RN4220 transformed with pGM11 (picaR, ΔicaR)
AK18
RN4220 transformed with pAK17
GM30
RN4220 Δica
GM33
GM30 (RN4220 Δica) transformed with pGM10 (picaR)
GM35
GM30 (RN4220 Δica) transformed with pGM11 (picaR, ΔicaR)
GM42
GM30 (RN4220 Δica) transformed with pGM12 (picaR)
GM44
GM30 (RN4220 Δica) transformed with pGM13 (picaR, ΔicaR)
GM46
GM30 (RN4220 Δica) transformed with pGM14 (picaR, ΔicaR, icaR)
GM49
GM30 (RN4220 Δica) transformed with pGM15 (picaR, ΔicaR, icaR)
GM57
GM30 (RN4220 Δica) transformed with pGM16 (picaR, ΔicaR, icaR)
GM59
GM30 (RN4220 Δica) transformed with pGM17 (picaR, ΔicaR, icaR)
GM61
GM30 (RN4220 Δica) transformed with pGM18 (picaR, ΔicaR, icaR)
GM63
GM30 (RN4220 Δica) transformed with pGM19 (picaR, ΔicaR, icaR)
GM65
GM30 (RN4220 Δica) transformed with pGM20 (picaR, ΔicaR, icaR)
GM67
GM30 (RN4220 Δica) transformed with pGM21 (picaR, ΔicaR, icaR)

#### Others
RP62A
S. epidermidis biofilm positive reference strain
TM300
S. carnosus biofilm negative reference strain

#### pAFS11
Original plasmid isolated from Rd11

#### iBASE6 mutant construction
pBASE6
Suicide mutagenesis vector
pAK17
pBASE carrying ica flanking region for ica deletion

#### ica complementation
pRB473
Staphylococcal shuttle vector
pGM10
pRB473 with ica operon from pAFS11 (icaR)
This work
pGM11
pRB473 with ica operon from RN4220 (icaR)
This work
pGM12
pRB473 with ica operon from RN4220 (icaR)
This work
pGM13
pRB473 with ica operon from RN4220 (icaR)
This work
pGM14
pRB473 with ica operon from RN4220 (icaR)
This work
pGM15
pRB473 with ica operon from RN4220 (icaR)
This work
pGM16
pRB473 with ica operon from RN4220 (icaR)
This work
pGM17
pRB473 with ica operon from RN4220 (icaR)
This work
pGM18
pRB473 with ica operon from RN4220 (icaR)
This work
pGM19
pRB473 with ica operon from RN4220 (icaR)
This work
pGM20
pRB473 with ica operon from RN4220 (icaR)
This work
pGM21
pRB473 with ica operon from RN4220 (icaR)
This work

** *** symbolized mutated palindromes.

### Construction of a Markerless icaADBC Mutant

The markerless ica mutant was obtained via allelic replacement with inducible counter-selection using the pBASE6 shuttle vector (Bae and Schneewind, 2006; Geiger et al., 2012). pBASE6 vector was linearized using primers SLIC_pBASE_R and SLIC_pBASE_F. Total deletion was achieved by overlapping PCR using as template gDNA from RN4220 with primers Flank_A_SLIC together with Flank_A_rev and Flank_B_rev together with Flank_B_SLIC. The amplicon was introduced into the linearized pBASE6 vector using the in vivo E. coli cloning (iVEC) method (Nozaki and Niki, 2019). As iVEC turned out to be more efficient in the presence of buffer, the ligation buffer from the QuickLigation™ Kit (NEB, #M2200S) was added to the reactions. Sanger sequencing was used to verify accuracy of all plasmids. To create plasmids pGM10 and pGM12, the icaR coding regions from plasmids pGM10 and pGM12 were amplified from pAFS11 and RN4220, respectively, and introduced in the linearized pRB473. Deletion of icaR coding regions from plasmids pGM10 and pGM12 (resulting in plasmids pGM11 and pGM13, respectively) was verified by PCR with oligonucleotides spanning the deletion region.

### Construction of Complementation Plasmids

All plasmids were created following the iVEC method (Nozaki and Niki, 2019). As iVEC turned out to be more efficient in the presence of buffer, the ligation buffer from the QuickLigation™ Kit (NEB, #M2200S) was added to the reactions. Sanger sequencing was used to verify accuracy of all plasmids. To create plasmids pGM10 and pGM12, the icaR coding regions from plasmids pGM10 and pGM12 were amplified from pAFS11 and RN4220, respectively, and introduced in the linearized pRB473. Deletion of icaR coding regions from plasmids pGM10 and pGM12 (resulting in plasmids pGM11 and pGM13, respectively) was
achieved by overlapping PCRs which amplified the respective vectors in two fragments that overlapped in the icaR deletion and multiple cloning site regions. Of note, this approach left putative icaR5' and 3' untranslated regions (UTRs) intact which might be involved in post-transcriptional regulation of icaR.

icaR_{pAF} was amplified from pAFS11 and introduced in the linearized pGM12, resulting in plasmid pGM14. icaR_{RN} was amplified from RN4220 and introduced in the linearized pGM10, resulting in plasmid pGM15. The vectors containing mutated sequences which alter the palindromes (pGM16 to pGM21) were generated by PCR site-directed mutagenesis amplifying the original vectors (see Table 2 for details) in two fragments that overlapped in the region containing the mutated palindrome and in the multiple cloning site region. In Table 2, the mutated palindrome sequences are shown as lower-case characters in the primer sequences.

### Preparation of Total RNA and qRT-PCR

Total RNA of bacteria was isolated as described previously (Lerch et al., 2019). Briefly, RNA was precipitated with 1x TABLE 2 | Oligonucleotides.

| Purpose | Template | Name | Sequence |
|---------|----------|------|----------|
| qRT-PCR | – | GM027 | ACGGATATTTAGTGGTGGG |
| icaA_{rev} | – | GM028 | TGCAAACCTCCTCTGAAATG |
| icaA_{pAF} | – | GM020 | AACAGAGGTAAAGCCAACGC |
| icaR_{pAF} | – | GM021 | ATTGGTGCATCTTGATCAACG |
| icaR_{RN} | – | GM016 | TCCCTGTATCTGCTCGGATTG |
| ica deletion mutant | pAK17 | Flank_A_SLM | GATCTGTCGACGATAACAGATACTATTGGAGATACT |
| | | Flank_A_rev | ATGGTGCATCTTGATCAACG |
| | | Flank_B_SLM | GCCATGAAAGCTGATACAATATG |
| | | Flank_B_rev | ATGATTTACCAATGCCAATGGGAGTGGGACA |
| | pBASE6 | SLIC_pBASE_R | TACGTGCAAGCATCGTCGCC |
| | | SLIC_pBASE_F | TCAAGCTGCAAGCATCGTCGCC |
| Deletion Confirmation | GM176 | TGCTGAAACATACAAACAATA |
| | GM177 | AAGGTAATCATGCAATATG |
| Complementation plasmids | pGM10 | pRB473 | SLIC_pRB473_R |
| | | SLIC_pRB473_F | TCCCTGAAAGCTGACAGAAGAATGACATACCA |
| | pAFS11 | GM154 | CTTGCATGTCGACGACAGAAGAATGACATACCA |
| | pGM12 | SLIC_icaCSc_R | TCCCTGAAAGCTGACAGAAGAATGACATACCA |
| | | SLIC_icaCSc_F | TCCCTGAAAGCTGACAGAAGAATGACATACCA |
| | RN4220 | GM178 | CTTGCATGTCGACGACAGAAGAATGACATACCA |
| | | GM179 | TCCCTGAAAGCTGACAGAAGAATGACATACCA |
| | GM15 | GM156 | GAGGCAAAATGAAATTCCAAACAAACTGATA |
| | | GM155 | GCTTTTGTATGAAATTCCAAACAAACTGATA |
| | | GM157 | AGCAAGTTTATGGGTCGCCCTCTAAAG |
| | | pRB473_MCS_F | GTTGTTACGAGCTGACCCCTCAG |
| | | pRB473_MCS_R | CTGCTCTACGAGCTGACCCCTCAG |
| | pGM13 | GM152 | SLIC_pGM13_F |
| | | SLIC_pGM13_R | AACATTTGCAAAATCATAATGAAATG |
| | | GM153 | AAAAAATGTGAAAAATATGAAATG |
| | | GM154 | ATGATTTACCAATGCCAATGGGAGTGGGACA |
| | pGM14 | GM186 | SLIC_pGM14_F |
| | | SLIC_pGM14_R | ATGATTTACCAATGCCAATGGGAGTGGGACA |
| | | GM187 | ATGATTTACCAATGCCAATGGGAGTGGGACA |
| | | GM188 | ATGATTTACCAATGCCAATGGGAGTGGGACA |
| | | GM189 | ATGATTTACCAATGCCAATGGGAGTGGGACA |
| | | GM190 | ATGATTTACCAATGCCAATGGGAGTGGGACA |
| | pGM15 | RN4220 | GM191 | ATGATTTACCAATGCCAATGGGAGTGGGACA |
| | | GM192 | ATGATTTACCAATGCCAATGGGAGTGGGACA |
| | | GM193 | ATGATTTACCAATGCCAATGGGAGTGGGACA |
| | | GM194 | ATGATTTACCAATGCCAATGGGAGTGGGACA |
| | | GM195 | ATGATTTACCAATGCCAATGGGAGTGGGACA |
| | | pGM16/pGM17/pGM18 | pGM10/pGM11/pGM15 | GM202 | ATAGTATATCtaaaagtAAGAAAAATAGGAAATG |
| | | | pRB473_MCS_F | GCTTTAAGGAGGCAAATGAATTGAAAGGATA |
| | | | pRB473_MCS_R | ATGATTTACCAATGCCAATGGGAGTGGGACA |
| | pGM19/pGM20/pGM21 | pGM10/pGM11/pGM15 | GM203 | ATAGTATATCtaaaagtAAGAAAAATAGGAAATG |
| | | | pRB473_MCS_F | TXTTTCAAGGAGGCAAATGAATTGAAAGGATA |
| | | | pRB473_MCS_R | ATGATTTACCAATGCCAATGGGAGTGGGACA |
| | | GM204 | ATGATTTACCAATGCCAATGGGAGTGGGACA |
| | | GM205 | ATGATTTACCAATGCCAATGGGAGTGGGACA |
| | | GM206 | ATGATTTACCAATGCCAATGGGAGTGGGACA |
| | | GM207 | ATGATTTACCAATGCCAATGGGAGTGGGACA |
volume isopropanol (Sigma-Aldrich, #I9516) for 10 minutes at room temperature. Pelleted RNA was washed with 70 % ethanol and solved in RNase-free double-distilled water (ddH₂O). The transcript abundance of icaA<sub>pAF</sub>, icaR<sub>pAF</sub> and icaA<sub>Rd11</sub> from three independent experiments was determined by real-time qRT-PCR. Thus, 5 µg of each RNA sample was treated with DNaseI (Thermofisher, #AM2235) for 45 min at 37°C and the reaction was stopped by phenol/chloroform/isoamylalcohol extraction (25:24:1, Carl Roth GmbH, #X985.2) with the aid of PLG heavy tubes (5 Prime, #2302830). RNA was precipitated overnight at -20°C with 4.67x volume ethanol/3M sodium acetate pH 6.5 (Thermofisher, #AM9740) (30:1 mix). Pelleted RNA was washed with 70 % ethanol, dissolved in 30 µl RNase-free ddH₂O and diluted 1:10. To check for efficiency of DNA digestion, a PCR was set up with the same amount of RNA (1 µl of 1:10) and same primers used for qRT-PCR. One-step qRT-PCR was performed using an amplification kit with SYBR Green (Power SYBR<sup>™</sup> Green RNA-to-CT<sup>™</sup> 1-Step Kit; Thermofisher, # 4389986) with the primers listed in Table 2 and run on Biorad CFX according to the manufacturer’s instructions. Transcript abundance was calculated using a logarithmic dilution series of one sample to generate a standard curve for each gene. Relative quantification of the genes of interest was expressed in relation to the expression of the constitutive reference gene gyrB (<i>gyrB</i>). The means were calculated from three biological replicates run in technical duplicates. Statistical analysis was performed using one-way ANOVA by employing the GraphPad Prism software package.

**Biofilm Assay**

Biofilm formation was tested on 96-well, polystyrene tissue culture plates (Greiner Bio-One, # 655180) as described previously (Christensen et al., 1985), using Trypticase<sup>™</sup> Soy Broth (BD BBL<sup>™</sup>, #211768) supplemented with 4% NaCl as growth medium. <i>S. epidermidis</i> RP62A and <i>S. carnosus</i> TM300 were used as positive and negative controls, respectively. For strains carrying resistance genes, antimicrobial agents were used at the following concentrations: 25 µg ml⁻¹ erythromycin (for both overnight and day culture) and 30 µg ml⁻¹ (for overnight culture) or 10 µg ml⁻¹ (for day culture) chloramphenicol. Bacterial overnight cultures were freshly diluted to OD₆₀ₐ of 0.05 and 200 µl filled in each well (two technical replicates per strain). To distinguish between total, protein and PIA matrix-mediated biofilm production, three tissue culture plates were set up in parallel and incubated at 30°C for 18 h. Cultures were then discarded and adherent cells washed twice with 1x PBS buffer. The control plate for measuring the total biofilm was dried and heat-fixed at 65°C for 1 h. To discern between PIA- and protein-mediated biofilm, biofilms were either treated with 1 mg ml⁻¹ proteinase K (Merck, #1245680500) for 4 h at 37°C or 40 mM NaIO₄ (Carl Roth GmbH, #2603.1) for 24 h at 4°C. Afterwards the plates were washed with 1x PBS, dried and heat-fixed. All three plates were stained with 10 µg ml⁻¹ crystal violet (Merck, #115940) for 2 min, washed twice with double-distilled water before measuring the absorbance at 492 nm (ELISA plate reader, Multiskan Ascent). The means were calculated from three biological replicates. Statistical analysis was performed using one-way ANOVA by employing the GraphPad Prism software package.

**RESULTS**

The Two ica Locus Copies in <i>S. aureus</i> Rd11 Are of Different Genetic Origin

We previously reported that database searches against the entire non-redundant nucleotide collection at NCBI (including whole-genome shotgun contigs) returned similarities of pAFS11 to an ica-like gene cluster present in some <i>S. sciuri</i> isolates [now <i>M. sciuri</i> (Madhiaiy et al., 2020)] (Feßler et al., 2017). Of note, the putative ica locus on pAFS11 was found to differ on nucleotide level from ica sequences present in <i>S. aureus</i> and <i>S. epidermidis</i> (Feßler et al., 2017). For further phylogenetic analysis, we therefore performed multiple sequence alignments of icaR/icaADBC nucleotide sequences from two <i>S. aureus</i> (i.e. RN4220 and ST398, to which Rd11 belongs) and two <i>S. epidermidis</i> (i.e. RP62A and O-47) strains as well as from the pAFS11 ica locus (referred to as ica<sub>pAF</sub> hereafter). Finally, based on the nucleotide BLAST query results, an ica-like locus from the CoNS species <i>S. sciuri</i> was included into the analysis as well. Average distances were calculated from the alignment data and the tree displayed in Figure 1A illustrates that ica<sub>pAF</sub> is most distantly related to the two <i>S. aureus</i>-derived ica loci. In addition, the two ica loci from <i>S. epidermidis</i> are highly divergent from ica<sub>pAF</sub>, while they are closer related to ica from <i>S. aureus</i>. Interestingly, however, the ica<sub>pAF</sub> nucleotide sequence is closely related to the ica-like locus from <i>S. sciuri</i> strain NS1 (Figures 1A, B). The data suggest that the two ica loci present in Rd11 are of different genetic origin, with ica<sub>pAF</sub> most likely being derived from another species for which <i>S. sciuri</i> (<i>M. sciuri</i>) is a putative candidate. Although, as expected, interspecies conservation on the nucleotide level was found to be low (Figure 1B), the ica<sub>pAF</sub> genes translate into amino acid sequences that are identified by the BLASTP algorithm as Ica-associated proteins with (again) some sequence differences between species. Thus, Figure 1C shows the percentage of identical amino acid positions upon pairwise alignments of Ica proteins from ica<sub>pAF</sub>, <i>S. aureus</i> RN4220 and <i>S. epidermidis</i> RP62A. While comparisons between RN4220 and RP62A revealed high conservation of <i>S. aureus</i> and <i>S. epidermidis</i> Ica proteins, identical amino acid positions were much lower between ica<sub>pAF</sub> and <i>S. aureus</i>-derived Ica proteins. In this respect, IcaR was the protein with the lowest conservation (29%), indicating that the two IcaR repressor proteins harbored by <i>S. aureus</i> Rd11 differ significantly on the protein sequence level (Figure 1C). Despite this apparent divergence, IcaR<sub>pAF</sub> exhibits a number of amino acid residues (marked by asterisks in the conservation histogram in Figure 1D) that are highly conserved in IcaR proteins of <i>S. aureus</i> and <i>S. epidermidis</i> as well (Figure 1D). These include the putative icaA operator-interacting residues shown for IcaR <i>S. epidermidis</i> (Jeng et al., 2008) (marked with red triangles on top of the sequence in Figure 1D).
Plasmid pAFS11 Has a Negative Effect on S. aureus PIA-Mediated Biofilm Formation

As mentioned above, we previously reported that S. aureus Rd11 carrying pAFS11 does not produce biofilm, nor does S. aureus RN4220 into which the plasmid was transformed, suggesting that ica_pAF on the plasmid might be inactive (Feßler et al., 2017). To challenge this hypothesis, we cloned the entire ica_pAF from pAFS11 onto the shuttle vector pRB473 (resulting in plasmid pica_pAF) to enable ready genetic manipulation of the locus. As a first step, we deleted the ica_pAF coding region from the vector, yielding plasmid pica_pAF ΔicaR. Both plasmids (with or without ica_pAF) were transformed into S. aureus RN4220 as recipient strain and biofilm assays were performed with the constructs and corresponding wild types (Figure 2). The biofilm assays allow to detect total biofilm formation as well as to differentiate between PIA and protein matrix-mediated biofilm production (see material and methods for details). We display here (and in the following figures) solely the data for PIA-mediated biofilm formation (Figures 2–5). The entire data sets on total, protein and PIA biofilm formation can be found in Supplementary Figure S1. The assays confirmed the PIA biofilm-negative phenotype of S. aureus Rd11 and revealed that the S. aureus RN4220 wild type is a weak, but detectable PIA biofilm producer. Upon acquisition of pAFS11, PIA biofilm formation of RN4220 did not increase, but on the contrary was even slightly reduced, although this reduction was statistically not significant (+pAFS11, Figure 2). The same phenomenon occurred when S. aureus RN4220 was transformed with plasmid pica_pAF, carrying the entire ica_pAF locus from pAFS11 (+pica_pAF, Figure 2). In contrast, however, PIA biofilm formation of RN4220 massively increased when the icaR_pAF gene was deleted from the ica_pAF copy on the vector (+pica_pAF ΔicaR, Figure 2). In the S. aureus RN4220 +pica_pAF ΔicaR strain, PIA biofilm levels even exceeded that of the S. epidermidis RP62A positive control and were much higher than in the RN4220 wild type, suggesting that the ica_pAF copy on the vector contributes to PIA production, but only when the IcaR_pAF repressor is absent. We conclude from this that pAFS11 may exert its negative effect on S. aureus PIA biofilm formation most likely via IcaR_pAF which also seems to negatively influence the ica locus on the RN4220 chromosome (hereinafter referred to as icaRN).

The ica Locus on pAFS11 Is Inactive Due to Efficient IcaR Repression

The data obtained so far strongly suggest that the ica_pAF locus is functional and capable to enable PIA synthesis. However, by our initial experimental set-up (i.e. by employing the S. aureus RN4220 wild type with an intact chromosomal icaRN locus) it was difficult to distinguish between ica_pAF- and icaRN-derived PIA production. Therefore, we constructed a markerless icaRN deletion mutant in RN4220 via allelic replacement, and transformed the resulting RN4220 Δica strain with plasmids pAFS11, pica_pAF and pica_pAF ΔicaR. Biofilm assays with the constructs confirmed loss of PIA production in the RN4220 Δica deletion mutant (Figure 3A). Providing the mutant with an entire ica_pAF locus either on plasmid pAFS11 or pica_pAF did not result in biofilm formation (Figure 3A). However, biofilm formation was triggered and highly significantly increased, when the icaR_pAF repressor-encoding gene was deleted from the ica_pAF locus (+pica_pAF ΔicaR, Figure 3A), indicating that the icaADBC_pAF genes of ica_pAF are indeed able to mediate PIA biofilm formation, once IcaR_pAF-dependent repression is alleviated. To further corroborate this assumption, we monitored transcription of icaA_pAF and icaR_pAF by qRT-PCR in the various constructs. In strain RN4220 Δica, transformed with either pAFS11 or pica_pAF, weak icaA_pAF transcription was detectable (Figure 3B). Upon deletion of icaR_pAF from the plasmid (+pica_pAF ΔicaR, Figure 3B), icaR_pAF transcription was no longer detectable (as expected) and icaA_pAF transcription levels massively increased (i.e. 200-fold) compared to the intact ica_pAF copy (+pica_pAF, Figure 3B). These findings are in agreement with the biofilm test results. From the combined data we conclude that (i) the icaRN locus on pAFS11 is functionally fully intact and (ii) icaADBC_pAF operon transcription is efficiently repressed by its cognate IcaR_pAF repressor.

IcaR From pAFS11 Represses the ica Locus in the S. aureus Chromosomal DNA and vice versa

Our initial experiments with wild type S. aureus RN4220 indicated that IcaR_pAF may also inhibit icaADBCRN expression on the RN4220 chromosome (Figure 2). To substantiate this hypothesis, we constructed another set of plasmids carrying (i)

![Figure 2](image-url)
the ica locus from RN4220 (picaRN), (ii) the ica locus from RN4220 lacking icaRN (picaRN_DicaRN) and (iii) the ica locus from RN4220 where we exchanged icaRN from RN4220 with icaRPAF from pAFS11 (picaRN_DicaRN_icaRPAF). All plasmids were transformed into the RN4220 Δica mutant background and the resulting strains were analyzed for their ability to form PIA biofilm (Figure 4A). Complementation of RN4220 Δica with its own icaRN locus restored PIA-mediated biofilm formation, and upon icaRN repressor gene deletion, PIA biofilm production significantly increased, demonstrating functionality of the vector-borne icaRN locus, including icaRN-mediated regulation (Figure 4A). Accordingly, qRT-PCR analysis confirmed that IcaRN efficiently represses transcription of its cognate icaADBCRN operon (Figure 4B). We then asked the question whether or not expression of the chromosomal icaADBCRN operon can undergo control by the foreign IcaRPAF repressor from pAFS11. Thus, we performed biofilm tests and quantitative transcription analyses with vector picaRN_DicaR_icaRPAF, in which icaADBCRN was combined with the icaRPAF gene from pAFS11. As shown in Figure 4, presence of icaRPAF significantly diminished PIA production and transcription of the icaADBCRN operon, suggesting the capability of IcaRPAF to control the icaADBCRN copy from RN4220 (Figure 4). Vice versa, we next investigated, if IcaRN from RN4220 can influence icaADBCPAF from pAFS11. For this purpose, we additionally constructed vector picaPAF_ΔicaRPAF_DicaRN which was transformed into the RN4220 Δica mutant background. Biofilm testing revealed a highly significant reduction of PIA production when icaADBCPAF was combined with icaRN, suggesting that IcaR from S. aureus RN4220 is indeed able to repress the icaADBCPAF genes from pAFS11 (Figure 4C).

A Palindrome Sequence in the icaA_{PAF} Upstream Region Is Required for IcaR-Mediated Biofilm Repression

The data obtained so far demonstrate that the pAFS11- and RN4220-derived IcaR repressors, which differ on amino acid level (Figure 1D), are able to control the icaADBC operon of the respective other ica locus copy. To understand the molecular prerequisites for the IcaR interactions with their DNA targets, we focused on the nucleotide sequence constraints known to be involved in IcaR binding. IcaR was previously shown to bind as a dimer to a specific palindromic sequence (ACCTANCTNNC/GNNAGNTAGGT) present in the icaA operator of S. epidermidis (Jeng et al., 2008). This palindrome is 22 nucleotides long and contains six mismatches (22,6) (Figure 5A, top). Of note, the sequence is highly conserved and is also present in the S. aureus icaA promoter region (Figure 5A, top). Surprisingly, although IcaR from S. aureus is clearly able to control PIA production from pAFS11, the S. aureus-like palindrome sequence stretch lacks in icaPAF (Figure 5A, bottom). Instead, the icaA_{PAF} upstream region displays two palindromes which differ at the nucleotide level from that of the known S. aureus/S. epidermidis recognition site. Thus, palindrome A (TNAAAATNNNTA/TANNAATTNTA) is 22 nucleotides long and harbors six mismatches (22,6), while palindrome B (CNAACNANCE/GNTNGTNTTAG) consists of 18 nucleotides with six mismatches (18,6) (Figure 5A, bottom). To investigate the putative involvement of these palindromes in IcaR function, we mutated either palindrome A or B in (i) a plasmid carrying the whole icaPAF, (ii) in an icaPAF plasmid with an icaRPAF deletion as well as (iii) in an icaRPAF deletion vector complemented with icaRN. The plasmids were again transformed into the RN4220 Δica mutant background and analyzed for PIA-mediated biofilm formation. Figure 5B demonstrates
that an altered palindrome A sequence did not influence icaR-mediated biofilm control. Thus, upon palindrome A mutation, PIA-mediated biofilm production remained repressed in picaRN, indicating that IcaR does not require this nucleotide sequence stretch for action. As expected, PIA production was derepressed when icaR was lacking in the palindrome A mutant (picaRN_icaR). Most importantly, however, icaR was still able to completely downregulate PIA-mediated biofilm production in this construct, confirming that palindrome A is not an interaction site for IcaR, neither for IcaR proteins derived from pAFS11 nor from RN4220 (Figure 5B). In contrast, mutation of palindrome B had a profound impact on biofilm regulation by IcaR. Firstly, PIA production was found to be deregulated and increased in an icaRN construct carrying an altered palindrome B nucleotide sequence (+picaRN), suggesting that control by the cognate IcaR is significantly impaired when integrity of this sequence stretch is disturbed (Figure 5B). Moreover, PIA-mediated biofilm production further increased in a palindrome B mutant in which icaR was deleted (+picaRN_icaR), which speaks in favor of residual IcaR repressor activity in the palindrome B mutant. Finally, when providing the mutant with IcaR from RN4220, biofilm repression was partially, but not fully restored which (again) is in good agreement with residual IcaR repression in the palindrome B mutant. Together, the combined data strongly suggest that IcaR from both pAFS11 and RN4220 require an intact palindrome B (but not palindrome A) for unfolding their repressor activity on the icaRN locus.

FIGURE 4 | IcaR controls expression of ica genes from RN4220 and vice versa. (A) Analysis of PIA biofilm production by static 96-well microtiter plate biofilm assays of strain RN4220 wild type and Δica alone or complemented with a plasmid carrying the ica operon of RN4220 (+picaRN) or with icaR deletion (+picaRN_icaR). The entire data sets on total, protein and PIA biofilm formation can be found in Supplementary Figure S1. (B) Quantification of icaRN and icaRN_RN transcripts by qRT-PCR of complemented strains from (A). The graph displays relative mRNA amounts using gyrB expression as reference. (C) Analysis of PIA biofilm production by static 96-well microtiter plate biofilm assays of RN4220 wild type and Δica alone or complemented with the ica operon of plasmid pAFS11 on a working plasmid (+picaRN) or the ica operon of plasmid pAFS11 with icaR deletion (+picaRN_icaR). The ica genes distinctive for each strain are depicted as symbols, with filled symbols indicating presence and empty symbols indicating absence of a given gene (as indicated in the legend). The means were calculated from three biological replicates run in duplicates. Statistical analysis was performed using one-way ANOVA by employing the GraphPad Prism software package. ns: P = 0.1234; *P = 0.0332; ****P < 0.0001.
DISCUSSION

Acquisition of mobile genetic elements (MGEs) is often beneficial for bacteria by providing novel metabolic and resistance traits. However, MGE carriage may also come at considerable cost for the recipient bacterial cell (Slater et al., 2008; San Millan and MacLean, 2017). Thus, resources will be required to replicate and maintain MGEs (e.g., plasmids) on which beneficial genes are located and their (inappropriate) expression may impose a metabolic burden, resulting in reduced fitness and competitiveness of MGE-bearing bacterial cells (Baltrus, 2013). Accordingly, bacteria have evolved sophisticated mechanisms to control both MGE uptake and maintenance as well as expression of horizontally acquired genes (Brantl, 2013; Kwong et al., 2017; Firth et al., 2018). In case of plasmid-mediated HGT, this often involves a regulatory crosstalk between chromosomal factors and the newly acquired plasmid (Huang et al., 1990; Charles and Nester, 1993; Baños et al., 2009). Interestingly, these control networks are not unidirectional and there is growing evidence to suggest that plasmids are able to influence chromosomal gene expression as well in a wide range of bacterial species [recently reviewed in (Vial and Hommais, 2020)]. In the study presented here, we extend these examples by the Gram-positive pathogen S. aureus and reveal that horizontally acquired and core genome genes have the capacity to mutually influence each other in this organism. Thus, we demonstrate that a transcription factor (i.e., IcaR) located on a large multi-resistance plasmid, is able to target a pathogenicity factor (i.e., icaADBC-mediated PIA biofilm formation) from the S. aureus core genome. Vice versa, the IcaR homolog from the S. aureus core genome was found to be able to silence transcription of plasmid-borne icaADBC genes, creating a

![Figure 5](image-url)
bi-directional regulatory crosstalk between plasmid- and chromosomally encoded factors that eventually hindered metabolically costly PIA-mediated biofilm formation.

PIA consists of N-acetyl-glucosamines (GlcNac) molecules, and ample sugar and energy supplies are fuellted into GlcNac synthesis to provide the building blocks of the exopolysaccharide. Accordingly, ica gene expression is intimately linked to central carbon flux control and energy balance (Vuong et al., 2005; Seidl et al., 2008; Zhu et al., 2009; Sadykov et al., 2011; Lindgren et al., 2014) which also involves the action of non-coding RNAs to appropriately adjust metabolic patterns (Rochat et al., 2018; Bronsky et al., 2019; Marincola et al., 2019; Schoenfelder et al., 2019). Presence of two fully functional ica gene clusters in strain S. aureus Rd11 is likely to represent a major metabolic challenge and the observed downregulation of PIA production in this strain makes sense to prevent metabolic overload. Paradoxically, it is just the additionally acquired ica$_{pAF}$ locus copy on plasmid pAFS11 that mediates a biofilm-negative phenotype. Indeed, acquisition of pAFS11 or the ica$_{pAF}$ locus alone abolished PIA biofilm formation in S. aureus (Figure 2). This effect is accomplished through the IcaR$_{pAF}$ repressor which can also target the chromosomal ica locus copy (Figure 4). Moreover, the tight self-control of the ica$_{pAF}$ copy on pAFS11 by its cognate IcaR$_{pAF}$ repressor further contributes to a biofilm-negative phenotype (Figures 2, 3). Remarkably, icaADBC$_{pAF}$ expression can be additionally repressed by IcaRN from the core genome (Figure 4C). Thus, although being different from the canonical IcaR recognition site known from S. epidermidis and S. aureus (Jeng et al., 2008), IcaRN interacts with a palindrome sequence present in the upstream region of icaADBC$_{pAF}$ (palindrome B in Figure 5A), revealing a certain flexibility of IcaR-like proteins in DNA target selection (Figure 5).

Phylogenetic analyses revealed that the two ica loci in Rd11 differ both on nucleotide and protein sequence levels and are only distantly related to each other (Figure 1). Thus, the horizontally acquired ica$_{pAF}$ copy on pAFS11 is likely to originate from an unknown bacterium for which the soil and animal dwelling species S. sciuri [recently re-classified as M. sciuri (Madhaiyan et al., 2020)] might represent a putative candidate (Feßler et al., 2017). But clearly more detailed investigations will be required to substantiate this hypothesis. Interestingly, inhibition of core genome-encoded exopolysaccharide production by plasmids seems to be a common theme in the bacterial world. Thus, in nitrogen-fixing Rhizobium tropici, exopolysaccharide production was found to be downregulated by the NrcR transcription factor, encoded on an acquired plasmid (Del Cerro et al., 2016), and in the nosocomial pathogen Acinetobacter baumannii, PNAG (PIA) production was described to be diminished upon acquisition of a multi-resistance plasmid (Venanzio et al., 2019). In the latter case, this large conjugative multiresistance plasmid facilitates its own transmission by downregulating chromosomally encoded type-6-secretion systems (T6SS) that usually hamper HGT (Venanzio et al., 2019). Together with the effect on PNAG production, the A. baumannii plasmids represent interesting examples for a plasmid-chromosome regulatory crosstalk that influences simultaneously both virulence and resistance traits.
suitable target to modulate PIA production (Jefferson et al., 2003; Schwartbeck et al., 2016). It will be interesting to explore if the ica$_R$/AF locus on pAFS11 might become subject to mutational variation. Long-term in vitro passage experiments together with surveillance of the evolution of pAFS11-like plasmids in field studies will be suitable approaches to give answers to how control of plasmid-borne ica locus expression will be integrated into the regulatory network of S. aureus.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

GM, AF, SS, and WZ: conceived and designed the experiments. GM, GJ and A-KK performed the experiments. GM, GJ, A-KK, FW and WZ analyzed the data. GM and WZ wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Conflict of Interest: 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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