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Important Contribution of the Novel Locus *comEB* to Extracellular DNA-Dependent *Staphylococcus lugdunensis* Biofilm Formation

Nithya Babu Rajendran,[a] Julian Eikmeier,[a] Karsten Becker,[a,b] Muzaffar Hussain,[a] Georg Peters,[a,b] Christine Heilmann[a,b]

Institute of Medical Microbiology[a] and Interdisciplinary Center for Clinical Research (IZKF),[b] University Hospital of Münster, Münster, Germany

The coagulase-negative species *Staphylococcus lugdunensis* is an emerging cause of serious and potentially life-threatening infections, such as infective endocarditis. The pathogenesis of these infections is characterized by the ability of *S. lugdunensis* to form biofilms on either biotic or abiotic surfaces. To elucidate the genetic basis of biofilm formation in *S. lugdunensis*, we performed transposon (Tn*917*) mutagenesis. One mutant had a significantly reduced biofilm-forming capacity and carried a Tn*917* insertion within the competence gene *comEB*. Site-directed mutagenesis and subsequent complementation with a functional copy of *comEB* verified the importance of *comEB* in biofilm formation. In several bacterial species, natural competence stimulates DNA release via lysis-dependent or -independent mechanisms. Extracellular DNA (eDNA) has been demonstrated to be an important structural component of many bacterial biofilms. Therefore, we quantified the eDNA in the biofilms and found diminished eDNA amounts in the *comEB* mutant biofilm. High-resolution images and three-dimensional data obtained via confocal laser scanning microscopy (CSLM) visualized the impact of the *comEB* mutation on biofilm integrity. The *comEB* mutant did not show reduced expression of autolysin genes, decreased autolytic activities, or increased cell viability, suggesting a cell lysis-independent mechanism of DNA release. Furthermore, reduced amounts of eDNA in the *comEB* mutant biofilms did not result from elevated levels or activity of the *S. lugdunensis* thermonuclease NucI. In conclusion, we defined here, for the first time, a role for the competence gene *comEB* in staphylococcal biofilm formation. Our findings indicate that *comEB* stimulates biofilm formation via a lysis-independent mechanism of DNA release.

The first published record of *Staphylococcus lugdunensis* dates back to 1988, when this species was identified and described as an infectious coagulase-negative *Staphylococcus* (CoNS) (1). Since then, many reports describing clinical cases of *S. lugdunensis* have emerged, underlining its significance as an important human pathogen and its special position among all other CoNS species (2, 3). This species can cause a variety of infections, ranging from mild skin abscess to aggressive, life-threatening infective endocarditis (4–6). Native valve endocarditis and device-related infections associated with catheters, prosthetic joints, or heart valves reflect the ability of *S. lugdunensis* to colonize biotic and abiotic surfaces, respectively, and produce biofilms (7, 8). Inside the host, biofilms offer protection against the host immune system and antibiotics (9). Distinct phases of biofilm formation include the attachment of the bacterial cells to a suitable surface, proliferation, and secretion of adhesive polymeric substances that glue the cells together, resulting in the formation of a multilayered biofilm (3, 9, 10).

While several factors involved in biofilm formation of the species *Staphylococcus aureus* and *Staphylococcus epidermidis* have been identified and extensively characterized during the past 2 decades, very little has been known about the mechanisms underlying *S. lugdunensis* biofilm formation (3, 10, 11). Available studies on the pathogenesis of *S. lugdunensis* focus on the characterization of adhesins and other protein factors like the von Willebrand factor (vWF)-binding protein (12), fibrinogen (Fg)-binding protein Fbl (13), autolysin AtlL (14), iron-regulated surface determinant (Isd) proteins (15), and sortase A (16). Some of these proteins have homologs in other staphylococci, whose functions are well established (3, 10). Frank and Patel reported that 15 different clinical isolates of *S. lugdunensis* predominantly formed a protein-dependent and polysaccharide intercellular adhesin (PIA)-independent biofilm despite the presence of the *icaADBC* operon (17). The *icaADBC* operon confers the production of PIA (also referred to as poly-*N*-acetylglucosamine; PNAG) on *S. epidermidis* and *S. aureus* as an essential component in PIA/PNAG-dependent biofilms (18, 19). A recent study also demonstrated that clinical *S. lugdunensis* isolates formed biofilms in a PIA-independent and protein-dependent manner and identified the surface protein IsdC as a mediator of biofilm formation under iron-restricted growth conditions (20).

In many bacterial species, including *S. aureus* and *S. epidermidis*, extracellular DNA (eDNA) has been demonstrated to be another important structural component of the biofilm matrix (21–25). Here, we used a random mutagenesis approach and identified a novel genetic locus, the competence gene *comEB*, involved in *S. lugdunensis* biofilm formation. Our further experiments suggested that *comEB* stimulates biofilm formation via a lysis-independent mechanism of DNA release representing a source of biofilm eDNA.

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Address correspondence to Christine Heilmann, heilmac@uni-muenster.de.

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TABLE 1 Oligonucleotide primers used in this study

Name and purpose	Sequence[a] (5′→3′)	Gene(s)
Disruption of *comEB*		
599CAKnockfor	CTGAATTCTCAGATTCACAAAAGGGAACAG (EcoRI)	
599EryKnockrev	CAGGATCCTAGGGACCTCTTTAGCTCCTTG (BamHI)	
599tn*917*for	CAGGATCCCGAAGGATCACTCATGGACTAA (BamHI)	
599CCKnockrev	GCACTGCAGATCTTAGTGCACTTGGTGCAAA (PstI)	
Complementation		
ComAF	CAGGATCCGAACAGCACGGCCAATTTAC (BamHI)	*comEB*
ComBR	CTGAATTCTGCAATATAAAACACTTAAATCACGA (EcoRI)	
ComAF	CAGGATCCGAACAGCACGGCCAATTTAC (BamHI)	*comEB*, *comEC*
ComER	CTGAATTCTTTTGAGGCATGCTTATAACTCC (EcoRI)	
Real-time PCR		
aroE1F	AACGAGCAGCAATCAATACC	*aroE*
aroE1R	TTCAATAATTCCCCCACCTG	
gyrBF	CACACGTATGAAGGCGGAAC	*gyrB*
gyrBR	CTACGGCCGTTAAACCTTCTC	
holAF	AAATTGGATTCAGCAACAATTACA	*holA*
holAR	GGTCTATCCCCTATGAACAACATT	
atlF	TGCATTTGTACATGCGTTTG	*atlL*
atlR	GCGCAAATGAAGCATAGTCA	
aalF	CCTTTACCAATAGCTGCACGA	*aal*
aalR	ACTGGCACAGCTTCAGGTTC	
nucF	AAACACGTCCATAGCGATCA	*nucI*
nucR	CGTTGATACGCCTGAAACAG	

[a] Restriction sites are underlined.

MATERIALS AND METHODS

Bacterial strains and media. The clinical *S. lugdunensis* isolates a19263 and w701 from the Institute of Medical Microbiology, University Hospital of Münster, were used for transposon and site-directed mutagenesis, respectively. The clinical strain *S. epidermidis* RP62A (18, 26), predominantly forms a PIA-dependent biofilm, and the biofilm-negative *Staphylococcus carnosus* TM300 (27) served as controls in studying the eDNA dependency of biofilm formation. *S. epidermidis* O-47 (28) and *S. aureus* SA113 (29), carrying the empty vector pCU1, were used as controls in detecting NucI activity. For cloning, *Escherichia coli* strains XL1 Blue or TG1 were used. *S. lugdunensis* was grown in either tryptic soy broth (TSB) or on tryptic soy agar, and *E. coli* was grown in Luria-Bertani (LB) broth or on LB agar. To analyze NucI activity, DNase test agar (Oxoid) was used according to the instructions of the manufacturer. Antibiotics were added to the growth media when appropriate.

Genetic manipulation, plasmids, complementation, and DNA sequencing. The plasmid pTV1ts (30), used for transposon Tn*917* mutagenesis, was introduced into strain a19263 by protoplast transformation (31) with slight modifications: early exponential a19263 cells were treated with lysostaphin at 37°C until the optical density at 578 nm (OD_{578}) dropped to 25% of the initial value. To determine the Tn*917* insertion site, an arbitrary PCR was performed as described previously (32). Primers were synthesized and DNA was sequenced by Eurofins MWG Operon (Ebersberg, Germany).

For site-directed mutagenesis, the replacement cassette (*comEB*::*ermB*) first was constructed in the shuttle plasmid pCU1 (33). The *ermB* cassette and recombination sites were amplified directly from the Tn*917* mutant using two separate PCRs: PCR1 (primer pair 599CAKnockfor and 599EryKnockrev) (Table 1) to obtain the 2,457-bp *comEA-comEB-ermB* fragment and PCR2 (primer pair 599tn*917*for and 599CCKnockrev) (Table 1) to obtain the 1,607-bp *orf3-comEB-comEC* fragment. Both fragments were sequentially ligated with pCU1, excised as a single 4,072-bp fragment, and then cloned into the EcoRI and PstI sites of the vector pBT2 (34), generating plasmid pBT2*comEB*::*ermB*. Subsequently, pBT2*comEB*::*ermB* was introduced into the strain w701 by protoplast transformation to disrupt the *comEB* gene by homologous recombination essentially as described previously (35). For complementation, DNA fragments encoding either *comEB* (883 bp) or *comEB-comEC* (3,147 bp), which were previously amplified from the genomic DNA of strain w701 using primer pairs ComAF and ComBR or ComAF and ComER, respectively (Table 1), were cloned into the EcoRI and BamHI sites of the shuttle vector pRB473 (36), generating pRB*comEB* or pRB*comEB/EC*, respectively.

Quantitative biofilm assay. Microtiter plate biofilm assays were performed as described earlier (28). In some experiments, biofilms were grown in the presence of DNase I (0.1 mg/ml). In the controls, the enzyme was replaced by the same volume of phosphate-buffered saline. Each assay was performed at least in triplicate.

Quantification of eDNA. Biofilm eDNA from 24-h biofilms was isolated as described previously (14) and quantified by UV-visible (Vis) spectrophotometry using a NanoPhotometer P330 (Implen) according to the instructions of the manufacturer. Each assay was performed in triplicate.

Confocal laser scanning microscopy (CLSM). Both 24-h and 48-h biofilms were grown on coverslips at 37°C. For the cultivation of 48-h biofilms, spent medium was exchanged for fresh medium thrice during growth. Afterwards, the biofilms were washed twice with 0.85% NaCl, stained with the live/dead staining kit (Invitrogen) according to the manufacturer's recommendations, and fixed with 10% neutral buffered formalin. The coverslips were mounted onto glass slides with Mowiol and observed with an LSM700 CLS microscope (Zeiss). Images were acquired with a 63× objective and a Z interval of 0.3 μm for 24-h biofilms and 1 μm for 48-h biofilms. Image analysis and processing was carried out with either the Zen Imaging software (Zeiss) or Fiji (37). Three-dimensional (3D) reconstructions were generated with Fiji.

Triton X-100-induced autolysis. For the autolysis assay, overnight cultures of *S. lugdunensis* were diluted in 25 ml fresh TSB medium to give a starting OD_{578} of 0.05. The cultures were shaken at 37°C and 160 rpm until an OD_{578} of 0.7 to 0.8 was reached. Five milliliters of each sample was

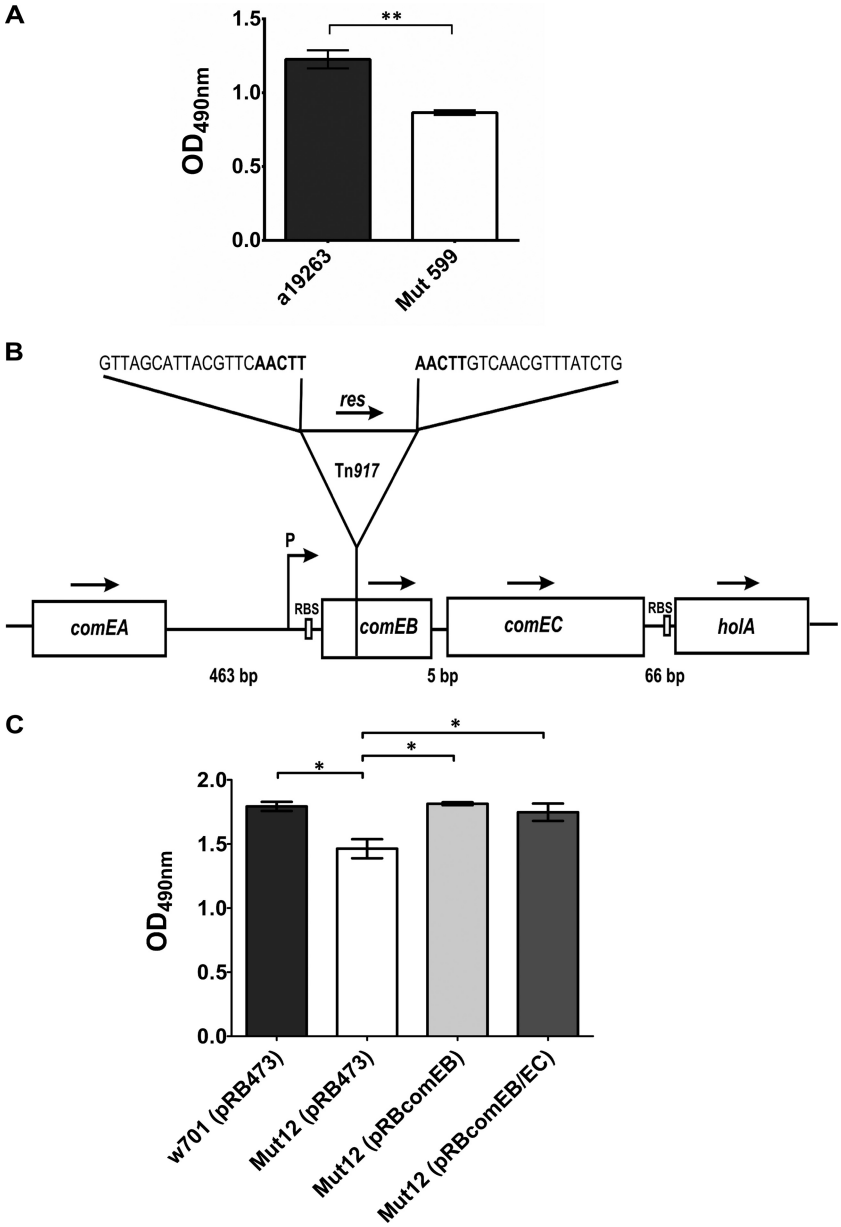

FIG 1 *comEB* is involved in biofilm formation. (A) Quantitative analysis of 24-h biofilms produced by the Tn*917* mutant Mut599 and its wild type. The OD_{490} values ± SEM are the averages from three independent experiments. **, $P \leq 0.01$. (B) The Tn*917* insertion site in Mut599 is located 64 bp downstream of the *comEB* start codon, which is part of a putative operon containing the *comEB*, *comEC*, and *holA* genes. The arrows mark the direction of transcription. P, putative promoter; RBS, ribosomal binding site; *res*, resolvase gene. The nucleotide sequences correspond to the regions flanking the transposon, with the nucleotides in boldface representing the characteristic 5-bp duplication. *comEB*, *comEC*, and *holA* putatively are transcribed from the common promoter upstream of *comEB*. (C) Quantitative analysis of 24-h biofilms produced by the clinical isolate *S. lugdunensis* w701 harboring the empty vector pRB473, its site-directed *comEB* mutant Mut12 containing pRB473, and the complemented mutants. For complementation, the vector pRB473 carrying *comEB-comEC* (pRB*comEB/EC*) or *comEB* alone (pRB*comEB*) was introduced into Mut12. The OD_{490} values ± SEM are averages from three independent assays. *, $P \leq 0.05$. The data were analyzed with one-way ANOVA and a Bonferroni's posttest.

pelleted. The cells were washed once with cold double-distilled water and resuspended in an equal volume of 0.05 M Tris-HCl containing 0.05% Triton X-100 (pH 7.2). The samples were incubated at 30°C for 3 h, and the OD_{578} was measured every 30 min.

Zymographical analysis of bacteriolytic activity. Surface-associated proteins for zymographical analysis were prepared essentially as described previously, except that overnight cultures of the bacteria were grown in TSB at 37°C and 160 rpm (38). One-milliliter samples were harvested at different time points of growth, and the bacterial cells were resuspended in 1 volume of SDS sample buffer. The samples were heated at 95°C for 10 min and centrifuged, and 10 μl of the supernatant containing the surface-associated autolysins was loaded on SDS gels (10% separation gel) and on corresponding zymogram gels (10% SDS gels containing 0.05% heat-inactivated *S. lugdunensis* cells in the separation gel). After electrophoresis, the zymogram gels were incubated overnight at 37°C in 25 mM Tris-HCl (pH 8) containing 1% (vol/vol) Triton X-100. The SDS and zymogram gels were stained with Coomassie brilliant blue R250 and methylene blue, respectively.

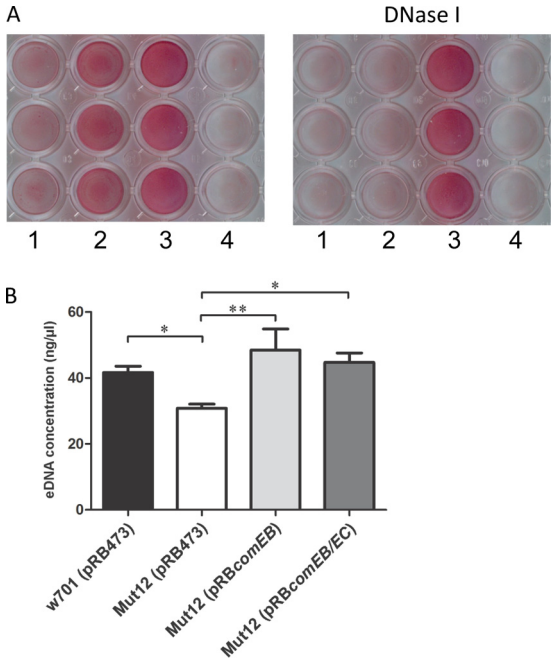

FIG 2 *S. lugdunensis* biofilm formation is eDNA dependent and *comEB* modulates eDNA levels in *S. lugdunensis* biofilms. (A) Biofilm formation in TSB in polystyrene microtiter plates in the presence (0.1 mg/ml) (right) or absence (left) of DNase I. Biofilms were stained with safranin. Lanes: 1, *S. lugdunensis* a19263; 2, *S. lugdunensis* w701; 3, *S. epidermidis* RP62A; 4, *S. carnosus* TM300. (B) Quantification of eDNA in biofilms. eDNA from 24-h biofilms was isolated and quantified by UV-Vis spectrophotometry. Mut12(pRB473) contained significantly less eDNA in the biofilm than its wild type, w701(pRB473), and the complemented mutants Mut12(pRB*comEB*) and Mut12(pRB*comEB/EC*). The values represented here are the averages from three independent experiments, and the error bars represent SEM. *, $P \leq 0.05$; **, $P \leq 0.01$. Statistical analysis was performed with one-way ANOVA and a Bonferroni's posttest.

Viability assay. Overnight-grown cultures of the *S. lugdunensis* strains were diluted in 150 ml fresh TSB to give a starting OD_{578} of 0.05. The cultures then were incubated at 37°C and 160 rpm. Serial dilutions of samples collected at different time points of growth were spread on blood agar plates, and the corresponding CFU were determined. Prior to dilution, the OD_{578} of each sample was measured.

Real-time PCR-based gene expression analysis. RNA was isolated from *S. lugdunensis* cells grown planktonically for 3 or 6 h or in a biofilm for 24 h using an RNeasy minikit (Qiagen) according to the manufacturer's protocol. One microgram of RNA was reverse transcribed to cDNA using the QuantiTect reverse transcription kit and then applied to SYBR green dye-based real-time PCR (SYBR green supermix; Bio-Rad) using the primers listed in Table 1. Melting curve analysis was performed for each reaction to ensure specificity, and the fold change values were calculated with the Bio-Rad iQ5 software using the housekeeping genes *aroE* and *gyrB* for normalization.

Statistical analysis. Data are given as means ± standard errors of the means (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA) and a Bonferroni's posttest; $P \leq 0.05$ was considered to indicate statistically significant differences.

Nucleotide sequence accession number. The GenBank accession number of the *comE* operon DNA sequence of strain *S. lugdunensis* w701 is KM232909.

RESULTS

Isolation of a Tn*917* insertion mutant producing decreased levels of biofilm. Approximately 5,000 transposon insertion mutants generated by Tn*917* mutagenesis of the biofilm-forming clinical isolate *S. lugdunensis* a19263 in a previously described procedure (28) were screened for an altered biofilm-forming phenotype using the quantitative biofilm assay. The mutant Mut599 formed less biofilm (average OD_{490}, 0.89 ± 0.02) compared to its wild type (average OD_{490}, 1.23 ± 0.06) (Fig. 1A). By means of arbitrary PCR and subsequent DNA sequencing, the Tn*917* insertion site was mapped to the competence gene *comEB*, which is part of an operon. Downstream of *comEB*, the *comE* operon contains the competence gene *comEC* and *holA*, encoding the putative DNA polymerase PolC δ subunit (Fig. 1B). Analysis of the DNA sequence suggested that the *comEC* gene is translationally coupled to the *comEB* gene, because *comEC* lacks its own ribosomal binding site (RBS).

Upon site-directed mutagenesis, the *comEB* disruption and the associated biofilm phenotype were successfully verified in a different genetic background, the clinical isolate *S. lugdunensis* w701. The resulting mutant was designated Mut12. Although the difference in biofilm formation between the wild type, w701(pRB473) (OD_{490}, 1.79 ± 0.04), and Mut12(pRB473) (OD_{490}, 1.46 ± 0.07) was not drastic, it was statistically significant (Fig. 1C). The Mut12 phenotype could be fully restored to the wild-type level of biofilm formation by complementation with either the *comEB* gene alone [Mut12(pRB*comEB*); OD_{490}, 1.81 ± 0.01] or with *comEB* together with *comEC* [Mut12(pRB*comEB/EC*); OD_{490}, 1.75 ± 0.07] (Fig. 1C).

***comEB* disruption decreases biofilm eDNA.** Because competence has been correlated with DNA release in different bacterial species (39–41) and eDNA is an important structural component in many bacterial biofilms (21–25, 42), we addressed the questions of whether eDNA is also an important structural component of *S. lugdunensis* biofilms and if the level of eDNA within the biofilms differs among Mut12 and its wild type. To analyze the eDNA dependency of the biofilm formation of the clinical *S. lugdunensis* isolates a19263 and w701, we performed biofilm assays and observed the influence of DNase I. Biofilm formation of *S. lugdunensis* a19263 and w701 in the presence of DNase I was almost completely abolished, clearly demonstrating that eDNA is an essential component of their biofilms (Fig. 2A). The same observation was made when preformed biofilms of *S. lugdunensis* a19263 and w701 were treated with DNase I (data not shown). In contrast, biofilm formation of the control strain *S. epidermidis* RP62A, predominantly forming a PIA-dependent biofilm (18), was largely unaffected by DNase I (Fig. 2A). Similarly, another recent study demonstrated that the biofilms of all 9 clinical *S. lugdunensis* strains tested were sensitive to DNase I treatment (20). We next quantified the eDNA content of 24-h biofilms. The biofilm eDNA concentration of mutant Mut12(pRB473) (30.8 ± 1.3 ng/μl) was significantly lower than that of the wild type, w701(pRB473) (41.7 ± 1.9 ng/μl) ($P \leq 0.05$), and the complemented mutants Mut12(pRB*comEB*) (48.5 ± 6.3 ng/μl) ($P \leq 0.01$) and Mut12(pRB*comEB/EC*) (44.7 ± 2.8 ng/μl) ($P \leq 0.05$) (Fig. 2B). The observed differences clearly indicated that the defect in biofilm formation was due to an eDNA loss mediated by *comEB* disruption.

Visualization of eDNA loss and its effect on biofilm integrity. To further qualitatively and quantitatively validate the decrease in the eDNA level, 24-h biofilms were stained with the live/dead staining kit and observed by CLSM. The nucleic acid stain SYTO9 (green fluorescence) penetrates membranes and stains the bacteria green, while the membrane-impermeable propidium iodide (PI; red fluorescence) stains membrane-compromised bacteria

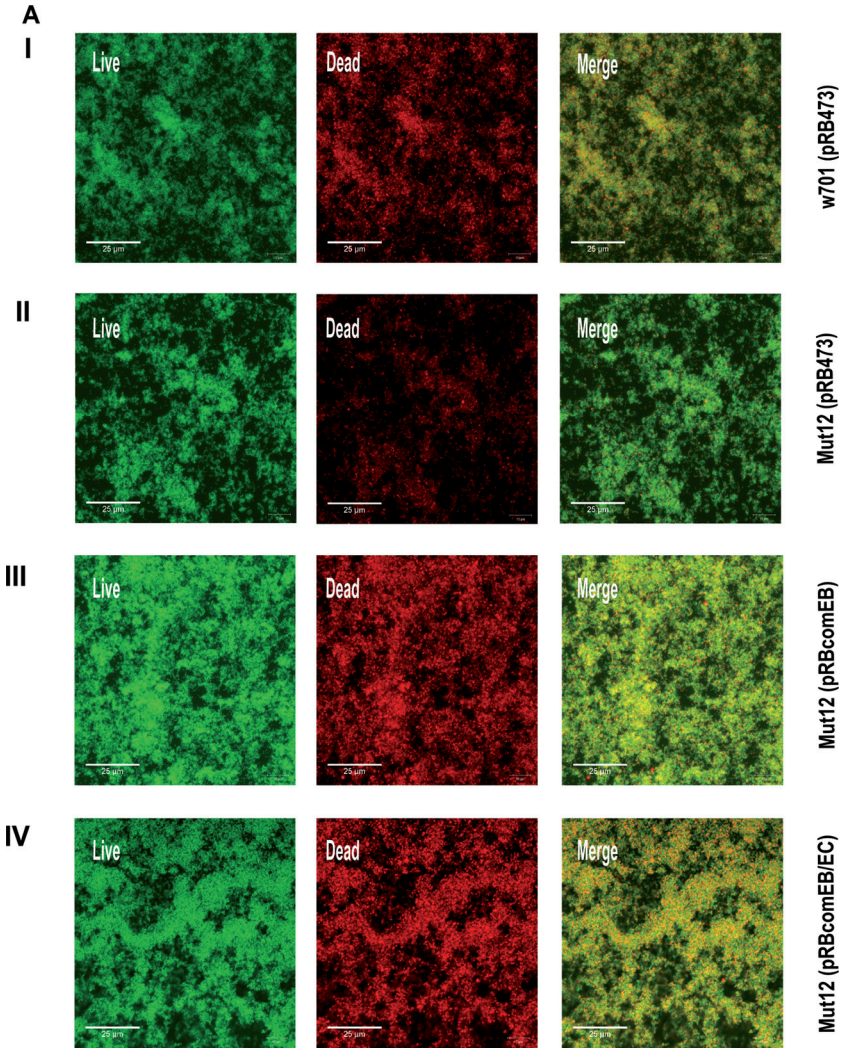

FIG 3 CLSM analysis of biofilm. (A) To visualize eDNA, mature 24-h biofilms grown on coverslips were stained with SYTO9 (live; green fluorescent) and PI (dead; red fluorescent) and are presented as maximum intensity projections. Colocalization occurs when live cells are closely associated with dead cells or are covered with eDNA, appearing yellow in the merged image (Merge). (B) To obtain 3D data, 48-h biofilms grown on coverslips were stained with the live/dead staining kit and 3D reconstructions were generated with z-stack images obtained with a z-slice interval of 1 μM. The images are representative of three independent experiments.

and extracellular nucleic acids red. By using both stains, it is possible to distinguish between live bacterial cells and dead bacteria/eDNA. This assay has been used to observe cell death and eDNA content of biofilms formed by various bacteria (21, 23, 25). Mut12(pRB473) (Fig. 3A, row II) showed diminished PI (red) staining compared to the wild type, w701(pRB473) (row I), and the complemented mutants Mut12(pRB*comEB*) (row III) and Mut12(pRB*comEB*/*EC*) (row IV). Since PI does not effectively distinguish between dead cells and eDNA, the lowered PI staining observed in the Mut12(pRB473) biofilm could be indicative of a decrease in both eDNA and dead cells. However, the viability of Mut12(pRB473) was not altered (see Fig. 4D), suggesting a decreased eDNA level.

eDNA is known to act as a cohesive agent that sticks the bacterial cells together and stabilizes the structure of a bacterial biofilm (21, 24, 43, 44). Consequently, the eDNA loss in Mut12 should negatively impact biofilm integrity. To detect potential structural alterations, 48-h biofilms grown on coverslips were stained and imaged by CLSM. The 3D reconstructions obtained from the image stacks illustrated a pronounced architectural weakness in Mut12. The biofilm produced by Mut12(pRB473) (Fig. 3B, row II) lacked the thickness that clearly defined the biofilms of w701(pRB473) (row I) and the complemented mutants Mut12(pRB*comEB*) (row III) and Mut12(pRB*comEB*/*EC*) (row IV). Using the COMSTAT program (45), this difference in thickness was quantified. The average thickness of the Mut12(pRB473) biofilm was 10.67 ± 1.12 μm, while that of w701(pRB473) was 23.89 ± 3.29 μm. The complemented mutants exhibited a biofilm thickness comparable to that of the wild type: 23.19 ± 1.81 μm for Mut12(pRB*comEB*) and 25.97 ± 1.90 μm for Mut12(pRB*comEB*/*EC*). Thus, it became evident that the *comEB* mutation resulted in decreased eDNA availability, which severely influenced biofilm integrity.

Autolytic activity in the *comEB* mutant is unaffected. DNA release by bacteria can occur via both lysis-dependent and lysis-

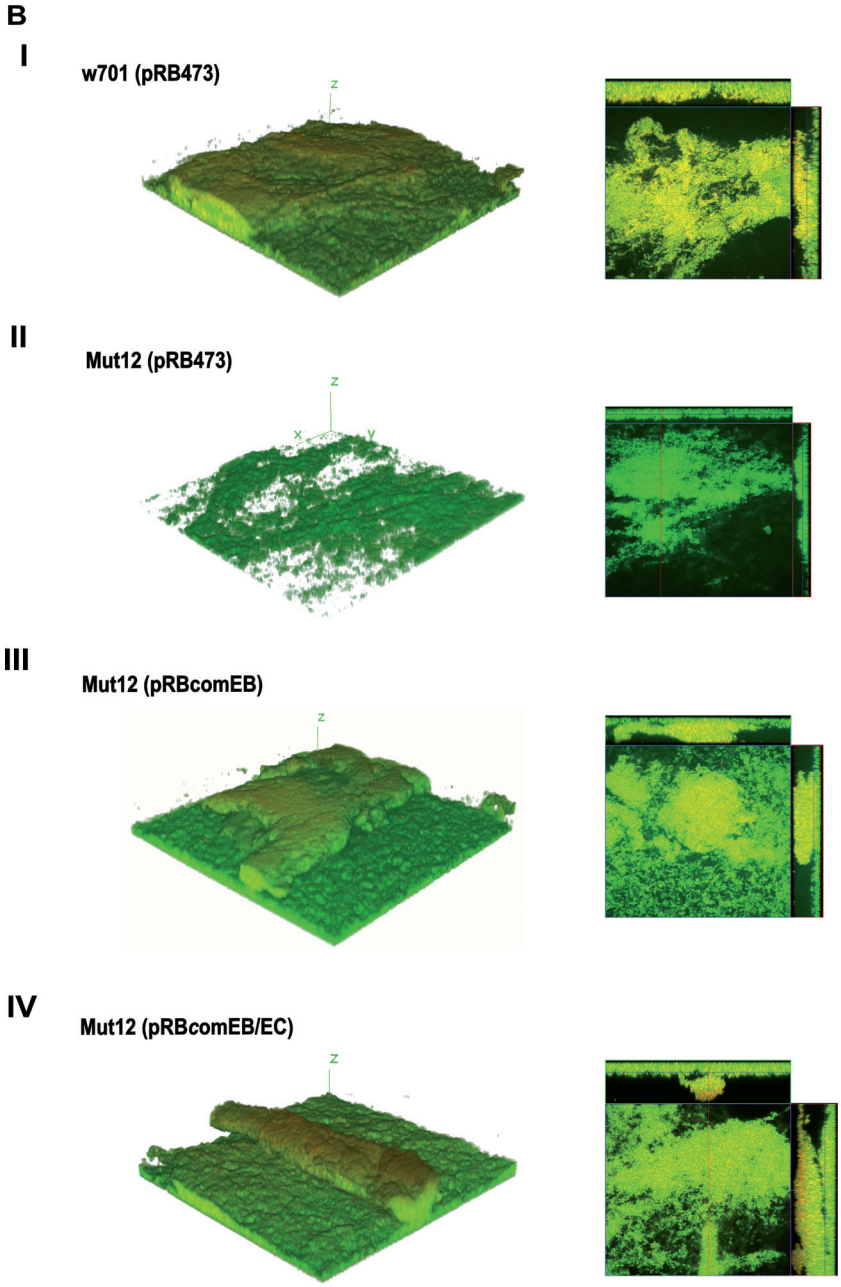

FIG 3 continued

independent mechanisms (21, 39, 41, 42, 46, 47). Lysis of bacterial cells is mediated by peptidoglycan hydrolases, also referred to as autolysins (48). To determine if *comEB* influences cell lysis, the expression of autolytic activities was analyzed. No marked differences were observed among Mut12(pRB473), its wild type, w701(pRB473), and the complemented mutants in both Triton X-100-induced autolysis assays (Fig. 4A) and zymographical analysis of autolytic activities (Fig. 4B, lower). In analogy to *S. aureus* and *S. epidermidis* (see below), clearing zones ranging from approximately 70 to 150 kDa probably represent AtlL-associated amidase and glucosaminidase activities, while the clearing zone around 35 kDa presumably is caused by Aal-associated bacteriolytic activity (Fig. 4B, lower). Moreover, no significantly differential expression of the *atlL* (SLUG_18190; available at the KEGG database [http://www.genome.ad.jp/kegg/]) and *aal* (SLUG_01420; available at the KEGG database) genes, encoding the autolysins AtlL and Aal, which are homologous to the major autolysins AtlA and Aaa from *S. aureus* (49, 50) and AtlE and Aae from *S. epidermidis* (38, 51), respectively, was detected by real-time PCR (Fig. 4C). Additionally, we analyzed the viability of Mut12(pRB473) compared to that of its wild type, w701(pRB473), and the complemented mutants by determining the CFU counts during growth, which did not indicate an altered cell viability (Fig. 4D).

***nucI* gene expression is not impaired by the *comEB* disruption.** Recently, it was shown that the *S. aureus* thermonuclease

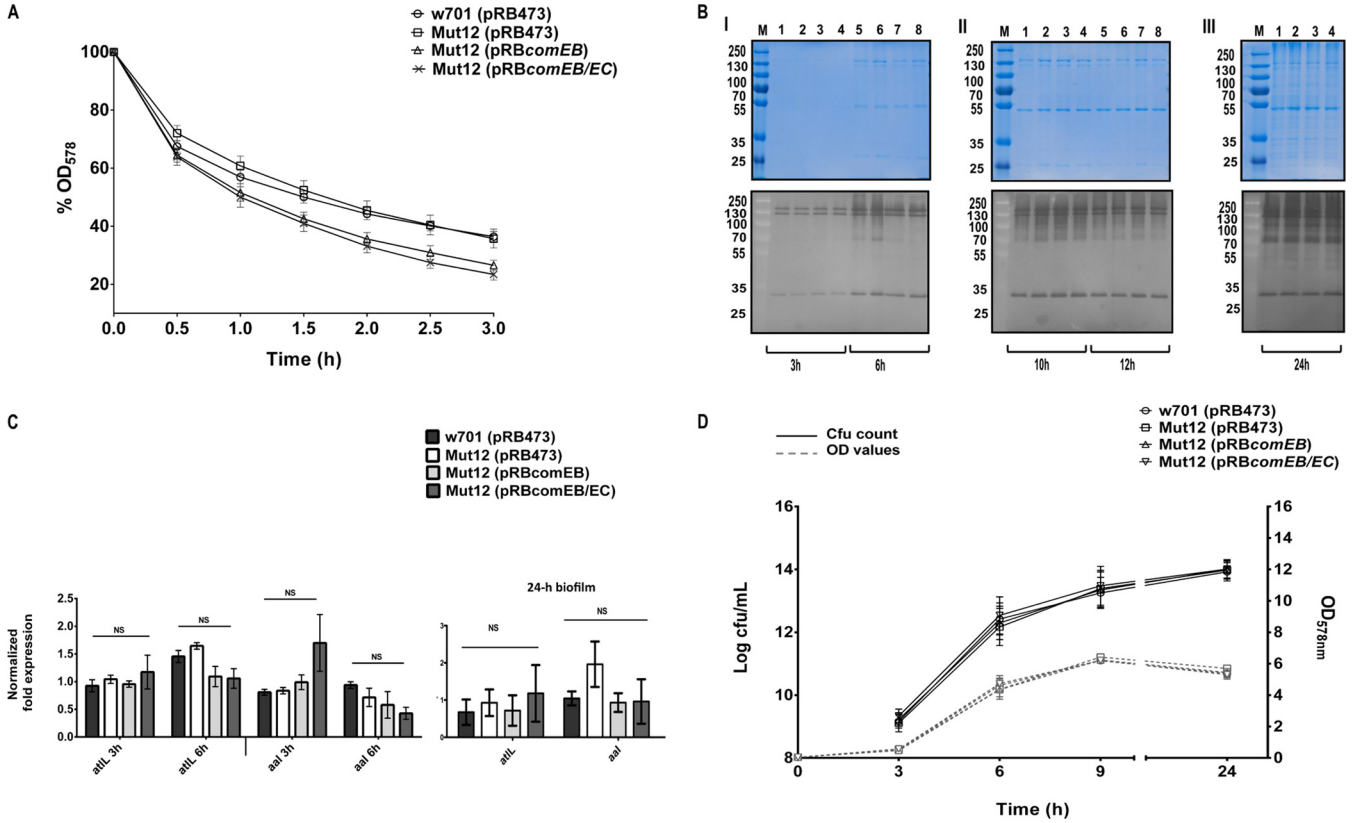

FIG 4 *comEB* does not impact the expression of autolytic activities. (A) Mid-log-phase cells were treated with Triton X-100, and autolysis was monitored by the drop in OD_{578}. The OD values are expressed in percentages, with the initial OD set to 100%. $n = 3$. Error bars indicate SEM. (B) Surface-associated proteins prepared from bacterial strains that were harvested at different time points of growth (3 h, 6 h [I], 10 h, 12 h [II], and 24 h [III]) were separated by SDS-PAGE on SDS gels and corresponding zymogram gels. Zymogram gels contained heat-inactivated *S. lugdunensis* cells in the separation gel. Bacteriolytic activities were observed as clear zones after overnight incubation in buffer. (Upper) SDS gels; (lower) corresponding zymogram gels. Molecular masses (in kilodaltons) of marker proteins (M) are indicated on the left. Lanes 1 and 5, w701(pRB473); 2 and 6, Mut12(pRB473); 3 and 7, Mut12(pRB*comEB*); 4 and 8, Mut12(pRB*comEB/EC*). SDS and zymogram gels are representative of three independent experiments. (C) The expression of the *S. lugdunensis* homologs of two major staphylococcal autolysin genes, *atlL* and *aal*, were analyzed by real-time PCR from cultures grown to mid-logarithmic (3 h) or stationary phase (6 h) or in 24-h biofilms. The values represent the averages from three independent experiments, and the error bars represent the SEM. The data were analyzed with one-way ANOVA and a Bonferroni's posttest. (D) Growth was initiated at a starting OD_{578} of 0.05 in TSB (37°C, 160 rpm) and monitored. At the indicated time points, the cultures were sampled, serially diluted, and plated on blood agar. Resulting colonies were counted after 24 h of incubation. The viability was expressed as log CFU/ml. $n = 3$. Error bars indicate SEM. NS, not significant.

Nuc has a negative impact on biofilm formation, because it degrades the eDNA that is necessary for maintaining the biofilm architecture (52). Thus, another possible cause of *comEB*-dependent, eDNA-based differential biofilm formation between Mut12 and its wild type is differentially expressed nuclease activities. While the *S. aureus* genome encodes two nucleases (53), analysis of the published *S. lugdunensis* genomes (54, 55) indicated the presence of a single gene (*nucI*) encoding the thermonuclease NucI (SLUG_15760; available at the KEGG database [http://www.genome.ad.jp/kegg/]). By using real-time PCR, no significant differences were observed in the *nucI* gene expression among the wild type, w701(pRB473), Mut12(pRB473), and the complemented mutants (Fig. 5A), suggesting that no altered nuclease expression accounted for the observed differences in biofilm formation. Moreover, the NucI activity observed as distinct clearing zones around bacterial growth on DNase agar was comparable among the wild type, w701(pRB473) (Fig. 5B, image 2), Mut12(pRB473) (image 1), and the complemented mutants Mut12(pRB*comEB*) (image 3) and Mut12(pRB*comEB/EC*) (image 4). For comparison, the positive-control *S. aureus* SA113(pCU1) (6), known to produce large amounts of nuclease activity, showed a pronounced clearing zone, while the negative-control *S. epidermidis* O-47(pCU1), which produces a nuclease in very small quantities, did not reveal a clearing zone (Fig. 5B).

DISCUSSION

S. lugdunensis is a human opportunistic pathogen (3, 56). Being part of the microbiota of the nasal cavity and of other skin areas particularly the lower abdomen and extremities (57, 58), it is not surprising that this species causes a wide range of foreign body-associated infections characterized by the formation of biofilms (2). However, factors contributing to *S. lugdunensis* biofilm formation have remained largely unknown. To identify genes involved in *S. lugdunensis* biofilm formation, we performed transposon mutagenesis and identified a mutant (Mut599) with a Tn*917* insertion in the competence gene *comEB* that produced less biofilm than the wild-type strain. Upon site-directed mutagenesis of the *comEB* gene (generating Mut12), the same biofilm pheno-

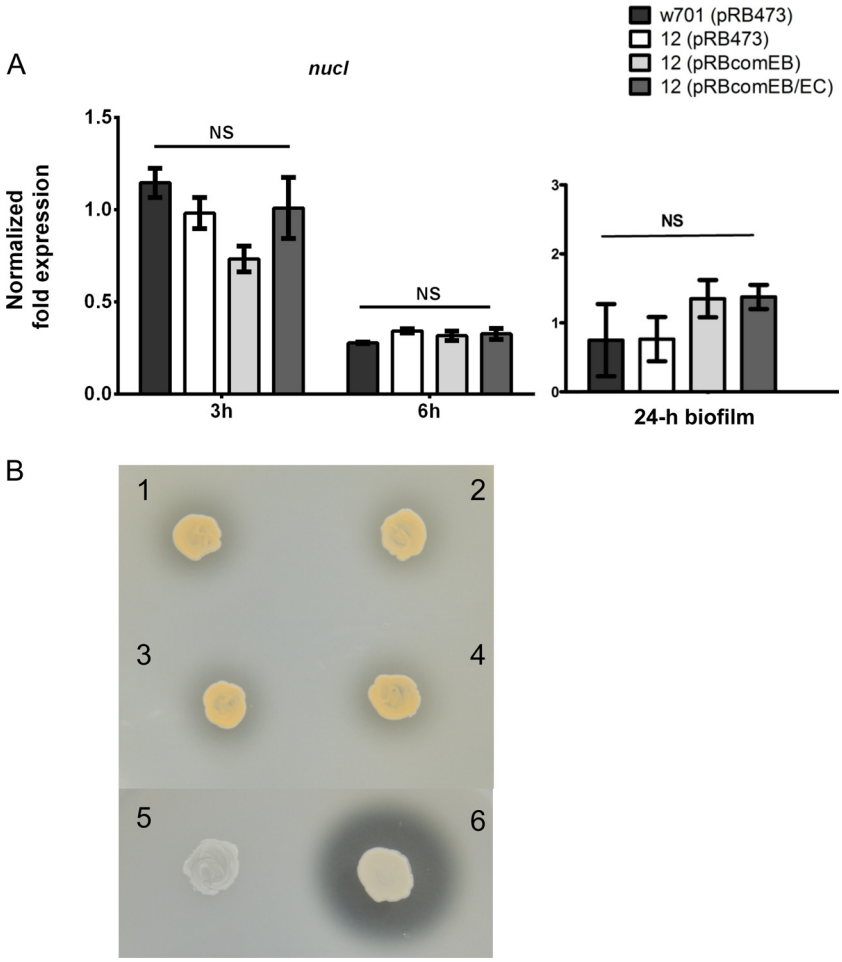

FIG 5 Expression (A) and activity (B) of the thermonuclease NucI is unchanged. (A) *nucI* gene expression was measured during the mid-logarithmic (3 h) and stationary (6 h) phases of growth or in 24-h-grown biofilms. The mRNA levels were quantified by real-time PCR and normalized against the relative quantities of the *aroE* and *gyrB* housekeeping gene transcripts. The data were analyzed with one-way ANOVA and a Bonferroni's posttest. (B) NucI activity, observed as clearing zones around bacterial growth on DNase agar, was similar among wild-type w701(pRB473) (2), Mut12(pRB473) (1), and the complemented mutants Mut12(pRB*comEB*) (3) and Mut12(pRB*comEB/EC*) (4). *S. aureus* SA113(pCU1) (6) and *S. epidermidis* O-47(pCU1) (5) served as positive and negative controls, respectively.

type was reproduced in a different genetic background, verifying that impaired biofilm formation is indeed due to the inactivation of the *comEB* gene and not due to secondary mutations. Moreover, complementation with *comEB* was sufficient to restore biofilm formation to wild-type levels, further verifying the implication of *comEB*.

comEB is part of the *comE* competence operon, which in *S. aureus* consists of the *comEA*, *comEB*, and *comEC* genes (59); thus, it largely resembles the organization of the *Bacillus subtilis comE* operon (60). In contrast, in *S. lugdunensis*, *comEB* and *comEC* form a transcriptional unit with *holA*, while *comEA* is encoded independently upstream of *comEB* (Fig. 1B). The *holA* gene putatively encodes the DNA polymerase PolC δ subunit, which is essential for DNA replication (61). Because (i) Mut12 exhibited the same growth characteristics as its wild type (Fig. 4D), (ii) *comEB* was sufficient to complement Mut12, and (iii) real-time PCR analysis indicated unaltered *holA* expression (see Fig. S1 in the supplemental material), we can rule out a potential involvement of *holA* in the observed biofilm phenotype.

Natural competence for DNA uptake has only recently been demonstrated in *S. aureus*, where it depends on the expression of a cryptic gene encoding the alternative σ factor SigH (62). In *S. aureus*, SigH is produced only under certain circumstances in a minor subpopulation and formerly was reported to induce the transcription of the *comG* and *comE* competence operons (59, 62). However, the situation seems to differ in *S. lugdunensis*, because in preliminary RT-PCR experiments we were able to detect *comEB* and *comEC* transcripts in the *S. lugdunensis* w701 wild type during mid-log and stationary growth phases (data not shown).

While in staphylococci the components required for the development of competence and the functions of *comEB* and *comEC* are currently largely undefined, such components and their regulation have been well studied in other bacterial species. In *B. subtilis*, both *comEB* and *comEC* belong to the late competence genes, and their gene products form the DNA uptake machinery (63). ComEC is the membrane channel protein that delivers the DNA into the cytosol, while ComEB has no clear role in the whole process of DNA uptake, although it is known to localize at the cell poles where the DNA uptake machinery assembles (60, 64). Analysis of the *S. lugdunensis* ComEC amino acid sequence at

HMMTOP (http://www.enzim.hu/hmmtop/) revealed the presence of 12 potential transmembrane helices, indicating its location within the membrane analogous to *B. subtilis* ComEC. However, *B. subtilis* ComEC has only 7 transmembrane helices (64).

The fact that *comEB* was sufficient to complement Mut12 indicates that either Mut12 still was able to translate *comEC* and to produce ComEC independently of *comEB* translation or that the function of ComEC in *S. lugdunensis* biofilm formation is dispensable. In order to determine a potential involvement of ComEC, we sought to analyze if Mut12 still produces ComEC. For this, we aimed to raise antibodies against ComEC. However, we were not successful in the expression and purification of ComEC from *E. coli* (data not shown), indicating that ComEC is toxic. Likewise, ComEC from *B. subtilis* has been found to be toxic to *E. coli* (60).

Because the induction of competence stimulates DNA release essential for biofilm formation in *Streptococcus pneumoniae* and *B. subtilis* (41, 46), we aimed to identify a possible correlation of the eDNA content of *S. lugdunensis* biofilms with the competence gene *comEB*. For this, we quantified the eDNA from mature biofilms, and indeed we did observe a significant reduction of biofilm eDNA in Mut12 compared to the level of its wild type. Furthermore, by staining the eDNA with PI, we were able to visualize the shortage of eDNA in the biofilm matrix of Mut12. Additionally, 3D images from 48-h biofilms were obtained, which confirmed the structural weakness of the Mut12 biofilm. Mutants with the inability to release DNA or factors that disturb the integrity of eDNA have been used in staphylococci before to demonstrate the negative outcome of a biofilm that is not supported by the eDNA scaffold (21, 22, 43, 52).

Competence triggers a lysis-dependent or lysis-independent DNA release in bacterial biofilms. Thus, possible origins of eDNA in the biofilm matrix may include cell lysis of a subpopulation in the biofilm, as shown for *S. epidermidis* (22), *S. aureus* (21), *S. pneumoniae* (46), and *Pseudomonas aeruginosa* (42), and specific secretion as identified in *Bacillus cereus* (25). Active DNA secretion that depends on a type IV secretion system also has been described in *Neisseria gonorrhoeae* (47). To analyze if the *comEB*-mediated, eDNA-based mechanism of biofilm formation involves lysis-dependent or lysis-independent mechanisms of DNA release, we determined autolysis, autolytic activities, and cell viability. Autolysis and autolytic activities of Mut12, its wild type, and the complemented mutants were comparable in Triton X-100-induced autolysis assays and zymograms, respectively (Fig. 4A and B). Moreover, we did not observe a change in cell viability (Fig. 4D). In agreement, in our recent study we did not observe significant differences in the eDNA content of preparations from the clinical *S. lugdunensis* isolate Sl253 and its *atlL* mutant (14), further supporting our findings that the release of DNA is lysis independent in *S. lugdunensis*. Consequently, *comEB*-mediated DNA release and biofilm formation also appear to be independent of the *cid-lrg* system, which has been reported to contribute to *S. aureus* cell lysis, genomic DNA release, and biofilm formation *in vitro* and *in vivo* (21). Considering further possible factors that may cause the loss of eDNA in Mut12, we also checked the expression and activity of the thermonuclease NucI and again did not observe any differences. Taken together, these results suggest normal cell lysis and nuclease activity in Mut12, indicative of a lysis-independent mechanism of DNA release mediated by *comEB*.

In conclusion, we propose a novel *comEB*-dependent and lysis-independent mechanism of DNA release that stimulates eDNA-based biofilm formation in *S. lugdunensis*. Because the *comE* operon has not been characterized in other staphylococcal species yet, we further hypothesize that this novel mechanism also represents a new source of biofilm eDNA in other relevant staphylococcal species, such as *S. aureus* and *S. epidermidis*. However, further research is necessary to characterize the underlying mechanisms involved in the *comEB*-dependent DNA release.

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REFERENCES

1. **Freney J, Brun Y, Bes M, Meugnier H, Grimont F, Grimont PAD, Nervi C, Fleurette J.** 1988. *Staphylococcus lugdunensis* sp. nov. and *Staphylococcus schleiferi* sp. nov., two species from human clinical specimens. Int J Syst Bacteriol **38:**168–172. http://dx.doi.org/10.1099/00207713-38-2-168.
2. **Ravaioli S, Selan L, Visai L, Pirini V, Campoccia D, Maso A, Speziale P, Montanaro L, Arciola CR.** 2012. *Staphylococcus lugdunensis*, an aggressive coagulase-negative pathogen not to be underestimated. Int J Artif Organs **35:**742–753.
3. **Becker K, Heilmann C, Peters G.** 2014. Coagulase-negative staphylococci. Clin Microbiol Rev **27:**870–926. http://dx.doi.org/10.1128/CMR.00109-13.
4. **Anguera I, Del Rio A, Miro JM, Matinez-Lacasa X, Marco F, Guma JR, Quaglio G, Claramonte X, Moreno A, Mestres CA, Mauri E, Azqueta M, Benito N, Garcia-de la Maria C, Almela M, Jimenez-Exposito MJ, Sued O, De Lazzari E, Gatell JM.** 2005. *Staphylococcus lugdunensis* infective endocarditis: description of 10 cases and analysis of native valve, prosthetic valve, and pacemaker lead endocarditis clinical profiles. Heart **91:**e10. http://dx.doi.org/10.1136/hrt.2004.040659.
5. **Böcher S, Tonning B, Skov RL, Prag J.** 2009. Staphylococcus lugdunensis, a common cause of skin and soft tissue infections in the community. J Clin Microbiol **47:**946–950. http://dx.doi.org/10.1128/JCM.01024-08.
6. **Zinkernagel AS, Zinkernagel MS, Elzi MV, Genoni M, Gubler J, Zbinden R, Mueller NJ.** 2008. Significance of *Staphylococcus lugdunensis* bacteremia: report of 28 cases and review of the literature. Infection **36:**314–321. http://dx.doi.org/10.1007/s15010-008-7287-9.
7. **Seifert H, Oltmanns D, Becker K, Wisplinghoff H, von Eiff C.** 2005. *Staphylococcus lugdunensis* pacemaker-related infection. Emerg Infect Dis **11:**1283–1286. http://dx.doi.org/10.3201/eid1108.041177.
8. **Koch LD, Knoll F, Hartmann G, Lhotta K.** 2011. Recurrent exit-site infection due to *Staphylococcus lugdunensis*–a virulent coagulase-negative *Staphylococcus*. Perit Dial Int **31:**372–373. http://dx.doi.org/10.3747/pdi.2010.00272.
9. **Kiedrowski MR, Horswill AR.** 2011. New approaches for treating staphylococcal biofilm infections. Ann N Y Acad Sci **1241:**104–121. http://dx.doi.org/10.1111/j.1749-6632.2011.06281.x.
10. **Heilmann C.** 2011. Adhesion mechanisms of staphylococci, p 105–123. *In* Linke D, Goldman A (ed), Bacterial adhesion chemistry, biology and physics: advances in experimental medicine and biology, vol 715. Springer Science and Business Media, New York, NY.
11. **Foster TJ, Geoghegan JA, Ganesh VK, Höök M.** 2014. Adhesion, invasion and evasion: the many functions of the surface proteins of Staphylococcus aureus. Nat Rev Microbiol **12:**49–62.
12. **Nilsson M, Bjerketorp J, Wiebensjo A, Ljungh A, Frykberg L, Guss B.** 2004. A von Willebrand factor-binding protein from *Staphylococcus lugdunensis*. FEMS Microbiol Lett **234:**155–161. http://dx.doi.org/10.1111/j.1574-6968.2004.tb09527.x.
13. **Mitchell J, Tristan A, Foster TJ.** 2004. Characterization of the fibrinogen-binding surface protein Fbl of *Staphylococcus lugdunensis*. Microbiology **150:**3831–3841. http://dx.doi.org/10.1099/mic.0.27337-0.

14. **Hussain M, Steinbacher T, Peters G, Heilmann C, Becker K.** 2015. The adhesive properties of the *Staphylococcus lugdunensis* multifunctional autolysin AtlL and its role in biofilm formation and internalization. Int J Med Microbiol **305:**129–139. http://dx.doi.org/10.1016/j.ijmm.2014.11.010.
15. **Haley KP, Janson EM, Heilbronner S, Foster TJ, Skaar EP.** 2011. *Staphylococcus lugdunensis* IsdG liberates iron from host heme. J Bacteriol **193:**4749–4757. http://dx.doi.org/10.1128/JB.00436-11.
16. **Heilbronner S, Hanses F, Monk IR, Speziale P, Foster TJ.** 2013. Sortase A promotes virulence in experimental *Staphylococcus lugdunensis* endocarditis. Microbiology **159:**2141–2152.
17. **Frank KL, Patel R.** 2007. Poly-N-acetylglucosamine is not a major component of the extracellular matrix in biofilms formed by *icaADBC*-positive *Staphylococcus lugdunensis* isolates. Infect Immun **75:**4728–4742. http://dx.doi.org/10.1128/IAI.00640-07.
18. **Heilmann C, Schweitzer O, Gerke C, Vanittanakom N, Mack D, Götz F.** 1996. Molecular basis of intercellular adhesion in the biofilm-forming *Staphylococcus epidermidis*. Mol Microbiol **20:**1083–1091. http://dx.doi.org/10.1111/j.1365-2958.1996.tb02548.x.
19. **Cramton SE, Ulrich M, Götz F, Döring G.** 2001. Anaerobic conditions induce expression of polysaccharide intercellular adhesin in *Staphylococcus aureus* and *Staphylococcus epidermidis*. Infect Immun **69:**4079–4085. http://dx.doi.org/10.1128/IAI.69.6.4079-4085.2001.
20. **Missineo A, Di Poto A, Geoghegan JA, Rindi S, Heilbronner S, Gianotti V, Arciola CR, Foster TJ, Speziale P, Pietrocola G.** 2014. IsdC from *Staphylococcus lugdunensis* induces biofilm formation under low-iron growth conditions. Infect Immun **82:**2448–2459. http://dx.doi.org/10.1128/IAI.01542-14.
21. **Rice KC, Mann EE, Endres JL, Weiss EC, Cassat JE, Smeltzer MS, Bayles KW.** 2007. The *cidA* murein hydrolase regulator contributes to DNA release and biofilm development in *Staphylococcus aureus*. Proc Natl Acad Sci U S A **104:**8113–8118. http://dx.doi.org/10.1073/pnas.0610226104.
22. **Qin Z, Ou Y, Yang L, Zhu Y, Tolker-Nielsen T, Molin S, Qu D.** 2007. Role of autolysin-mediated DNA release in biofilm formation of *Staphylococcus epidermidis*. Microbiology **153:**2083–2092. http://dx.doi.org/10.1099/mic.0.2007/006031-0.
23. **Guiton PS, Hung CS, Kline KA, Roth R, Kau AL, Hayes E, Heuser J, Dodson KW, Caparon MG, Hultgren SJ.** 2009. Contribution of autolysin and sortase A during *Enterococcus faecalis* DNA-dependent biofilm development. Infect Immun **77:**3626–3638. http://dx.doi.org/10.1128/IAI.00219-09.
24. **Seper A, Fengler VH, Roier S, Wolinski H, Kohlwein SD, Bishop AL, Camilli A, Reidl J, Schild S.** 2011. Extracellular nucleases and extracellular DNA play important roles in *Vibrio cholerae* biofilm formation. Mol Microbiol **82:**1015–1037. http://dx.doi.org/10.1111/j.1365-2958.2011.07867.x.
25. **Vilain S, Pretorius JM, Theron J, Brozel VS.** 2009. DNA as an adhesin: *Bacillus cereus* requires extracellular DNA to form biofilms. Appl Environ Microbiol **75:**2861–2868. http://dx.doi.org/10.1128/AEM.01317-08.
26. **Christensen GD, Simpson WA, Bisno AL, Beachey EH.** 1982. Adherence of slime-producing strains of *Staphylococcus epidermidis* to smooth surfaces. Infect Immun **37:**318–326.
27. **Schleifer KH, Fischer U.** 1982. Description of a new species of the genus *Staphylococcus*: *Staphylococcus carnosus*. Int J Syst Bacteriol **32:**153–156. http://dx.doi.org/10.1099/00207713-32-2-153.
28. **Heilmann C, Gerke C, Perdreau-Remington F, Götz F.** 1996. Characterization of Tn*917* insertion mutants of *Staphylococcus epidermidis* affected in biofilm formation. Infect Immun **64:**277–282.
29. **Iordanescu S, Surdeanu M.** 1976. Two restriction and modification systems in *Staphylococcus aureus* NCTC8325. J Gen Microbiol **96:**277–281. http://dx.doi.org/10.1099/00221287-96-2-277.
30. **Youngman PJ, Perkins JB, Losick R.** 1983. Genetic transposition and insertional mutagenesis in *Bacillus subtilis* with *Streptococcus faecalis* transposon *Tn*917. Proc Natl Acad Sci U S A **80:**2305–2309. http://dx.doi.org/10.1073/pnas.80.8.2305.
31. **Götz F, Schumacher B.** 1987. Improvements of protoplast transformation in *Staphylococcus carnosus*. FEMS Microbiol Lett **40:**285–288. http://dx.doi.org/10.1111/j.1574-6968.1987.tb02040.x.
32. **Knobloch JK, Nedelmann M, Kiel K, Bartscht K, Horstkotte MA, Dobinsky S, Rohde H, Mack D.** 2003. Establishment of an arbitrary PCR for rapid identification of Tn*917* insertion sites in *Staphylococcus epidermidis*: characterization of biofilm-negative and nonmucoid mutants. Appl Environ Microbiol **69:**5812–5818. http://dx.doi.org/10.1128/AEM.69.10.5812-5818.2003.
33. **Kozlowski M, Thatte V, Lau PC, Visentin LP, Iyer VN.** 1987. Isolation and structure of the replicon of the promiscuous plasmid pCU1. Gene **58:**217–228. http://dx.doi.org/10.1016/0378-1119(87)90377-5.
34. **Brückner R.** 1997. Gene replacement in *Staphylococcus carnosus* and *Staphylococcus xylosus*. FEMS Microbiol Lett **151:**1–8. http://dx.doi.org/10.1016/S0378-1097(97)00116-X.
35. **Marlinghaus L, Becker K, Korte M, Neumann S, Gatermann SG, Szabados F.** 2012. Construction and characterization of three knockout mutants of the *fbl* gene in *Staphylococcus lugdunensis*. APMIS **120:**108–116. http://dx.doi.org/10.1111/j.1600-0463.2011.02819.x.
36. **Brückner R, Wagner E, Götz F.** 1993. Characterization of a sucrase gene from *Staphylococcus xylosus*. J Bacteriol **175:**851–857.
37. **Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A.** 2012. Fiji: an open-source platform for biological-image analysis. Nat Methods **9:**676–682. http://dx.doi.org/10.1038/nmeth.2019.
38. **Heilmann C, Hussain M, Peters G, Götz F.** 1997. Evidence for autolysin-mediated primary attachment of *Staphylococcus epidermidis* to a polystyrene surface. Mol Microbiol **24:**1013–1024. http://dx.doi.org/10.1046/j.1365-2958.1997.4101774.x.
39. **Steinmoen H, Knutsen E, Havarstein LS.** 2002. Induction of natural competence in *Streptococcus pneumoniae* triggers lysis and DNA release from a subfraction of the cell population. Proc Natl Acad Sci U S A **99:**7681–7686. http://dx.doi.org/10.1073/pnas.112464599.
40. **Lorenz MG, Gerjets D, Wackernagel W.** 1991. Release of transforming plasmid and chromosomal DNA from two cultured soil bacteria. Arch Microbiol **156:**319–326. http://dx.doi.org/10.1007/BF00263005.
41. **Zafra O, Lamprecht-Grandio M, de Figueras CG, Gonzalez-Pastor JE.** 2012. Extracellular DNA release by undomesticated *Bacillus subtilis* is regulated by early competence. PLoS One **7:**e48716. http://dx.doi.org/10.1371/journal.pone.0048716.
42. **Allesen-Holm M, Barken KB, Yang L, Klausen M, Webb JS, Kjelleberg S, Molin S, Givskov M, Tolker-Nielsen T.** 2006. A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms. Mol Microbiol **59:**1114–1128. http://dx.doi.org/10.1111/j.1365-2958.2005.05008.x.
43. **Mann EE, Rice KC, Boles BR, Endres JL, Ranjit D, Chandramohan L, Tsang LH, Smeltzer MS, Horswill AR, Bayles KW.** 2009. Modulation of eDNA release and degradation affects *Staphylococcus aureus* biofilm maturation. PLoS One **4:**e5822. http://dx.doi.org/10.1371/journal.pone.0005822.
44. **Bose JL, Lehman MK, Fey PD, Bayles KW.** 2012. Contribution of the *Staphylococcus aureus* Atl AM and GL murein hydrolase activities in cell division, autolysis, and biofilm formation. PLoS One **7:**e42244. http://dx.doi.org/10.1371/journal.pone.0042244.
45. **Heydorn A, Nielsen AT, Hentzer M, Sternberg C, Givskov M, Ersboll BK, Molin S.** 2000. Quantification of biofilm structures by the novel computer program COMSTAT. Microbiology **146**(Part 10)**:**2395–2407. http://dx.doi.org/10.1099/00221287-146-10-2395.
46. **Trappetti C, Potter AJ, Paton AW, Oggioni MR, Paton JC.** 2011. LuxS mediates iron-dependent biofilm formation, competence, and fratricide in *Streptococcus pneumoniae*. Infect Immun **79:**4550–4558. http://dx.doi.org/10.1128/IAI.05644-11.
47. **Hamilton HL, Dominguez NM, Schwartz KJ, Hackett KT, Dillard JP.** 2005. *Neisseria gonorrhoeae* secretes chromosomal DNA via a novel type IV secretion system. Mol Microbiol **55:**1704–1721. http://dx.doi.org/10.1111/j.1365-2958.2005.04521.x.
48. **Rice KC, Bayles KW.** 2008. Molecular control of bacterial death and lysis. Microbiol Mol Biol Rev **72:**85–109. http://dx.doi.org/10.1128/MMBR.00030-07.
49. **Hirschhausen N, Schlesier T, Peters G, Heilmann C.** 2012. Characterization of the modular design of the autolysin/adhesin Aaa from *Staphylococcus aureus*. PLoS One **7:**e40353. http://dx.doi.org/10.1371/journal.pone.0040353.
50. **Hirschhausen N, Schlesier T, Schmidt MA, Götz F, Peters G, Heilmann C.** 2010. A novel staphylococcal internalization mechanism involves the major autolysin Atl and heat shock cognate protein Hsc70 as host cell receptor. Cell Microbiol **12:**1746–1764. http://dx.doi.org/10.1111/j.1462-5822.2010.01506.x.
51. **Heilmann C, Thumm G, Chhatwal GS, Hartleib J, Uekötter A, Peters G.** 2003. Identification and characterization of a novel autolysin (Aae) with

adhesive properties from *Staphylococcus epidermidis*. Microbiology **149:** 2769–2778. http://dx.doi.org/10.1099/mic.0.26527-0.

52. **Kiedrowski MR, Kavanaugh JS, Malone CL, Mootz JM, Voyich JM, Smeltzer MS, Bayles KW, Horswill AR.** 2011. Nuclease modulates biofilm formation in community-associated methicillin-resistant *Staphylococcus aureus*. PLoS One **6:**e26714. http://dx.doi.org/10.1371/journal.pone.0026714.
53. **Tang J, Zhou R, Shi X, Kang M, Wang H, Chen H.** 2008. Two thermostable nucleases coexisted in *Staphylococcus aureus*: evidence from mutagenesis and in vitro expression. FEMS Microbiol Lett **284:**176–183. http://dx.doi.org/10.1111/j.1574-6968.2008.01194.x.
54. **Tse H, Tsoi HW, Leung SP, Lau SK, Woo PC, Yuen KY.** 2010. Complete genome sequence of *Staphylococcus lugdunensis* strain HKU09-01. J Bacteriol **192:**1471–1472. http://dx.doi.org/10.1128/JB.01627-09.
55. **Heilbronner S, Holden MT, van Tonder A, Geoghegan JA, Foster TJ, Parkhill J, Bentley SD.** 2011. Genome sequence of *Staphylococcus lugdunensis* N920143 allows identification of putative colonization and virulence factors. FEMS Microbiol Lett **322:**60–67. http://dx.doi.org/10.1111/j.1574-6968.2011.02339.x.
56. **Frank KL, Del Pozo JL, Patel R.** 2008. From clinical microbiology to infection pathogenesis: how daring to be different works for *Staphylococcus lugdunensis*. Clin Microbiol Rev **21:**111–133. http://dx.doi.org/10.1128/CMR.00036-07.
57. **Kaspar U, Kriegeskorte A, Schubert T, Peters G, Rudack C, Pieper DH, Wos-Oxley M, Becker K.** 28 April 2015. The culturome of the human nose habitats reveals individual bacterial fingerprint patterns. Environ Microbiol.
58. **Bieber L, Kahlmeter G.** 2010. *Staphylococcus lugdunensis* in several niches of the normal skin flora. Clin Microbiol Infect **16:**385–388. http://dx.doi.org/10.1111/j.1469-0691.2009.02813.x.
59. **Morikawa K, Inose Y, Okamura H, Maruyama A, Hayashi H, Takeyasu K, Ohta T.** 2003. A new staphylococcal sigma factor in the conserved gene cassette: functional significance and implication for the evolutionary processes. Genes Cells **8:**699–712. http://dx.doi.org/10.1046/j.1365-2443.2003.00668.x.
60. **Inamine GS, Dubnau D.** 1995. ComEA, a *Bacillus subtilis* integral membrane protein required for genetic transformation, is needed for both DNA binding and transport. J Bacteriol **177:**3045–3051.
61. **Bruck I, Georgescu RE, O'Donnell M.** 2005. Conserved interactions in the *Staphylococcus aureus* DNA PolC chromosome replication machine. J Biol Chem **280:**18152–18162. http://dx.doi.org/10.1074/jbc.M413595200.
62. **Morikawa K, Takemura AJ, Inose Y, Tsai M, Nguyen TL, Ohta TT, Msadek T.** 2012. Expression of a cryptic secondary sigma factor gene unveils natural competence for DNA transformation in *Staphylococcus aureus*. PLoS Pathog **8:**e1003003. http://dx.doi.org/10.1371/journal.ppat.1003003.
63. **Dubnau D.** 1991. Genetic competence in *Bacillus subtilis*. Microbiol Rev **55:**395–424.
64. **Draskovic I, Dubnau D.** 2005. Biogenesis of a putative channel protein, ComEC, required for DNA uptake: membrane topology, oligomerization and formation of disulfide bonds. Mol Microbiol **55:**881–896.