# IsdC from *Staphylococcus lugdunensis* induces biofilm formation in low-iron growth conditions

**Antonino Missineo[1,§,†], Antonella Di Poto[1,†], Joan A Geoghegan[2], Simonetta Rindi[1], Simon Heilbronner[2],Valentina Gianotti[1], Carla Renata Arciola[3], Timothy J Foster[2], Pietro Speziale[1]*, Giampiero Pietrocola[1]**

[1]Department of Molecular Medicine, Unit of Biochemistry, Viale Taramelli 3/b, 27100 Pavia, Italy

[2]Department of Microbiology, Moyne Institute of Preventive Medicine, Trinity College Dublin 2, Ireland

[3]Istituti Ortopedici Rizzoli Laboratorio Patologia delle Infezioni Associate all'Impianto, Via di Barbiano 1/10, 40136 Bologna, Italy

[§]Present address: IRBM Science Park S.p.A. Via Pontina Km. 30.600 Pomezia, Roma 00040, Italy

*Address correspondence: Pietro Speziale, Department of Molecular Medicine, Unit of Biochemistry, Viale Taramelli 3/b, 27100 Pavia, Italy. Tel: 0039 0382 987787 Fax: 0039 0382 423108
E-mail: pspeziale@unipv.it

[†]A. Missineo and A. Di Poto contributed equally to this work.

## ABSTRACT

*Staphylococcus lugdunensis* is a coagulase-negative *Staphylococcus* that is a commensal of humans and an opportunistic pathogen. It can cause a spectrum of infections including those that are associated with the ability to form biofilm such as endocarditis or indwelling medical devices. The genome sequences of two strains revealed the presence of orthologues of the *ica* genes that are responsible for synthesis of poly-N-acetyl glucosamine (PNAG) that is commonly associated with biofilm in other staphylococci. However, here we discovered that biofilm formed by a panel of *S. lugdunensis* isolates growing in iron-restricted medium was susceptible to degradation by proteases and not by metaperiodate suggesting that the biofilm matrix comprised proteins and not PNAG. When the iron concentration was raised to 1mM biofilm formation by all strains tested was greatly reduced. A mutant of strain N920143 lacking the entire locus that encodes iron-regulated surface determinant (Isd) proteins was defective in biofilm formation under iron-limited conditions. A IsdC null mutant was defective whereas IsdK, IsdJ and IsdB mutants formed biofilm to the same level as the parental strain. Expression of IsdC was required both for the primary attachment to unconditioned polystyrene and for the accumulation phase of biofilm involving cell-cell interactions. Purified recombinant IsdC protein formed dimers in solution and *Lactococcus lactis* cells expressing only IsdC adhered to immobilized recombinant IsdC but not to IsdJ, IsdK or IsdB. This is consistent with a specific homophilic interaction between IsdC molecules on neighbouring cells contributing to accumulation of *S. lugdunensis* biofilm *in vivo*.


## INTRODUCTION

Coagulase-negative staphylococci (CoNS) and *Staphylococcus aureus* are the predominant etiological agents of medical device-related infections, largely owing to their ability to form biofilm. Biofilms are defined as communities of bacteria encased in a self-synthesized extracellular

polymeric matrix (1) growing attached to biological or abiotic surfaces. Staphylococci in biofilms are resistant to antibiotics (2) and host immune responses (3), reducing the efficacy of available antimicrobials. The formation of biofilm is a complex, multifactorial process. Initially, bacteria adhere directly to the surface of implanted device or to devices coated with the host matrix components. In *S. aureus* biofilm the major autolysin Atl mediates primary attachment to plastic surfaces by promoting release of DNA from bacterial cells (4, 5), while adherence to surfaces conditioned by host plasma proteins is promoted by surface protein adhesins such as the fibrinogen-binding clumping factor A or fibronectin binding proteins (6). This process is followed by proliferation, accumulation and intercellular interactions mediated by the *icaADBC*-encoded polysaccharide intercellular adhesins (PIA) (7) or surface proteins such as Bap (8), SasG (9), SasC (10), protein A (11), or fibronectin-binding proteins (FnBPs) (12, 13). Likewise, biofilm formation by *S. epidermidis* is dependent on PIA or proteinaceous components such as Aap (14, 15) or SesC (16).

*Staphylococcus lugdunensis* is a coagulase-negative species with enhanced virulence compared with the other CoNS (17). *S. lugdunensis* causes a severe form of native valve endocarditis (18, 19), infections of prosthetic heart valves (20), intravascular catheters (21), prosthetic joints (22) and ventriculoperitoneal shunts (23). This pathogenic potential is largely attributed to the ability of this bacterium to form biofilm. A previous study by Frank and Patel (24) demonstrated that despite the presence of *icaADBC* orthologues in *S. lugdunensis*, PIA is not the major component of the extracellular matrix of biofilms formed *in vitro* by this species. Rather, *S. lugdunensis* biofilms appear to be composed of proteins. *S. lugdunensis* expresses a fibrinogen-binding protein (Fbl) (25), a member of the family of MSCRAMM (Microbial Surface Components Recognizing Adhesive Matrix Molecules) family that is closely related to ClfA, which does not appear to have any role in biofilm formation (26) and a yet uncharacterized von Willebrand factor binding protein (27).

Uniquely for CoNS, *S. lugdunensis* contains a cluster of genes with similarity both in terms of organization and sequence to the iron-regulated surface determinant (*isd*) locus of *S. aureus* (28). Both systems are expressed under iron-restricted conditions (28, 29). Four of the *S. lugdunensis* Isd proteins are anchored to the cell wall peptidoglycan by sortases. In *S. aureus* and *S. lugdunensis*, the Isd proteins cooperate to capture heme and transfer it across the wall to a membrane-bound transporter, which delivers it to the cytoplasm, where heme is degraded to recover iron (30). There is evidence that surface-exposed Isd proteins may have additional roles in colonization and pathogenesis of both species. For example, IsdJ from *S. lugdunensis* (29) and IsdA from *S. aureus* (31) are multifunctional proteins which recognize and bind several host proteins and can confer resistance to skin fatty acids. In this study we investigated biofilm formation *in vitro* by a collection of *S. lugdunensis* isolates grown in low iron conditions and assessed the role of *isd* locus in biofilm formation by this important pathogen.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions**. The microorganisms used in this study are reported in Table 1. *S. aureus* V329 (8) and SA113 (32) were kindly donated by Dr. J.R. Penades (Universidad Cardenal Herrera-CEU, Moncada, Valencia, Spain). The clinical isolate of *S. epidermidis* 5179R (14) was provided by Dr. H. Rohde (University Medical Centre Hamburg-Eppendorf, Hamburg, Germany). *S. epidermidis* RP62A was originally isolated from a patient with intravascular catheter-associated sepsis by Muller *et al*. (33). Staphylococci were grown in TSB (trypticase soy broth) (Difco, Detroit, MI, USA) or in RPMI 1640 (Biowest, Nuaillé, France) supplemented with 2 mM glutamine (Lonza Srl, Bergamo, Italy) at 37°C for 16-18 h with high shaking (200 rpm). *L. lactis* transformants were grown in M17 medium (Difco) containing 0.5 % glucose and 10% lactose at 30°C.

106 **Construction of *S. lugdunensis* mutants.** *S. lugdunensis* N920143 mutants deficient in individual
107 genes *isdB*, *isdC*, *isdJ* and *isdK* and the mutant deficient in the entire *isd* locus were reported earlier
108 (29) (Table1). Additional mutants deficient in *srtB* and the autolysin *atlI* were created using the
109 thermosensitive vector pIMAY and allelic exchange. A detailed protocol of the procedure is
110 described elsewhere (34). Primers used for the construction of deletion cassettes are summarized in
111 Supplementary Table 1.
112
113 **Construction and expression of *isdC* in *S. lugdunensis* Δ*isd*.** In order to create a *S. lugdunensis*
114 strain expressing IsdC in the absence of any other Isd proteins, the *isdC* gene was cloned into the
115 anhydrotetracycline-inducible vector pRMC2 (35). The gene was PCR amplified with the primers
116 isdC-pRF and isdC-pRR using pNZ8048 *isdC*-LPQTG as the template. *S. lugdunensis* Δ*isd* is
117 deficient in sortase B which will prevent cell wall sorting of the wild type IsdC protein harboring a
118 NPQTS motif. The IsdC-LPQTG protein will be sorted by sortase A.
119 The *isdC*-LPQTG gene was cloned into pRMC2 using sequence and ligase independent
120 cloning (SLIC) (36,37). This method involves PCR amplification of the vector backbone and of the
121 insert. Amplification of the vector backbone generates a linear product with blunt ends at the site
122 required for the cloning. Primers incorporate short identical DNA sequences in vector and insert.
123 Treatment of both PCR products with T4 polymerase (3'-5' exonuclease activity) creates single
124 stranded, 5'-overhangs in both vector and insert. The DNA fragments are assembled *in vitro*
125 (without ligation) and used to transform *E. coli*.
126 Primers pRA and pRB were used to amplify the pRMC2 backbone (Supplementary Table
127 1). Identical sequences (20-25 nucleotides) were integrated in the primers for the amplification of
128 the insert (isdC-pRF / isdC-pRR, Supplementary Table 1). 10 ng of plasmid DNA was used as
129 template for the amplification of the plasmid backbone with Phusion polymerase (Finnzymes,
130 Keilaranta 16 A, 02150 Espoo, Finland ). The PCR products were purified and the vector product
131 was digested with DpnI to remove methylated template DNA. 1 µg of vector and insert DNA was

132 treated with T4 polymerase in a final volume of 40 µl of NEB Buffer 2 (New England Biolabs,
133 Ipswich, MA, USA) with highly purified BSA (100 µg/ml) (New England Biolabs), 5 mM
134 dithiothreitol (DTT), 200 mM urea and 3 units T4 DNA polymerase (New England Biolabs),
135 incubated for 20 min at 23°C and the reaction stopped by the addition of 25 mM EDTA and
136 subsequent incubation for 20 min at 73°C.

137 5 µl of vector DNA and 5 µl of insert DNA were mixed and the single-stranded overhangs
138 were allowed to anneal. The tube was placed in a PCR machine for 10 min at 65°C followed by a
139 slow decrease in temperature from 65°C to 25°C with a 1 min hold for each degree. 2.5 µl of the
140 reaction was used to transform *E. coli* to isolate pRMC2 isdC-LPQTG which was confirmed by
141 DNA sequencing. The plasmid was transformed into *S. lugdunensis* Δ*isd* (34). Induction of IsdC-
142 LPXTG expression from pRMC2 in *S. lugdunensis* Δ*isd* was carried out by adding
143 anhydrotetracycline (0.125 µg/ml) (Sigma) to exponential growing cultures.

144

145 **Construction of *Lactococcus lactis* expressing IsdC**. To express IsdC in the surrogate host *L.*
146 *lactis*, the *isdC* gene was amplified (IsdC-F / IsdC-R) and cloned into the nisin inducible expression
147 vector pNZ8048 (38). *L. lactis* was transformed with the recombinant plasmid as described earlier
148 (39).

149 IsdC possesses a NPQTS sorting signal at its C-terminus and is therefore anchored to the cell wall
150 by the transpeptidase sortase B which is encoded within the *isd* operon. *L. lactis* does not encode
151 sortase B which will prevent sorting of IsdC to the cell wall. To allow sorting, pNZ8048-*isdC* was
152 isolated and DNA encoding the NPQTS signal was exchanged to LPQTG using 5'-phosphorylated
153 primers (LPXTG-A/LPXTG-B) and inverse PCR. The primers allowed the amplification of the
154 entire plasmid and introduced the required nucleotide substitutions in the cell wall anchoring region
155 of *isdC*. The PCR product was treated with T4 ligase to allow circularization of the plasmid and
156 transformed into *L. lactis*. The resulting plasmid (pNZ8048*isdC*-LPQTG) was confirmed by DNA
157 sequencing. Primers are summarized in Supplementary Table 1. Induction of IsdC-LPXTG

158 expression from pNZ8048 in *L. lactis* was carried out by adding nisin (0.4 ng/ml) to exponential
159 growing cultures.

160

161 **Biofilm formation.** Overnight cultures of staphylococci were diluted 1:200 in TSB containing
162 0.3% glucose ($TSB_{0.3\%glucose}$) or RPMI supplemented with 0.3% glucose ($RPMI_{0.3\%glucose}$) and 2 mM
163 glutamine. Aliquots (200 µl) of the diluted bacterial suspensions were added to 96-well flat-bottom
164 sterile polystyrene microplates (Costar. Corning, NY, NY) and incubated statically for 24 h at 37°C.
165 Biofilm formation was detected by the method of Christensen *et al*. (40). Briefly, biofilms formed
166 on the plates were gently washed twice with phosphate-buffered saline (PBS) (137 mM NaCl, 2.7
167 mM KCl, 4.3 mM $Na_2HPO_4$, pH 7.4) to remove planktonic and loosely adhering bacteria. Adherent
168 cells were fixed with 96% ethanol for 10 min and stained with 0.1% crystal violet for 15 min and,
169 after several washings, the wells were air dried. For a quantitative estimation of biofilm density,
170 bound crystal violet was solubilised with 10% glacial acetic acid and the absorbance of the
171 solubilised dye was read at 595 nm in a microplate reader (model 680) (Bio-Rad Laboratories, Inc.,
172 Hercules, CA). To test the role of iron on biofilm formation, *S. lugdunensis* N920143, N940025,
173 N940113 and N940135 were cultured in $RPMI_{0.3\%glucose}$ / 2 mM glutamine supplemented with 1000
174 µM $FeCl_3$ in 96-well flat-bottom sterile polystyrene microplates, and treated as described above.
175 Iron-depleted growth medium was obtained by treatment of RPMI with divalent metal chelator
176 Chelex 100 following the manufacturer's instructions (Bio-Rad).

177

178 **Enzymatic and chemical treatment of biofilms**. Chemical and enzymatic treatments of biofilms
179 were carried out as described previously (41, 42). Briefly, the biofilms grown in microtiter plates
180 were rinsed with 200 µl 0.9% NaCl and then treated for 2 h at 37 °C with 100 µl of 10 mM sodium
181 metaperiodate (Sigma, St. Louis MO) in 50 mM sodium acetate buffer, pH 4.5. Alternatively,
182 biofilms were incubated with 100 µl proteinase K (Sigma) at 1 mg/ml in 20 mM Tris buffer

containing 100 mM NaCl, pH 7.5 or 100 µl DNase I (Sigma) at 2 mg/ml in PBS. Enzymes or sodium metaperiodate were replaced with the appropriate amounts of buffer in the controls. To rule out the possibility that DNase I could be contaminated with proteases, the enzyme was incubated with albumin for 2 h and the mixture subjected to SDS-PAGE: in these conditions no difference in the electrophoretic mobility of DNase I-treated and untreated samples of albumin was observed.

**Expression and purification of recombinant proteins.** Recombinant His-tagged proteins were expressed and purified by $Ni^{2+}$ chelate chromatography as described previously (29) (Table1). *E. coli* strain TOPP3 (Stratagene, La Jolla, CA), used for the expression of recombinant His-tagged proteins, was grown in Luria-Bertani (LB) broth (Difco) supplemented with ampicillin (100µg/ml) (Sigma) at 37°C for 18 h with shaking (150 rpm). Overnight cultures were diluted 1:100 in LB medium and grown at 37°C, with shaking, until the $OD_{600nm}$ reached 0.5–0.6. Expression was induced by adding isopropyl-thio-β-D-galactoside (Inalco, Milan, Italy) to a final concentration of 1 mM. Bacteria were harvested by centrifugation at 1700x*g* for 20 min, and lysed by passage through a French press. The cell debris was removed by centrifugation (20000x*g*), and the filtered supernatant (0.45 µm membrane) was applied to a 1 ml $Ni^{2+}$-Sepharose His Trap HP column (GE Healthcare, Buckinghamshire, UK). Fusion proteins were eluted with twenty column volumes of 0.00-500 mM imidazole (Sigma) gradient in 20 mM sodium phosphate, 0.5 M NaCl buffer, pH 7.4. Fractions corresponding to the recombinant protein were pooled and extensively dialyzed against PBS. Protein concentrations were determined with a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA).

**Primary attachment assay.** The attachment assay was performed as reported by Geoghegan *et al.* (9). Briefly, bacteria were grown overnight in RPMI/2 mM glutamine, diluted in $RPMI_{0.3\%glucose}$/2 mM glutamine, and approximately 300 CFU in 100 µl was spread on the base of empty petri dishes. Dishes were incubated upright at 37°C for 30 min, washed three times with 5 ml of sterile PBS, and

covered with TSB agar. Bacterial plate counts were run in parallel, and the percent attachment was calculated. Each experiment was repeated three times. Statistical significance was determined with Student's *t* test, using Graph Pad software.

**Aggregation assays**. The aggregation assay was based on the method described by Geoghegan *et al.* (9). Bacteria were grown overnight in RPMI/2mM glutamine and diluted in $RPMI_{0.3\%glucose}$ /2 mM glutamine, to an optical density at 600 nm ($OD_{600}$) of 1.0. Tubes were incubated statically at 37°C up to 24 h. One ml of broth was removed from the top of the tube at the indicated times, and the $OD_{600}$ was measured. The remaining culture was vortexed to resuspend the cells, and the $OD_{600}$ was measured again. The percent aggregation was calculated using the following formula: 100 x [($OD_{600}$ of vortexed sample - $OD_{600}$ before vortexing) / ($OD_{600}$ of vortexed sample)]. Statistical significance was determined with Student's *t* test, using Graph Pad software.

**Gel filtration chromatography.** The size exclusion chromatography experiment was performed using a Superose 12 HR 10/30 column (GE Healthcare) connected to an AKTA design™ chromatography system (GE Healthcare). 100 µl of 100 µM IsdC was loaded onto a gel filtration column equilibrated in PBS with or without 100 µM $FeCl_3$ and eluted with one column volume (24 ml) at a flow rate of 0.5 ml/min. Recorded data were analyzed using UNICORN 5.10 control software (GE Healthcare). The Mr of IsdC was determined from the calibration curve (plot of Kav versus log Mr) once its Kav value was calculated from the measured elution volume.

**Preparation of bacterial lysates.** The lysates from *S. lugdunensis* and *L. lactis* strains were prepared as previously described (29) with minor modifications. Briefly, bacterial cultures were harvested by centrifugation, washed in PBS and adjusted to an optical density at 600 nm of 10. A 1 ml portion of the bacterial suspension was pelleted and resuspended in 250 µl of digestion buffer (50 mM Tris-HCl, 20 mM $MgCl_2$, 30% (w/v) raffinose pH 7.5), containing complete mini protease

inhibitors (Roche). Cell wall proteins were solubilised by digestion with lysostaphin (500 µg/ml) (*S. lugdunensis*) or a combination of mutanolysin (1000 U/ml) and lysozyme (900 µg/ml) (*L. lactis)* at 37° C for 30 or 15 min, respectively. Protoplasts were harvested by centrifugation (5000x*g*, 15 min), and the supernatants were subjected to SDS-PAGE/Western blotting.

**Western immunoblotting.** Whole cell lysates of transformants of *L. lactis* were subjected to 12.5% polyacrylamide gel electrophoresis and then electroblotted onto a nitrocellulose membrane (GE HealthCare). The membrane was treated with a solution containing 5% (w/v) dried milk in PBS, washed and incubated with anti-IsdC rabbit IgG for 1 h at 22°C. Following additional washings with 0.5% (v/v) Tween 20 in PBS, the membrane was incubated for 1 h with HRP-conjugated goat anti-rabbit IgG. The membrane was treated with ECL detection reagents 1 and 2 as recommended by the manufacturer (GE HealthCare) and exposed to an X-ray film for 30-60 sec.

**Attachment of *L. lactis* transformants to Isd proteins.** Isd proteins (1 µg/well) were coated in microtiter wells in bicarbonate buffer overnight. *L. lactis* (pNZ8048*isdC*) and *L. lactis* (pNZ8048) ($5x10^8$ cells/well) were added to the wells and incubated for 1 h at 22°C. After extensive washing with PBS adhering cells were fixed with 25% formaldehyde (Sigma), stained with 2.5% crystal violet and the $A_{595}$ measured.

**Statistical methods.** Continuous data were expressed as means and SD. Two-group comparisons were performed by student's *t* test. One-way ANOVA followed by Bonferroni's post-hoc tests was exploited for comparison of three or more groups. Analyses were performed using Graph Pad Prism 4.0 (Graph Pad Software Inc., San Diego, CA, USA). Two-tailed P values $< 0.05$ were considered statistically significant.

## RESULTS

### *S. lugdunensis* clinical isolates form proteinaceous biofilm in iron-restricted conditions

It was reported previously that *S. lugdunensis* strains growing in rich broth formed biofilm that was predominantly proteinaceous in that preformed biofilm could be disrupted by proteases but not by periodate (24). In order to investigate the nature of *S. lugdunensis* biofilm in more detail we tested a panel of clinical isolates along with *S. aureus* and *S. epidermidis* controls where the composition of the biofilm matrix is known to be composed predominantly of protein (V329, 5179R) or polysaccharide (SA113, RP62A) growing in iron replete TSB or in iron-deficient RPMI. The controls formed protein or polysaccharide dependent biofilms as previously reported.

All *S. lugdunensis* strains tested formed biofilm when growing in both media. Glucose concentrations above 0.25% supported higher levels of biofilm formation compared to lower concentrations ($p < 0.001$, data not shown). We confirmed the previous report that biofilms formed by *S. lugdunensis* growing in TSB-glucose were susceptible to detachment by proteinase K but not sodium metaperiodate (Fig. 1). We also found that biofilms formed by *S. lugdunensis* growing in RPMI were susceptible to protease and not to periodate. Furthermore, DNase caused significant detachment of *S. lugdunensis* biofilm formed under both growth conditions. DNase also detached the control protein-dependent biofilm but not that involving a polysaccharide matrix (Fig. 1).

### Involvement of the IsdC protein in *S. lugdunensis* biofilm.

To determine if *S. lugdunensis* biofilm formed during growth in RPMI was influenced by the availability of iron, three strains were tested in RPMI supplemented with 1 mM $FeCl_3$. Interestingly, the addition of iron reduced the biofilm density suggesting the involvement of proteins whose expression was regulated by iron (Fig. 2). *S. lugdunensis* is the only species of coagulase-negative staphylococcus that harbours an iron regulated surface determinant (Isd) locus.

284 It is only expressed under iron-limited conditions and is responsible for the acquisition of iron from
285 haemoglobin and heme *in vivo* (28, 29). To determine if the *isd* locus is involved in biofim
286 formation, *S. lugdunensis* N920143 wild type and a mutant where the entire *isd* locus is deleted
287 (Δ*isd*) were compared. A schematic representation of the *isd* locus and mutations are shown in Fig.
288 3. The level of biofilm formed by the *isd* mutant was the same as that formed by the wild-type strain
289 in the presence of $FeCl_3$. This indicates that one or more proteins expressed by the *isd* locus are
290 involved in biofilm formation in iron limited conditions (Fig. 4). The *S. lugdunensis isd* locus
291 expresses four cell envelope-associated proteins IsdC, IsdB, IsdJ and IsdK (29). Mutants lacking
292 each of the Isd proteins were tested for biofilm formation in RPMI. Notably, only the IsdC mutant
293 was defective and showed the same low level of biofilm formation as the wild-type supplemented
294 with 1 mM $FeCl_3$ or the Δ*isd* mutant. To exclude the possibility that the reduction in biofilm
295 formation was due to a difference in growth in RPMI the growth curve for the wild type and each of
296 the mutants were compared and found to be superimposable. A revertant strain where the *isdC* gene
297 had been restored to wild-type expressed biofilm normally indicating that the *isdC* mutation and not
298 a secondary mutation is responsible.

299 IsdC is known to be surface-exposed and anchored to cell wall peptidoglycan by sortase B
300 (29). Consistent with this observation, a sortase B mutant (Δ*srtB*) gene formed biofilm at a level
301 comparable to that of the *isdC* mutant. In contrast, an *atlI* mutant expressed biofilm at a level
302 comparable to the wild type strain. Furthermore, the Δ*isd* mutant (lacking the entire *isd* locus),
303 carrying plasmid pRMC2 bearing an *isdC* gene engineered to express IsdC containing a sortase A
304 recognition sequence (pRMC2*isdC*-LPXTG), expressed a similar level of biofilm to that of *S.*
305 *lugdunensis* N920143 wild type. This shows that IsdC alone is necessary and sufficient to promote
306 biofilm formation in *S. lugdunensis*. (Fig. 4).

307 To examine further the effect of iron on biofilm development, *S. lugdunensis* N920143 was
308 grown in Chelex 100-pretreated RPMI supplemented with various amounts of $FeCl_3$ and tested for

biofilm formation. The levels of IsdC detected by Western immunoblotting were the same under depleted and low-iron conditions (from 0 to 50 μM) and virtually missing in 250-2000 μM $FeCl_3$ (Fig. 5). A high level of biofilm formation was observed with bacteria growing in the low-iron environment (1-50 μM), whereas significantly reduced biofilm was detected either in $FeCl_3$-depleted conditions or in the presence of high iron concentrations (≥ 250 μM). In conclusion, for the IsdC-dependent biofilm formation critical concentrations of $FeCl_3$ are required.

We also tested the effect of pH on IsdC expression by planktonic cells of *S. lugdunensis* N920143 and on biofilm formation. The same level of IsdC was detected by Western immunoblotting of lysates of staphylococcal cells grown at pH 5-8 (Fig. 6, panel A), while the biofilm level was significantly enhanced when the pH of the medium was 6.0 or 6.5 compared to 5.5, 7.0, 7.5 or 8.0 (Fig. 6, panel B). A similar trend was found when the effect of pH on biofilm formation by the *isdC* mutant was examined. These conditions also promote protein-dependent biofilm formation in *S. aureus* (12). Together these data indicate that different pHs do not affect IsdC expression but somehow influence the levels of biofilm formation.

**Role of IsdC in attachment and accumulation during biofilm formation.**

Biofilm formation in static growth conditions occurs in several stages beginning with attachment of individual cells to a surface followed by growth and the accumulation of cells in a multi-layered complex held together by proteins or polysaccharide. In our experiments bacteria must attach to the surface of a polystyrene dish. To test attachment, bacterial cells were incubated in dishes, washed and immobilized in molten agar before incubation overnight to allow colonies to develop. For the wild type strain N920143 73% of the added cells attached whereas for the *isdC* mutant only 36.7% attached. The reverted control strain *isdC*r adhered at a similar level as the wild type. To investigate if IsdC contributes to cell-cell aggregation and thus possibly to biofilm accumulation, suspensions of RPMI-grown bacteria were allowed to settle for up to 24h. The

density of cells at the top of the suspension was measured periodically. The density of the *isdC* mutant was significantly lower than the wild-type or the restored mutant after 6 and 8 h but the densities were the same after 24h. This indicates that IsdC is involved both in the initial attachment of bacterial cells and also in the accumulation phase.

**IsdC forms homodimers.**

To test the hypothesis that the ability of IsdC to promote the accumulation phase of biofilm formation by iron-starved bacteria could be due to a homophilic interaction between IsdC proteins attached to adjacent cells, the ability of the protein to dimerize in solution was examined. A preparation of purified recombinant IsdC was subjected to gel filtration chromatography on a Superose 12 HR column equilibrated with or without $FeCl_3$ (Fig. 7). Two major peaks were eluted from the column equilibrated with 100 μM $FeCl_3$, an early peak corresponding to IsdC dimers (Mr = 59 kDa) and a retarded peak with a molecular mass of 29.5 kDa which corresponds to the IsdC monomer. Conversely, a single peak of 29.5 kDa was obtained from the column equilibrated and eluted with PBS only. Thus an equilibrium exists between the monomeric and dimeric forms of IsdC in solution.

To determine if IsdC protein that is attached to the surface of a bacterial cell could interact with other IsdC molecules, the *isdC* gene was cloned into the nisin-inducible *Lactococcus lactis* expression vector pNZ8048. The sortase B cleavage motif NPQTS was changed to LPQTG in order to provide a substrate for sortase A in *L. lactis*. After induction, expression of IsdC was detected in the cell wall fraction derived from protoplasts of *L. lactis* by Western immunoblotting (Fig 8, panel A) indicating that IsdC was sorted to the cell wall. When the ability of *L. lactis* expressing IsdC to adhere to immobilized Isd proteins was tested, *L. lactis* (pNZ8048*isdC*) adhered to immobilized IsdC at a four-fold higher level than the control strain bearing the empty vector, but not significantly to IsdB, IsdJ or IsdK. A slight promotion of adherence to IsdB was noted but this was

359 not statistically significant. These data are consistent with the conclusion that IsdC homophilic
360 interactions contribute to the accumulation phase of *S. lugdunensis* biofilm formation under iron-
361 restricted conditions (Fig 8, panel B). As reported for biofilm formation by *S. lugdunensis* N920143
362 (Fig. 6, panel B), the interaction of *L. lactis* (pNZ8048*isdC*) with immobilized IsdC was enhanced
363 at pH values of 6.0 and 6.5 and decreased at pH 5.0, 5.5, 7.0, 7.5 and 8.0 (Fig. 8, panel C).
364 Consistent with low iron-induced biofilm formation (Fig. 5), we also found that attachment of *L.*
365 *lactis* pNZ8048*isdC* to surface-coated IsdC requires the presence of $FeCl_3$ (≥ 1μM). Significantly
366 reduced levels of IsdC homophilic interactions were observed when bacterial attachment was
367 performed in iron-depleted conditions (Fig. 8, panel D).

368

369 **Inhibition by recombinant Isd proteins and antibodies.**

370 The soluble recombinant Isd proteins or specific antisera raised against each of the Isd
371 proteins were added to the $RPMI_{0.3\%glucose}$/2 mM glutamine medium at the same time as the
372 inoculum and incubated for 24 h before the density of biofilm formed was measured. Only IsdC
373 protein or anti-IsdC serum caused a significant (~ 35%) reduction in biofilm formation (Fig. 9).
374 Although complete inhibition was not achieved we can be confident of the specificity of the effect
375 which is consistent with an important role in biofilm formation.

376

377 **DISCUSSION**

378 The identification of surface proteins that are able to induce biofilm development in the
379 absence of polysaccharides is one of the most unexpected results obtained recently in the field of
380 staphylococcal biofilm studies. The existence of alternative mechanisms promoting biofilm
381 development suggests that different staphylococcal strains might involve a specific component to

form a particular type of biofilm matrix. *S. aureus* can opt for at least five different surface proteins, Bap (8), protein A (11), SasG (9), SasC (10) and FnBPs (12, 13) to achieve protein-mediated biofilm. Moreover, functional amyloids composed of phenol soluble modulins (PSMs) stabilize *S. aureus* biofilms (43). A similar proteinaceous biofilm has been demonstrated with *S. epidermidis* based on the expression of proteins such as Aap (14) or SesC (16, 44). Furthermore, the same staphylococcal strain can switch between a proteinaceous or polysaccharide-based biofilm depending on the growth conditions (45).

Investigating biofilm production by a collection of strains of *S. lugdunensis*, we confirmed the proteinaceous nature of biofilms in iron-rich (TSB) medium (24) and demonstrated for the first time that biofilm formed in iron-restricted conditions (RPMI) was also protein-dependent. We found that proteins expressed by the *isd* locus are involved in biofilm development and we identified the IsdC protein as the main factor responsible of cell-to-cell interactions and biofilm formation in iron-restricted conditions. IsdC contributed both to the attachment of cells to a polystyrene surface and to the accumulation phase. Thus, IsdC can be added to the growing list of proteinaceous factors involved in staphylococcal biofilm formation.

Surprisingly, for maximal biofilm formation, both IsdC expression and iron-restricted conditions were required, whereas *S. lugdunensis* grown in completely iron-depleted conditions (chelex-treated RPMI) or in RPMI supplemented with excess amounts of $FeCl_3$ ($\geq$ 250 µM) developed a significantly reduced biofilm. Thus iron plays a dual role in IsdC-dependent biofilm formation. Iron restriction is required to induce IsdC expression and a low level of iron is required to support the formation of biofilm while high levels (>250 µM) are inhibitory. Given that *S. lugdunensis* biofilm is dependent on IsdC and that IsdC has been demonstrated to bind heme (29), it is also possible that IsdC-associated heme is involved in biofilm formation, although it cannot be excluded that the requirement for iron could be unrelated to heme binding. The fact that IsdC is only expressed under iron-restricted conditions and the ability of *S. lugdunensis* strains also to form

proteinaceous biofilm under iron-replete conditions implies that biofilm formation in TSB is promoted by proteinaceous factors distinct from those involved in RPMI growth medium. It would be interesting to determine if the protein(s) involved are anchored by sortase A

The observation that IsdC confers primary attachment to polystyrene and production of cell aggregates in the accumulation phase of biofilm, parallels the behaviour observed for Bap (8) and SasC (10) of *S. aureus*. The aggregation activity observed for IsdC is also promoted by staphylococcal proteins such as Aap (14), SasG (9), and FnBPs (13).

The structural characterization of Aap and SasG in biofilm development has been recently defined (46, 47). Aap and SasG comprise an N-terminal A region and repeated B domains towards the c-terminus. Proteolytic removal of the A domain of Aap or limited partial cleavage within the B domains of SasG is required for biofilm formation. This presumably reduces steric hindrance by the N-terminal region and allows the B repeats from opposing cells to engage. The length of the B region was shown to be critical for biofilm development because only SasG (or Aap) constructs with five or more B-repeats supported biofilm formation, while constructs with four or fewer B-repeats did not. Structural studies revealed that Aap B-repeats of the appropriate length adopt an elongated, rope-like structure coordinated by zinc ions and wrap around one another in an antiparallel fashion to form bundles of fibres that establish homophilic interactions and have the potential to interconnect neighbour cells (46, 47).

Homophilic protein-protein interactions may be also operational in the biofilm promoted by IsdC. Indeed IsdC forms dimers in solution in the presence of $FeCl_3$ and immobilized recombinant IsdC specifically interacts with IsdC expressed on the surface of *L. lactis*. Moreover, as reported for biofilm formation by *S. lugdunensis,* this interaction is dependent on the pH, suggesting that a pH-induced changes in the conformation of IsdC contribute to both biofilm formation and homophilic interactions.

Due to the absence of tandem repeats in IsdC, alternative structures/mechanisms should be envisaged in IsdC-mediated biofilm formation. For example, the N-terminal subdomain N2N3 of the A region of the fibronectin-binding protein FnBPA and the N-terminal domain of SasC, but not their repetitive regions, mediate biofilm formation in *S. aureus* (13, 10). Thus, there are two different scenarios, a first one involving Aap and SasG which requires a Zn-dependent interaction between repeats and a second one where a direct Zn-dependent binding of the A domain of FnBPA on one cell to the A domain on another one is needed. The possibility that the A domain of FnBPA bound to a different ligand on the adjacent cell surface cannot be excluded. However, this was not backed up by *in vitro* studies with recombinant proteins as in the case of IsdC.

Although *in vitro* evidence indicates the involvement of homophilic interactions, it cannot be excluded that IsdC *in vivo* might establish heterophilic interactions with proteinaceous or non-proteinaceous components expressed on the surface or localized in the extracellular matrix of staphylococcal cells. Moreover, due to the reduced but not abolished ability of the Δ*isd* mutant of *S. lugdunensis* strains to develop biofilms and the partial inhibition by soluble IsdC protein or anti-IsdC serum, it is plausible that additional, yet undefined factors may contribute. The residual biofilm in the Δ*isd* mutant and Δ*isdC* mutant was the same and appears to be proteinaceous (data not shown). It would be interesting to determine whether sortase A anchored proteins (other than IsdB or IsdJ) could play a role in this process. This would require construction of a double Δ*isd srtA* mutant.

Several questions on the role of IsdC in biofilm formation remain unanswered. For example, it should be of interest to investigate the molecular details of IsdC-mediated biofilm formation and whether the putative heme-binding NEAT domain is involved. This would require the production of recombinant mutant proteins and assessment of their ability to form dimers. Mutant strains with an altered potential to bind heme could also be constructed and concurrent evaluation of their capability to form biofilm performed. In connection with this, it could be also interesting to

investigate the reasons why only IsdC, but not IsdB, IsdJ or IsdK, is involved in biofilm formation, despite similarity between their NEAT domains (29).

*S. lugdunensis* is an important cause of infections associated with indwelling medical devices. In general, device-related staphylococcal infections, including those associated with *S. lugdunensis* infections, are difficult to treat because staphylococci form biofilm and are protected from antimicrobial agents and the host's immune system. As a consequence, device removal can be required to resolve the infection. Thus, it will be of interest to determine if IsdC-promoted biofilm contributes to virulence in animal models of foreign body infection. This is particularly important if one considers that due to the very low concentrations of free iron in the body fluids, IsdC should be expressed on the surface of *S. lugdunensis* cells and could play a role in biofilm formation under these conditions. This study provides initial clues about a protein that could be targeted to prevent developing of biofilm in conditions resembling those present in the body fluids.

## ACKNOWLEDGMENTS

We acknowledge funding from Fondazione CARIPLO (Grant Vaccines 2009-3546) to PS. Research in Dublin was supported by the IRCSET Embark Scholarship and by Science Foundation Ireland (Programme Investigator grant 08/IN.1/B1854).

## FIGURE LEGENDS

FIG. 1 Characterization of *S. lugdunensis* biofilm matrix composition. Biofilms were grown in sterile 96-well polystyrene plates in TSB (panels A, B and C) or RPMI (Panels D, E and F) supplemented with 0.3% glucose, for 24 h at 37°C and the adherent cells were treated with proteinase K (panels A and D), sodium metaperiodate (panels B and E) or DNAase I (panels C and F). After washing, cells were stained with 0.1% crystal violet and the absorbance measured at 595

480 nm. Bars are the averages of measurements taken from four wells. Error bars represent the standard
481 deviations. Controls for protein-dependent biofilm (*S. aureus* V329 and *S. epidermidis* 5179R) and
482 PIA-dependent biofilm (*S. aureus* SA113 and *S. epidermidis* RP62a) were included in experiments
483 to test sensitivity to proteinase K, DNAase and sodium metaperiodate. This experiment was
484 performed three times with similar results

485

486 FIG. 2 Biofilm formation by *S. lugdunensis* clinical isolates grown in RPMI with or without 100μM
487 $FeCl_3$. Bacteria were grown for 24 hrs in RPMI and diluted 1:200 in RPMI-glucose (0.3%, w/v) in
488 the presence or absence of 1 mM $FeCl_3$. Diluted bacteria (200 μl) were added to sterile 96-well
489 polystyrene plates and statically incubated at 37°C for 24 h. Wells were washed three times with
490 phosphate-buffered saline and fixed with 96% ethanol. Adherent cells were stained with 0.1% (w/v)
491 crystal violet and the absorbance measured at 595 nm. Bars are the averages of measurements taken
492 from four wells. Error bars represent the standard deviations. This experiment was performed three
493 times with similar results. P values were determined using the two-tailed, two-sample unequal
494 variance Student's *t* test in Graph Pad.

495

496 FIG. 3 Schematic diagram of the *isd* locus and mutations. The open boxes denote individual genes
497 and the arrows the direction of their transcription. Encoded NEAT motifs are shown as small black
498 boxes. Angled dashes indicate the beginning and the end of the *isd* locus. Mutations are indicated
499 by horizontal bi-headed arrows.

500

501 FIG. 4 Effect of *isd* locus deletion on biofilm formation by *S. lugdunensis* in iron-limiting
502 conditions. *S. lugdunensis* N920143 WT and its deletion mutants covering the entire *isd* locus or
503 single *isd* genes were grown overnight in RPMI. Experimental conditions for biofilm formation by

504 the strains and biofilm detection are identical to those reported in Fig 2. The effect of 1 mM
505 $FeCl_3$ on biofilm formation is also reported. This experiment was performed four times with
506 similar results. P values were determined using the two-tailed, two-sample unequal variance
507 Student's *t* test in Graph Pad.

508

509 FIG.5 Iron-dependent biofilm formation by *S. lugdunensis* N920143. Bacteria grown overnight in
510 Chelex 100-treated RPMI were diluted in the same medium supplemented with increasing
511 amounts of $FeCl_3$ (final concentrations from 0 to 2000 μM), added to sterile 96-well polystyrene
512 plates and statically incubated at 37°C for 24 h. Experimental conditions for biofilm detection are
513 identical to those reported in Fig 2. The upper part of the figure shows the detection by Western
514 blotting of IsdC protein in the lysates obtained from cultures grown overnight plancktonically in
515 different $FeCl_3$ concentrations. This experiment was performed three times with similar results. P
516 values were determined using the two-tailed, two-sample unequal variance Student's *t* test in
517 Graph Pad.

518

519 FIG. 6 Effect of pH on the IsdC expression and biofilm formation by *S. lugdunensis* N920143.
520 Panel A, *S. lugdunensis* N920143 was grown overnight in RPMI adjusted to the indicated pH and
521 the corresponding lysates subjected to Western immunoblotting. IsdC protein transblotted onto
522 nitrocellulose filter was detected with rabbit anti-IsdC IgG, followed by HRP-conjugated
523 secondary goat anti-rabbit IgG. Panel B, *S. lugdunensis* N920143 and its *isdC* deletion mutant
524 were grown overnight in RPMI, diluted 1:200 in the same medium adjusted to different pHs,
525 added to sterile 96-well polystyrene plates and statically incubated at 37°C for 24 h. Experimental
526 conditions for biofilm detection are identical to those reported in Fig 2. This experiment was

performed three times with similar results. P values were determined using the two-tailed, two-sample unequal variance Student's *t* test in Graph Pad.

FIG. 7 Analysis of IsdC interactions by size exclusion chromatography. 100 µl of IsdC at a concentration of 100 µM was loaded onto a gel filtration column of Superose 12 HR and eluted with one column volume (Vt= 24 ml) of PBS with or without 100 µM $FeCl_3$ at a flow rate of 0.5 ml/min. Molecular weight determination was carried out by comparing elution volumes of the two peaks marked as IsdC1 and IsdC2, with the values obtained for several known calibration standards. The Mr of IsdC1 or IsdC2 was determined from the calibration curve (plot of Kav versus log Mr) once their Kav value were calculated from the measured elution volumes [Kav= (elution volume - void volume)/(column volume - void volume)] (inset).

FIG. 8. Homophilic and heterophilic interactions of IsdC expressed by *L. lactis* with surface-coated Isd proteins. Panel A, validation of IsdC expression by *L. lactis*. Expression of IsdC by *L. lactis* (pNZ8084*isdC*) and *L. lactis* (pNZ8084) was demonstrated by Western immunoblotting. IsdC protein in the solubilised cell wall lysates was detected with rabbit anti-IsdC IgG, followed by HRP-conjugated secondary goat anti-rabbit IgG. Panel B, adhesion of *L. lactis* expressing IsdC to Isd proteins. Microtiter wells were coated with recombinant IsdB, IsdC, IsdK and IsdJ in a bicarbonate buffer overnight (1 µg/well). *L. lactis* (pNZ8084*isdC*) and *L. lactis* (pNZ8084) ($5x10^8$ cells/well) were added to the wells and the mixtures incubated for 1 h. After washing with PBS, adherent cells were fixed, stained with crystal violet and measured at 595 nm. Panel C, effect of pH on the adherence of *L. lactis* (pNZ8084*isdC*) and *L. lactis* (pNZ8084) to immobilized IsdC protein. Bacterial cells ($5x10^8$ cells/well) suspended in growth medium were adjusted to the indicated pHs and added to the wells. Panel D, effect of iron concentrations on the adherence of *L.*

551 *lactis*. Bacterial cells ($5x10^8$ cells/well) suspended in growth medium were supplemented with
552 increasing amounts of $FeCl_3$ and added to the wells. Incubation of the mixtures as described in
553 panels C and D was carried out for 1 h and detection of attached bacteria was performed as
554 reported in panel B. Results shown in panels B, C and D are the mean values of triplicate samples.
555 Error bars show the standard deviation. P values were determined using the two-tailed, two-sample
556 unequal variance Student's t test in Graph Pad.

557

558 FIG. 9. Effect of soluble Isd proteins or anti-Isd IgG on the biofilm formation by *S. lugdunensis*
559 920143. Overnight cultures of *S. lugdunensis* 920143 grown in RPMI were diluted into fresh
560 RPMI-glucose and 200 µl of mixtures were allowed to form biofilm for 24 h in the presence of 5
561 µM Isd proteins (panel A) or 2 µg/ml of pre-immune or immune IgG raised against each Isd protein
562 (panel B). Adherent cells were stained with crystal violet and the absorbance measured at 595 nm.
563 Bars are the averages of measurements taken from four wells. Biofilm values are expressed as
564 percentage of biofilm level obtained from cultures grown in the absence of any potential inhibitor.
565 Error bars represent the standard deviations. This experiment was performed three times with
566 similar results. P values were determined using the two-tailed, two-sample unequal variance
567 Student's t test in Graph Pad.

568

569 **REFERENCES**

570 1. **Götz F.** 2002. Staphylococcus and biofilms. Mol. Microbiol. **43**:1367-1378.
571 2. **Stewart PS, Costerton JW.** 2001. Antibiotic resistance of bacteria in biofilms. Lancet
572 **358**:135-138.

3. **Vuong C, Voyich JM, Fischer ER, Braughton KR, Whitney AR, DeLeo FR, Otto M.** 2004. Polysaccharide intercellular adhesin (PIA) protects *Staphylococcus epidermidis* against major components of the human innate immune system. Cell. Microbiol. **6**: 269-275.
4. **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. doi:10.1371/journal.pone.0042244.
5. **Houston P, Rowe SE, Pozzi C, Waters EM, O'Gara JP.** 2011. Essential role for the major autolysin in the fibronectin-binding protein-mediated *Staphylococcus aureus* biofilm phenotype. Infect. Immun. **79**: 1153-1165.
6. **Vaudaux PE, François P, Proctor RA, McDevitt D, Foster TJ, Albrecht RM, Lew DP, Wabers H, Cooper SL.** 1995. Use of adhesion-defective mutants of *Staphylococcus aureus* to define the role of specific plasma proteins in promoting bacterial adhesion to canine arteriovenous shunts. Infect. Immun. **63**: 585-590.
7. **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.
8. **Cucarella C, Solano C, Valle J, Amorena B, Lasa I, Penadés JR.** 2001. Bap, a *Staphylococcus aureus* surface protein involved in biofilm formation. J. Bacteriol. **183**: 2888-2896.
9. **Geoghegan JA, Corrigan RM, Gruszka DT, Speziale P, O'Gara JP, Potts JR, Foster TJ.** 2010. Role of surface protein SasG in biofilm formation by *Staphylococcus aureus*. J. Bacteriol. **192**: 5663-5673.
10. **Schroeder K, Jularic M, Horsburgh SM, Hirschhausen N, Neumann C, Bertling A, Schulte A, Foster S, Kehrel BE, Peters G, Heilmann C.** 2009. Molecular characterization of a novel *Staphylococcus aureus* surface protein (SasC) involved in cell aggregation and biofilm accumulation. PLoS One **4**:e7567. doi:10.1371/journal.pone.0007567.

11. **Merino N, Toledo-Arana A, Vergara-Irigaray M, Valle J, Solano C, Calvo E, Lopez JA, Foster TJ, Penadés JR, Lasa I.** 2009. Protein A-mediated multicellular behavior in *Staphylococcus aureus*. J. Bacteriol. **191**: 832-843.
12. **O'Neill E, Pozzi C, Houston P, Humphreys H, Robinson DA, Loughman A, Foster TJ, O'Gara JP.** 2008. A novel *Staphylococcus aureus* biofilm phenotype mediated by the fibronectin-binding proteins, FnBPA and FnBPB. J. Bacteriol. **190**: 3835-3850.
13. **Geoghegan JA, Monk IR, O'Gara JP, Foster TJ.** 2013. Subdomains N2N3 of fibronectin binding protein A mediate *Staphylococcus aureus* biofilm formation and adherence to fibrinogen using distinct mechanisms. J. Bacteriol. **195**: 2675-2683.
14. **Rohde H, Burdelski C, Bartscht K, Hussain M, Buck F, Horstkotte MA, Knobloch JK, Heilmann C, Herrmann M, Mack D.** 2005. Induction of *Staphylococcus epidermidis* biofilm formation via proteolytic processing of the accumulation-associated protein by staphylococcal and host proteases. Mol. Microbiol. **55**: 1883-1895.
15. **Banner MA, Cunniffe JG, Macintosh RL, Foster TJ, Rohde H, Mack D, Hoyes E, Derrick J, Upton M, Handley PS.** 2007. Localized tufts of fibrils on *Staphylococcus epidermidis* NCTC 11047 are comprised of the accumulation-associated protein. J. Bacteriol. **189**: 2793-2804.
16. **Shahrooei M, Hira V, Stijlemans B, Merckx R, Hermans PW, Van Eldere J.** 2009. Inhibition of *Staphylococcus epidermidis* biofilm formation by rabbit polyclonal antibodies against the SesC protein. Infect. Immun. **77**: 3670-3678.
17. **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.
18. **Vandenesch F, Etienne J, Reverdy ME, Eykyn SJ.** 1993. Endocarditis due to *Staphylococcus lugdunensis*: report of 11 cases and review. Clin. Infect. Dis. **17**:871-876.

19. **Patel R, Piper KE, Rouse MS, Uhl JR, Cockerill FR 3rd, Steckelberg JM.** 2000. Frequency of isolation of *Staphylococcus lugdunensis* among staphylococcal isolates causing endocarditis: a 20-year experience. J. Clin. Microbiol. **38**: 4262-4263.

20. **Anguera I, Del Río A, Miró JM, Matínez-Lacasa X, Marco F, Gumá JR, Quaglio G, Claramonte X, Moreno A, Mestres CA, Mauri E, Azqueta M, Benito N, García-de la María C, Almela M, Jiménez-Expósito 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.

21. **Ebright JR, Penugonda N, Brown W.** 2004. Clinical experience with *Staphylococcus lugdunensis* bacteremia: a retrospective analysis. Diagn. Microbiol. Infect. Dis. **48**: 17-21.

22. **Sampathkumar P, Osmon DR, Cockerill FR.** 2000. Prosthetic joint infection due to *Staphylococcus lugdunensis*. Mayo Clin. Proc. **75**: 511-512.

23. **Sandoe JA, Longshaw CM.** 2001. Ventriculoperitoneal shunt infection caused by *Staphylococcus lugdunensis*. Clin. Microbiol. Infect. **7**: 385-387.

24. **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.

25. **Geoghegan JA, Ganesh VK, Smeds E, Liang X, Höök M, Foster TJ.** 2010. Molecular characterization of the interaction of staphylococcal microbial surface components recognizing adhesive matrix molecules (MSCRAMM) ClfA and Fbl with fibrinogen. J. Biol. Chem. **285**: 6208-6216.

26. **Szabados F, Marlinghaus L, Korte M, Neumann S, Kaase M, Gatermann SG.** 2011. Fbl is not involved in the invasion of eukaryotic epithelial and endothelial cells by *Staphylococcus lugdunens*is. FEMS Microbiol. Lett. **324**: 48-55.

649 27. **Nilsson M, Bjerketorp J, Wiebensjö A, Ljungh A, Frykberg L, Guss B.** 2004. A von
650 Willebrand factor-binding protein from *Staphylococcus lugdunensis*. FEMS Microbiol. Lett.
651 **234**: 155-161.
652 28. **Heilbronner S, Holden MT, van Tonder A, Geoghegan JA, Foster TJ, Parkhill J,**
653 **Bentley SD.** 2011. Genome sequence of *Staphylococcus lugdunensis* N920143 allows
654 identification of putative colonization and virulence factors. FEMS Microbiol. Lett. **322**: 60-
655 67.
656 29. **Zapotoczna M, Heilbronner S, Speziale P, Foster TJ.** 2012. Iron-regulated surface
657 determinant (Isd) proteins of *Staphylococcus lugdunensis*. J. Bacteriol. **194**: 6453-6467.
658 30. **Haley KP, Janson EM, Heilbronner S, Foster TJ, Skaar EP.** 2011. *Staphylococcus*
659 *lugdunensis* IsdG liberates iron from host heme. J. Bacteriol. 193: 4749-4757.
660 31. **Clarke SR, Mohamed R, Bian L, Routh AF, Kokai-Kun JF, Mond JJ, Tarkowski A,**
661 **Foster SJ.** 2007. The *Staphylococcus aureus* surface protein IsdA mediates resistance to
662 innate defenses of human skin. Cell. Host Microbe. **1**: 199-212.
663 32. **Cramton SE, Gerke C, Schnell NF, Nichols WW, Götz F**. 1999. The intercellular
664 adhesion (ica) locus is present in *Staphylococcus aureus* and is required for biofilm
665 formation. Infect. Immun. **67**: 5427-5433.
666 33. **Muller E, Takeda S, Shiro H, Goldmann D, Pier GB.** 1993. Occurrence of capsular
667 polysaccharide/adhesin among clinical isolates of coagulase-negative staphylococci. J.
668 Infect. Dis. **168**: 1211-1218.
669 34. **Heilbronner S, Hanses F, Monk IR, Speziale P, Foster TJ.** (2013). Sortase A promotes
670 virulence in experimental *Staphylococcus lugdunensis* endocarditis. Microbiology. **159**:
671 2141-2152.
672 35. **Corrigan RM, Foster TJ.** (2009). An improved tetracycline-inducible expression vector for
673 *Staphylococcus aureus*. Plasmid **61**, 126-129.

36. **Li MZ, Elledge SJ.** (2007). Harnessing homologous recombination in vitro to generate recombinant DNA via SLIC. Nat Methods **4**, 251-256.

37. **Li MZ, Elledge SJ.** (2012). SLIC: a method for sequence- and ligation-independent cloning. Methods Mol Biol **852**, 51-59.

38. **Bahey-El-Din M, Griffin BT, Gahan CG.** (2008). Nisin inducible production of listeriolysin O in *Lactococcus lactis* NZ9000. Microb. Cell Fact. **7**, 24. doi: 10.1186/1475-2859-7-24

39. **Monk IR, Casey PG, Hill C, Gahan CG.** (2010). Directed evolution and targeted mutagenesis to murinize *Listeria monocytogenes* internalin A for enhanced infectivity in the murine oral infection model. BMC Microbiol. **10**, 318. doi: 10.1186/1471-2180-10-318

40. **Christensen GD, Simpson WA, Younger JJ, Baddour LM, Barrett FF, Melton DM, Beachey EH.** 1985. Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. J. Clin. Microbiol. **22**: 996-1006.

41. **Chaignon P, Sadovskaya I, Ragunah Ch, Ramasubbu N, Kaplan JB, Jabbouri S.** 2007. Susceptibility of staphylococcal biofilms to enzymatic treatments depends on their chemical composition. Appl. Microbiol. Biotechnol. **75**: 125-132.

42. **Izano EA, Amarante MA, Kher WB, Kaplan JB.** 2008. Differential roles of poly-N-acetylglucosamine surface polysaccharide and extracellular DNA in *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. Appl. Environ. Microbiol. **74**: 470-476.

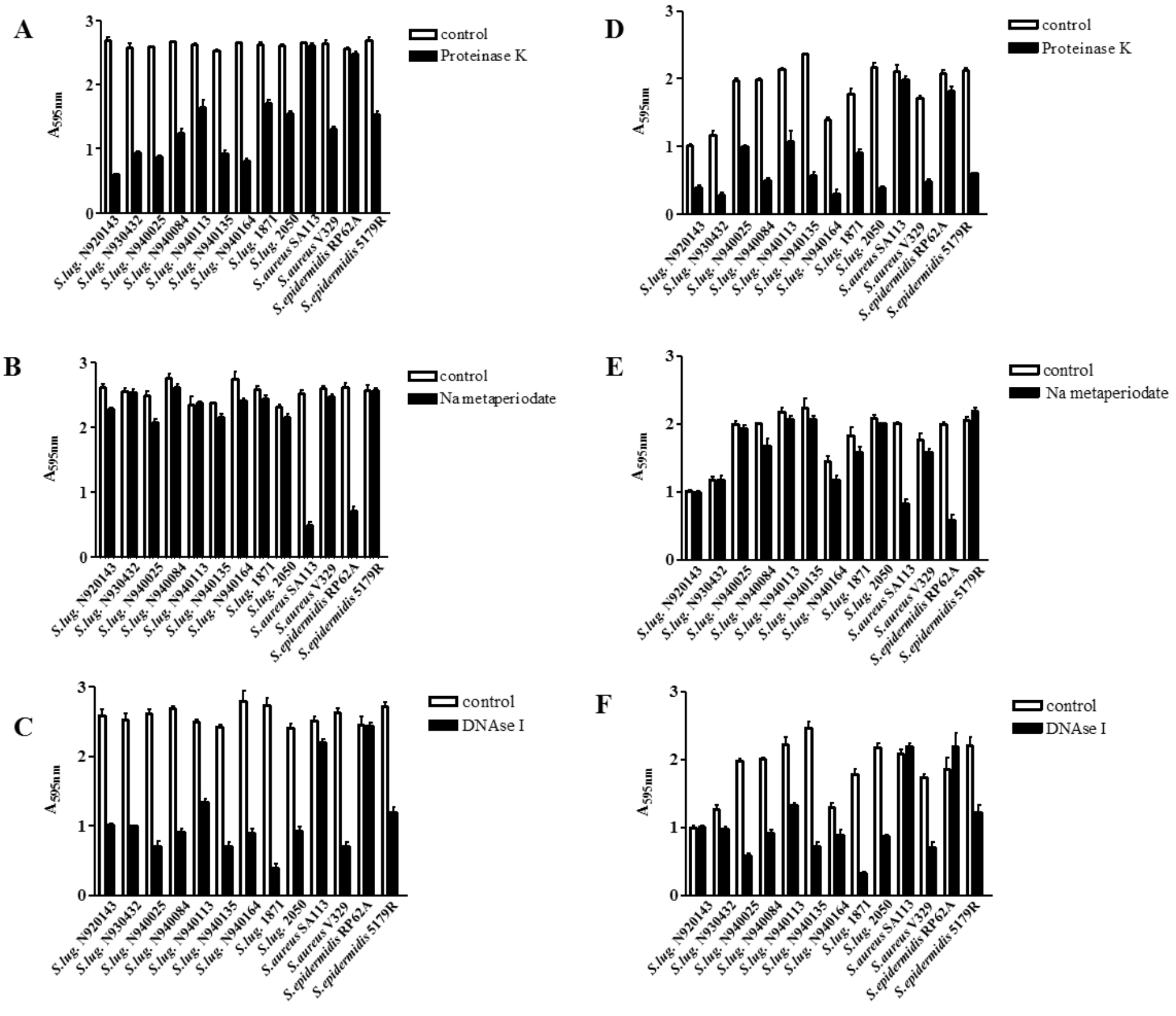
43. **Schwartz K, Syed AK, Stephenson RE, Rickard AH, Boles BR.** 2012. Functional amyloids composed of phenol soluble modulins stabilize *Staphylococcus aureus* biofilms. PLoS Pathog. **8**: e1002744.

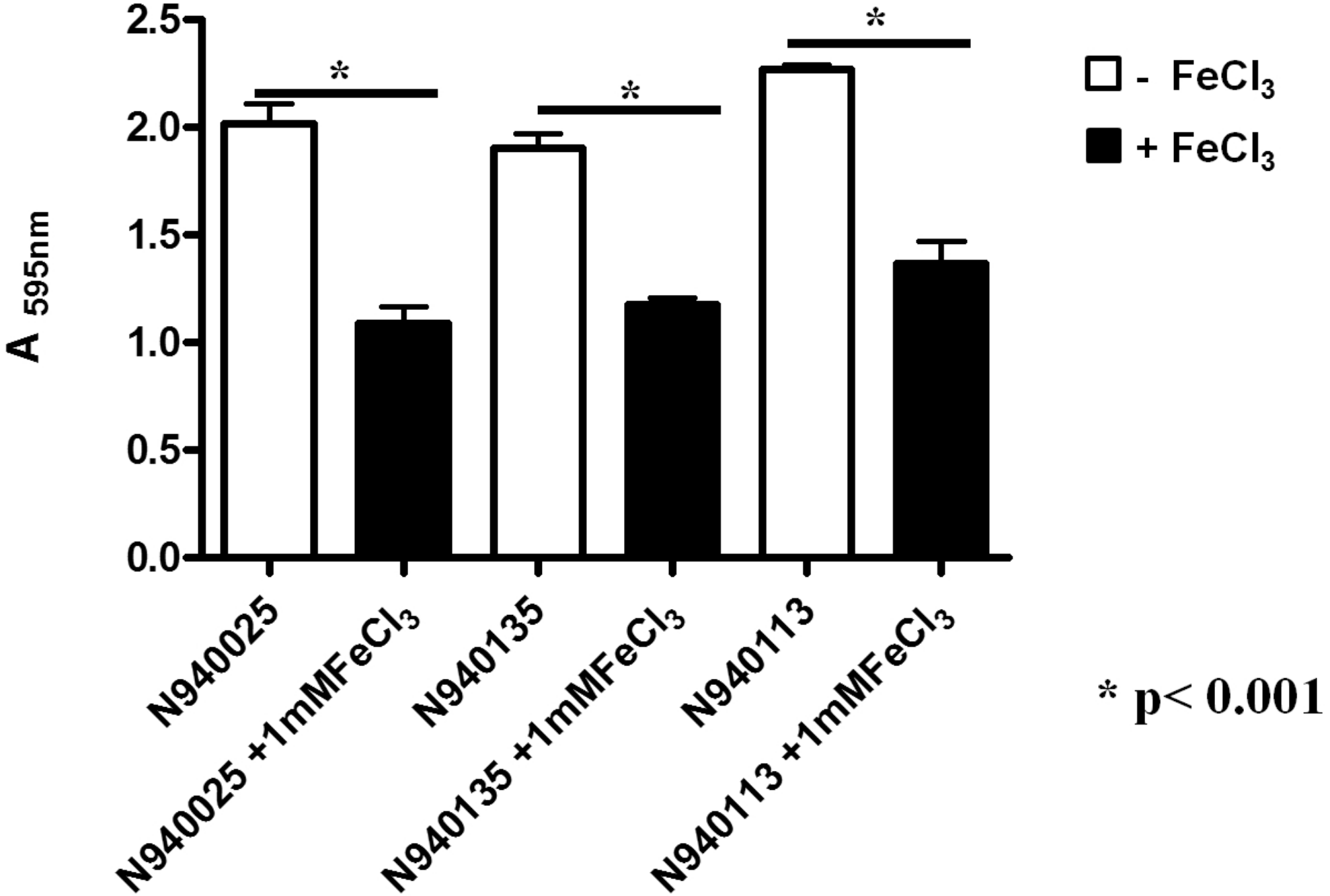
44. **Shahrooei M, Hira V, Khodaparast L, Khodaparast L, Stijlemans B, Kucharíková S, Burghout P, Hermans PW, Van Eldere J.** 2012. Vaccination with SesC decreases *Staphylococcus epidermidis* biofilm formation. Infect. Immun. **80**: 3660-3668.

45. **Vergara-Irigaray M, Valle J, Merino N, Latasa C, García B, Ruiz de Los Mozos I, Solano C, Toledo-Arana A, Penadés JR, Lasa I.** 2009. Relevant role of fibronectin-binding proteins in Staphylococcus aureus biofilm-associated foreign-body infections. Infect. Immun. **77**: 3978-3991.

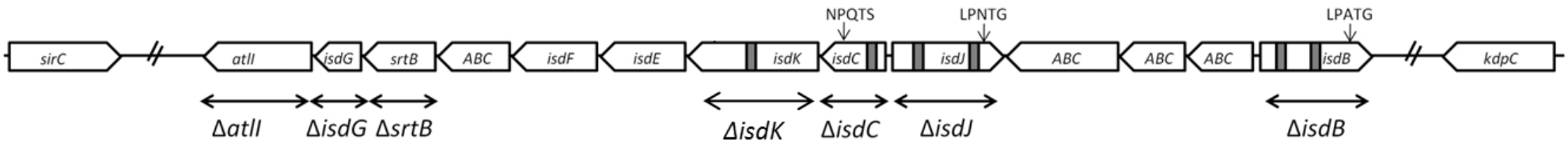
46. **Conrady DG, Wilson JJ, Herr AB.** 2013. Structural basis for $Zn^{2+}$-dependent intercellular adhesion in staphylococcal biofilms. Proc. Natl. Acad. Sci. U S A. **110**: E202-E211.

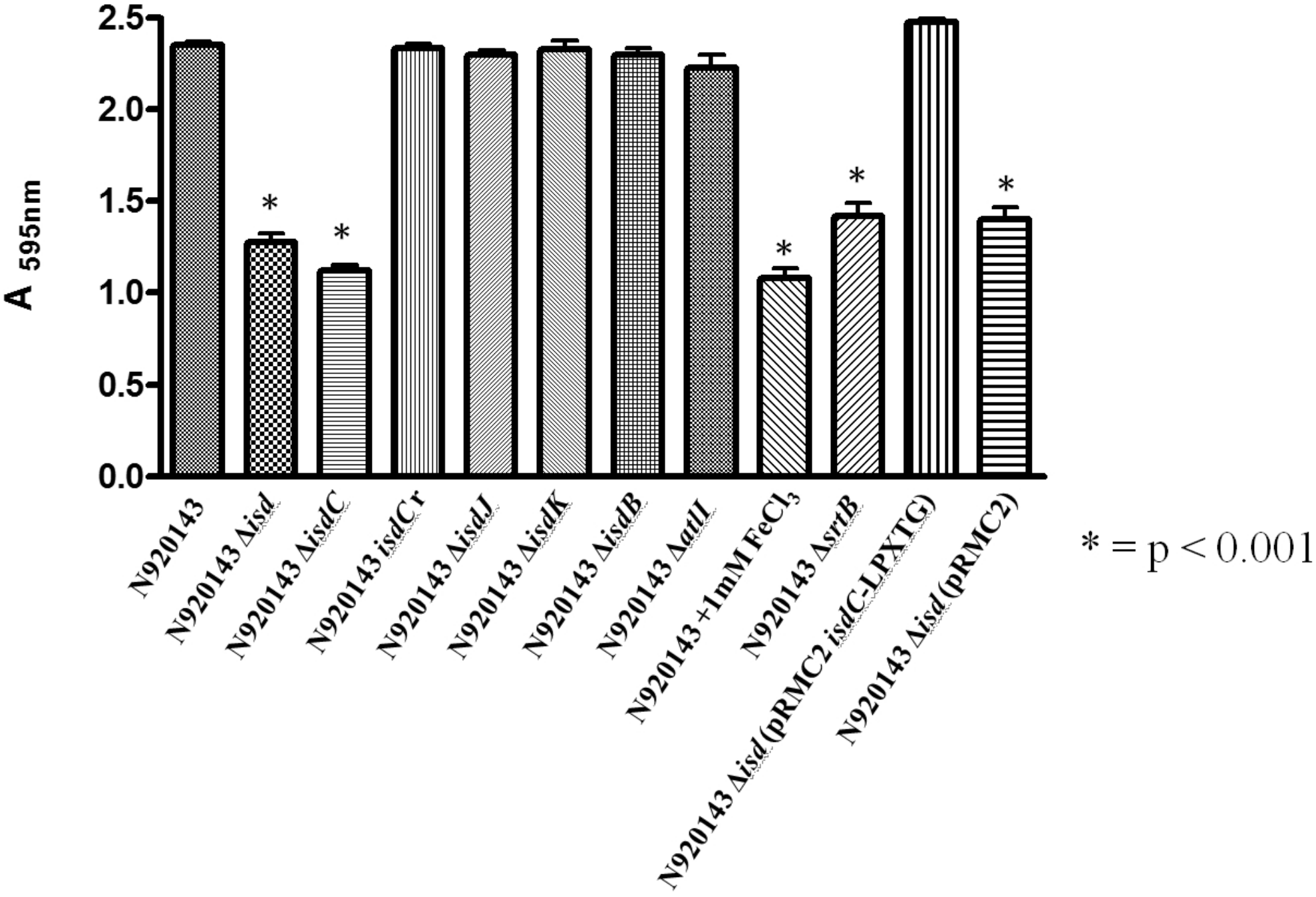
47. **Gruszka DT, Wojdyla JA, Bingham RJ, Turkenburg JP, Manfield IW, Steward A, Leech AP, Geoghegan JA, Foster TJ, Clarke J, Potts JR.** 2012. Staphylococcal biofilm-forming protein has a contiguous rod-like structure. Proc. Natl. Acad. Sci. U S A. **109**: E1011-E1018.

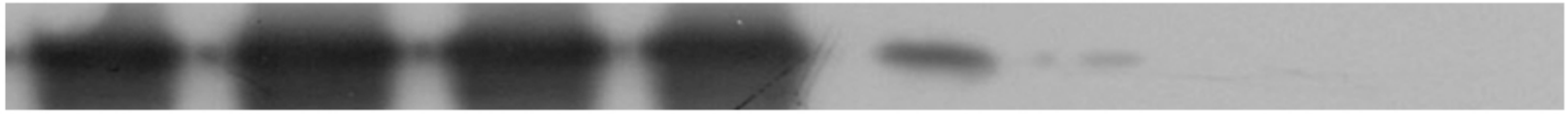

A
B
C
D
E
F
control
Proteinase K
Na metaperiodate
DNAse I
A595nm
0
1
2
3
S.lug. N920143
S.lug. N930432
S.lug. N940025
S.lug. N940084
S.lug. N940113
S.lug. N940135
S.lug. N940164
S.lug. 1871
S.lug. 2050
S.aureus SA113
S.aureus V329
S.epidermidis RP62A
S.epidermidis 5179R

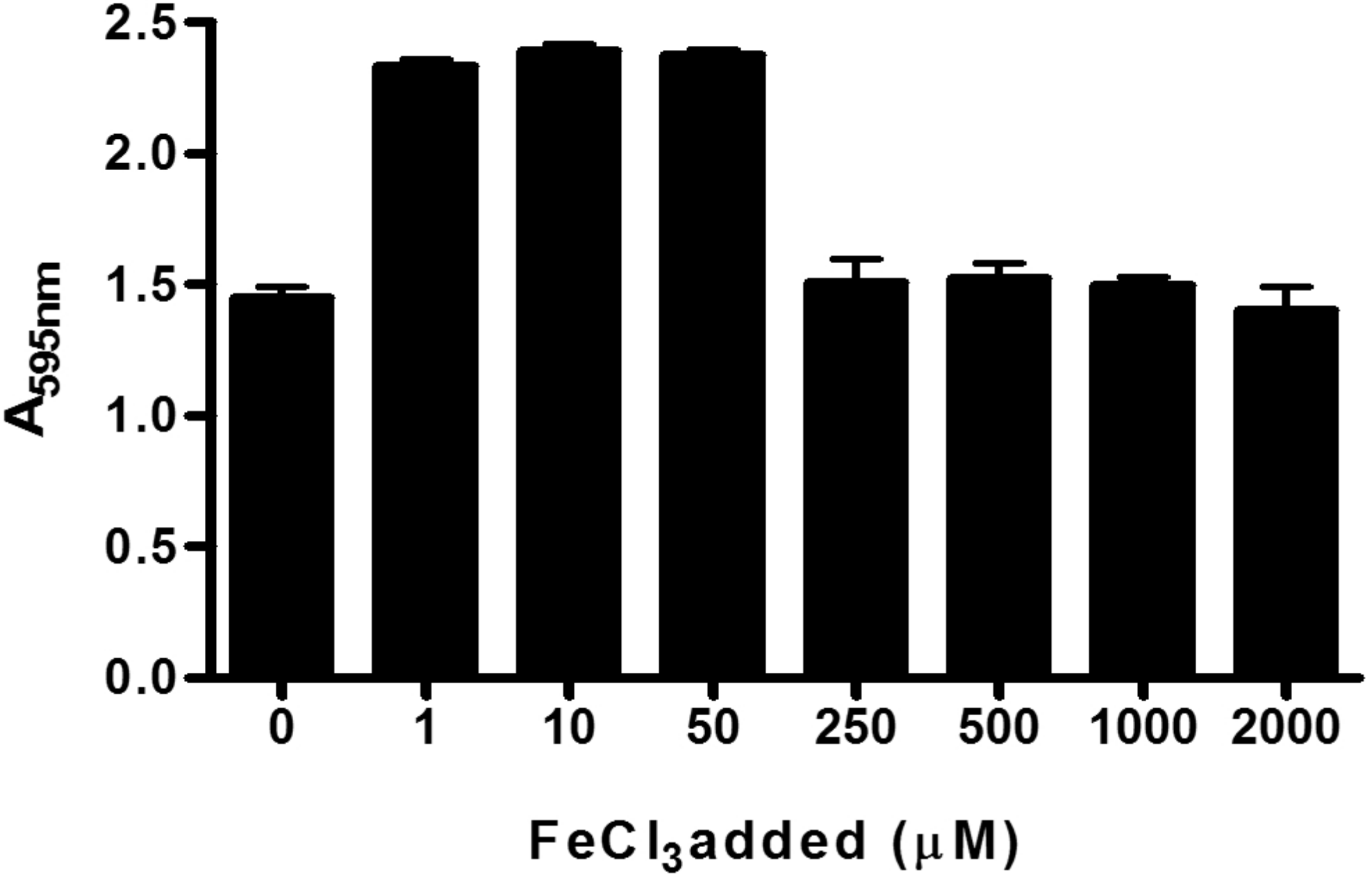

2.5
2.0
1.5
1.0
0.5
0.0
A 595nm
*
*
*
- FeCl3
+ FeCl3
N940025
N940025 +1mMFeCl3
N940135
N940135 +1mMFeCl3
N940113
N940113 +1mMFeCl3

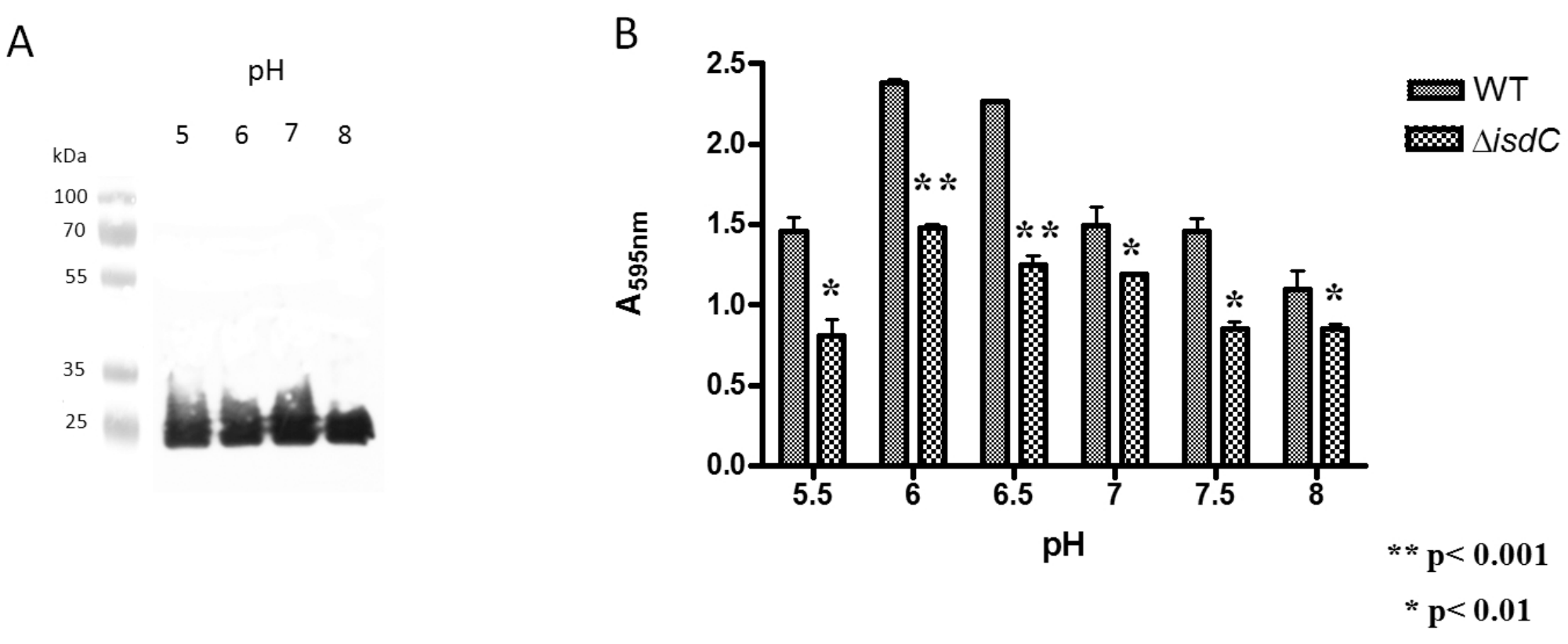
* p< 0.001

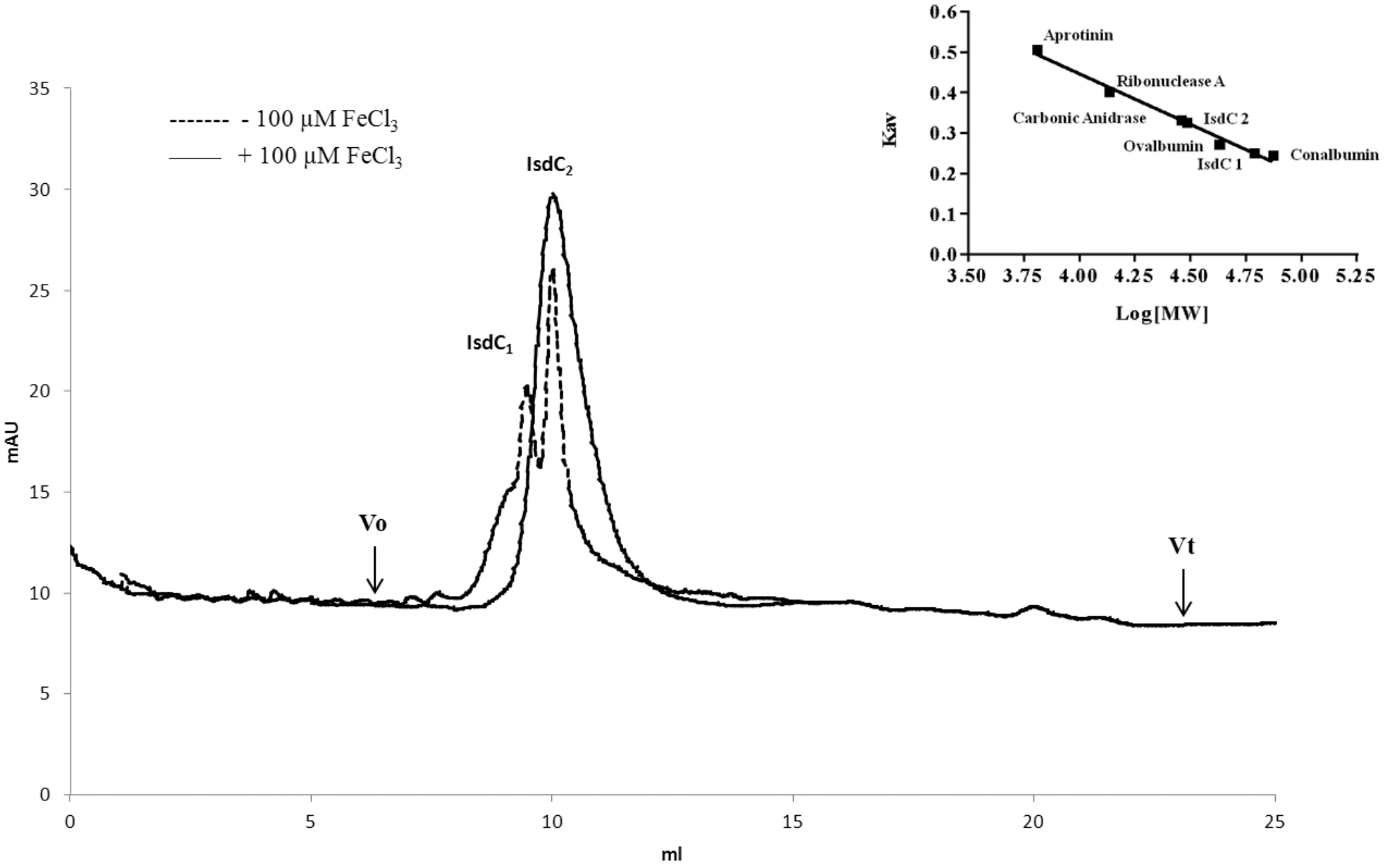

NPQTS
LPNTG
LPATG
sirC
atlI
isdG
srtB
ABC
isdF
isdE
isdK
isdC
isdJ
ABC
ABC
ABC
isdB
kdpC
ΔatlI
ΔisdG ΔsrtB
ΔisdK
ΔisdC
ΔisdJ
ΔisdB
Δisd – deletion of the entire locus

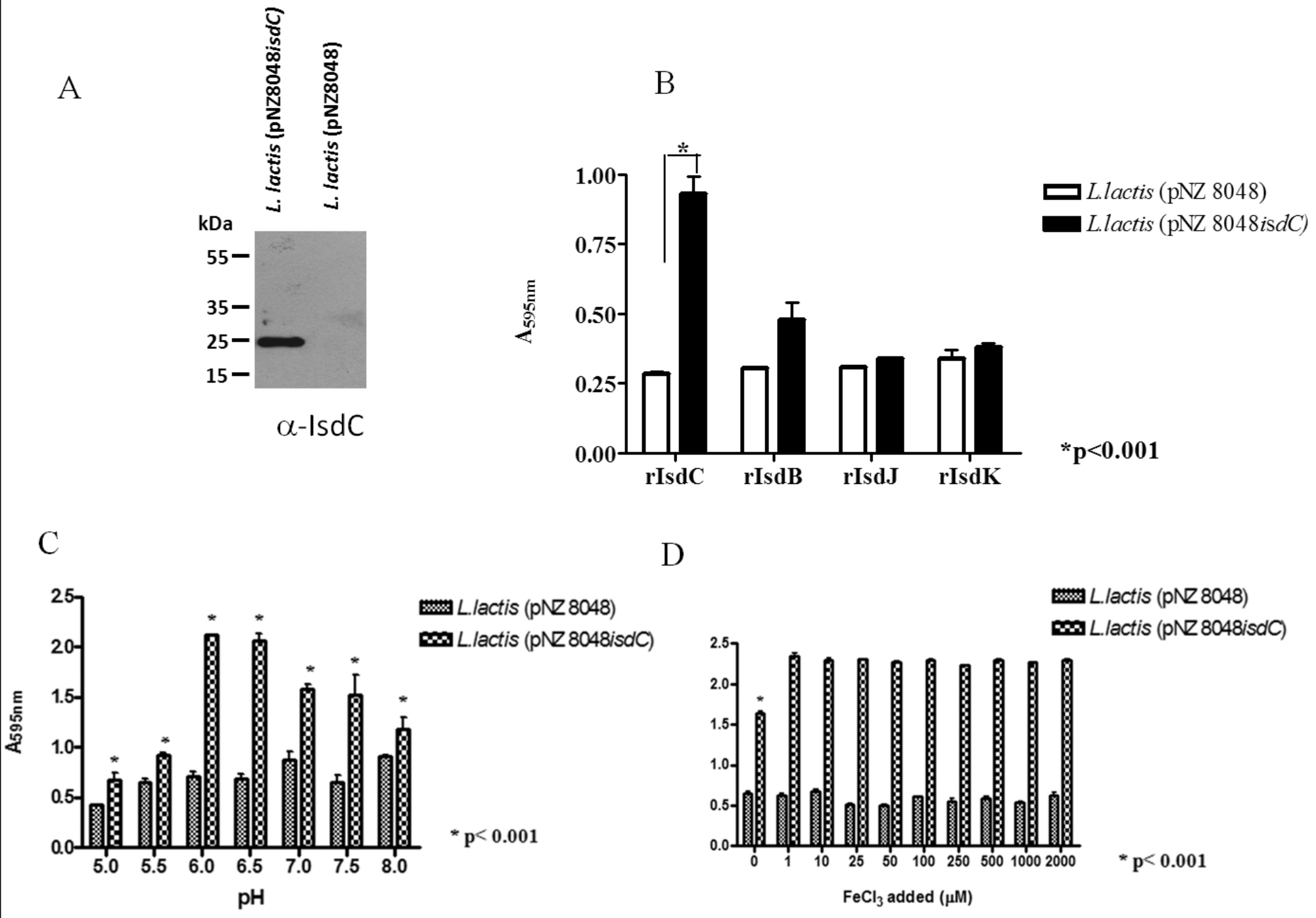

A 595nm
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
*
*
N920143
N920143 Δisd
N920143 ΔisdC
N920143 isdCr
N920143 ΔisdJ
N920143 ΔisdK
N920143 ΔisdB
N920143 ΔatlL
N920143 +1mM FeCl3
N920143 ΔsrtB
N920143 Δisd (pRMC2 isdC-LPXTG)
N920143 Δisd (pRMC2)
* = p < 0.001




2.5
2.0
1.5
1.0
0.5
0.0
$A_{595nm}$
0
1
10
50
250
500
1000
2000
$FeCl_3$ added (μM)


A
pH
5
6
7
8
kDa
100
70
55
35
25
B
2.5
2.0
1.5
1.0
0.5
0.0
$A_{595nm}$
WT
ΔisdC
*
**
**
*
*
*
5.5
6
6.5
7
7.5
8
pH
** p< 0.001
* p< 0.01


------- - 100 µM FeCl3
—— + 100 µM FeCl3
IsdC2
IsdC1
Vo
Vt
mAU
ml
0
5
10
15
20
25
30
35
Kav
Log[MW]
Aprotinin
Ribonuclease A
Carbonic Anidrase
IsdC 2
Ovalbumin
IsdC 1
Conalbumin
0.0
0.1
0.2
0.3
0.4
0.5
0.6
3.50
3.75
4.00
4.25
4.50
4.75
5.00
5.25


A
L. lactis (pNZ8048isdC)
L. lactis (pNZ8048)
kDa
55
35
25
15
α-IsdC
B
*
1.00
0.75
0.50
0.25
0.00
A595nm
L.lactis (pNZ 8048)
L.lactis (pNZ 8048isdC)
rIsdC
rIsdB
rIsdJ
rIsdK
*p<0.001
C
2.5
2.0
1.5
1.0
0.5
0.0
A595nm
L.lactis (pNZ 8048)
L.lactis (pNZ 8048isdC)
5.0
5.5
6.0
6.5
7.0
7.5
8.0
pH
* p< 0.001
D
L.lactis (pNZ 8048)
L.lactis (pNZ 8048isdC)
2.5
2.0
1.5
1.0
0.5
0.0
0
1
10
25
50
100
250
500
1000
2000
FeCl3 added (μM)
* p< 0.001

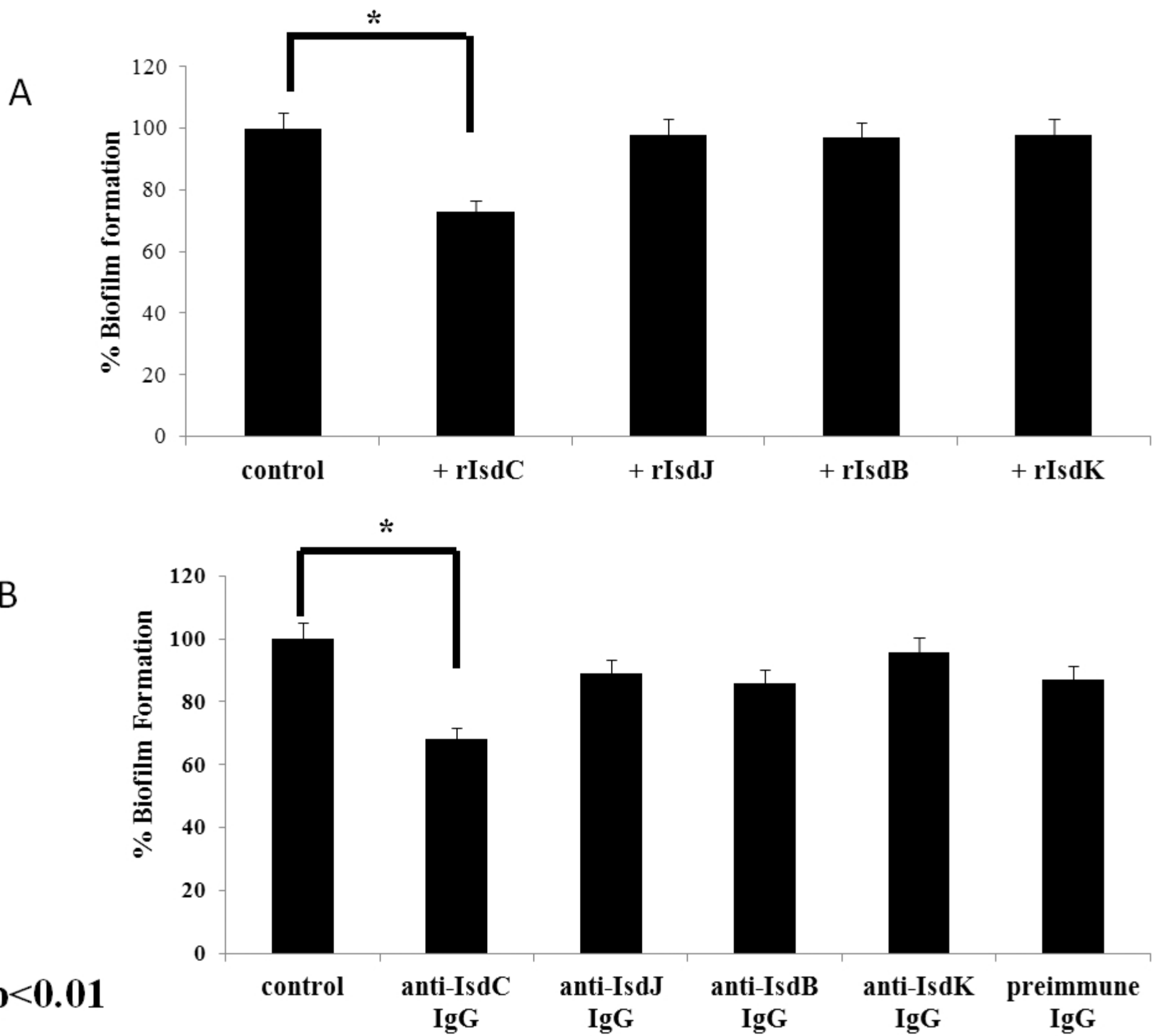

A
*
120
100
80
60
40
20
0
% Biofilm formation
control
+ rIsdC
+ rIsdJ
+ rIsdB
+ rIsdK
B
*
120
100
80
60
40
20
0
% Biofilm Formation
control
anti-IsdC IgG
anti-IsdJ IgG
anti-IsdB IgG
anti-IsdK IgG
preimmune IgG
* p<0.01

TABLE 1 Bacterial strains and plasmids

| Strain or plasmid | Relevant characteristics[a] | Source or reference |
|---|---|---|
| Strains | | |
| *Staphylococcus lugdunensis* | | |
| N920143 | Human breast abscess isolate | 28 |
| N930432 | Human endocarditis isolate | F. Vandenesch (unpublished data) |
| N940025 | Human endocarditis isolate | F. Vandenesch (unpublished data) |
| N940084 | Finger pulp infection isolate | This study |
| N940113 | Vertebral infection isolate | This study |
| N940135 | Perineal infection isolate | This study |
| N940164 | Perineal infection isolate | This study |
| 1871 | Orthopaedic infection with internal fixation system | This study |
| 2050 | Orthopaedic infection with knee implant | This study |
| N920143 Δ*isdB* | *isdB* null mutation in N920143 | 29 |
| N920143 Δ*isdC* | *isdC* null mutation in N920143 | 29 |
| N920143 Δ*isdJ* | *isdJ* null mutation in N920143 | 29 |
| N920143 Δ*isdK* | *isdK* null mutation in N920143 | 29 |
| N920143 Δ*isd* | *isd* null mutation in N920143 | 29 |
| N920143 Δ*atlI* | *atlI* null mutation in N920143 | This study |
| N920143 Δ*srtB* | *srtB* null mutation in N920143 | This study |
| *Staphylococcus aureus* | | |
| SA113 | ATCC 35556, restriction deficient | 31 |
| V329 | Isolate obtained from a bovine subclinical phenotype | 8 |
| *Staphylococcus epidermidis* | | |
| RP62A | ATCC 35984, isolated from a patient with sepsis | 32 |
| 5179R | A clinical isolate obtained from cerebrospinal fluid infection | 14 |
| *Lactococcus lactis* NZ9000 | *L. lactis* subsp. *cremoris* MG1363 carrying nisin resistance cassette | 33 |
| *Escherichia coli* | | |
| TOPP3 | *E. coli* cloning and protein purification host | Stratagene |
| Plasmids | | |
| pQE30 | *E. coli* cloning and expression vector.Ap$^r$ | Stratagene |
| pQE30 *isdB* | pQE30 encoding residues 45 to 655 of IsdB | 29 |
| pQE30 *isdC* | pQE30 encoding residues 30 to 190 of IsdC | 29 |
| pQE30 *isdJ* (45-610) | pQE30 encoding residues 45 to 610 of IsdJ | 29 |
| pQE30 *isdK* | pQE30 encoding residues 35 to 426 of IsdK | 29 |
| pNZ8048 | *L. lactis* shuttle vector containing the PnisA promoter and start codon in NcoI site. Cm$^r$allowing nisin-inducible expression of insert. | 33 |
| pNZ8048*isdC*-LPQTG | pNZ8048 encoding *isdC*, for controlled expression of sortase A-anchored IsdC in *L. lactis* | This study |
| pRMC2 | Inducible expression vector for staphylococci Ap$^r$ Cm$^r$ | 35 |
| pRMC2 *isdC*-LPQTG | pRMC2 encoding *isdC*, for controlled expression of sortase A-anchored IsdC in *S. lugdunensis* Δ*isd* | This study |

[a]Ap$^r$ ampicillin resistance, Cm$^r$ chloramphenicol resistance