Sortase A Promotes Virulence in Experimental *Staphylococcus lugdunensis* Endocarditis

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ABSTRACT

Staphylococcus lugdunensis is a commensal of humans and an opportunistic pathogen. It can cause an aggressive form of infective endocarditis in healthy humans akin to *Staphylococcus aureus*. The virulence of *S. lugdunensis* N920143 was compared to *S. aureus* in an experimental rat endocarditis model. It caused a milder course of disease with lower levels of bacteraemia and smaller endocardial vegetations than *S. aureus* strain Newman. Vegetations were of similar size, however, when compared to the *S. aureus* MRSA strain COL. The organism is difficult to transform with plasmids and little is known about virulence factors and the molecular mechanisms of pathogenicity. We report a method for electroporation of *S. lugdunensis* with plasmid DNA and demonstrated that the low efficiency of transformation is in part due to a type I restriction modification system because deletion of a putative *hsdR* gene by allelic exchange allowed a higher frequency of transfer. A deletion mutant lacking sortase A was found to be significantly less virulent than wild type in the endocarditis model. Mutants defective in surface proteins Fbl and vWbl were not significantly different from the wild-type but showed trends towards reduced virulence.

INTRODUCTION

The coagulase negative staphylococcus (CoNS) *S. lugdunensis* was first described by Freney *et al*. in 1988 in Lyon, France (Freney *et al.*, 1988). It is a skin commensal that is predominantly isolated from moist areas such as the perineum, the inguinal fold and under the large toenail (Bieber & Kahlmeter, 2010). Described as a “wolf in sheep’s clothing” (Frank *et al.*, 2008), *S. lugdunensis* behaves in many ways more like the coagulase positive *S. aureus* than the other CoNS including having an apparent elevated degree of virulence. *S.*

lugdunensis causes a wide range of different infections including abscesses and wound infections but is particularly associated with severe cases of infectious endocarditis (IE). Around 1 % of IE cases are reported to be caused by *S. lugdunensis* (Anguera *et al.*, 2005) with mortality rates ranging from 38 % (Liu *et al.*, 2010) to 42% (Anguera *et al.*, 2005). *S. lugdunensis* most frequently causes left sided native valve endocarditis involving mitral and/or aortic valves (Frank *et al.*, 2008; Liu *et al.*, 2010).

S. lugdunensis can be easily misidentified as *S. aureus* due to its similar colony morphology, haemolytic activity and its ability to agglutinate latex particles coated with fibrinogen (Zbinden *et al.*, 1997). This raises the question as to whether *S. lugdunensis* infections might be more prevalent than reported. Furthermore, *S. lugdunensis* is a CoNS and might therefore be interpreted as a contaminant in clinical samples. This might lead to a delay in treatment of infected patients which could be a reason for increased mortality.

S. lugdunensis has the potential to express up to 9 proteins that are covalently anchored to the cell wall peptidoglycan by sortases. The fibrinogen binding protein Fbl (Geoghegan *et al.*, 2010; Mitchell *et al.*, 2004; Nilsson *et al.*, 2004a) is closely related to clumping factor A (ClfA) of *S. aureus*, a protein which contributes to the pathogenesis of experimental septic arthritis, to kidney abscess formation in mice and to endocarditis in rats (Josefsson *et al.*, 2001; Moreillon *et al.*, 1995). Previously described *fbl* mutants of *S. lugdunensis* were unable to adhere to Fg suggesting that it is the only Fg binding MSCRAMM of *S. lugdunensis* (Marlinghaus *et al.*, 2012). The von Willebrand factor binding protein (vWbl) contains an Arg-Glu-Asp (RGD) motive which is found in many integrin binding proteins and has a region consisting of 10 imperfect repeats of a 67 amino acids that binds to von Willebrand factor (vWf) (Nilsson *et al.*, 2004b). (Schematic diagrams of Fbl and vWbl are shown in Fig. S1).

Prior to this study genetic manipulation had only been reported in *S. lugdunensis* following introduction of plasmids by protoplast transformation (Marlinghaus *et al.*, 2012). An objective of this project was to establish a convenient genetic system involving electroporation and efficient vectors for performing allelic exchange. Transformation of Gram-positive bacteria can be difficult because of the thick cell wall acting as a physical barrier (Löfblom *et al.*, 2007) and restriction endonuclease (R-M) systems. The major barriers to DNA transfer into *S. aureus* and *S. epidermidis* from *Escherichia coli* are the type IV restriction enzymes SauUSI and McrR, respectively, which recognize cytosine methylated DNA (Corvaglia *et al.*, 2010; Monk *et al.*, 2012; Xu *et al.*, 2011). In addition *S. aureus* has the potential to express two type I restriction systems consisting of three subunits, HsdS (specificity), HsdM (methylation) and an HsdR endonuclease (Veiga & Pinho, 2009) which reduces uptake from *E. coli* and limits DNA transfer between closely related lineages (McCarthy & Lindsay, 2012; McCarthy *et al*., 2012).

In this study, we describe a method for the preparation of *S. lugdunensis* electrocompetent cells and use efficient allelic exchange to isolate a restriction deficient mutant. We isolated mutants of *S. lugdunensis* N920143 deficient in Fbl, vWbl and sortase A and tested their ability to cause endocarditis in a rat infection model.

METHODS

Bacterial strains, growth conditions and reagents. All strains are listed in Table 1. *S. lugdunensis* was grown in tryptic soy broth (TSB) or agar (TSA) (Difco) or RPMI-1640 (Sigma) to create iron-restricted conditions. *E. coli* strains were grown in L-broth or L-agar

(Difco). Unless stated otherwise, strains were grown at 37 °C. Unless indicated otherwise, reagents were obtained from Sigma.

Transformation of *S. lugdunensis*. Electrocompetent *S. lugdunensis* cells were prepared according to a protocol described for *S. carnosus* (Löfblom *et al.*, 2007) with only minor differences. In brief, 50 ml TSB was inoculated with cells from an overnight culture to an OD_{600} = 0.5 and incubated shaking at 37 °C for 60 min. Cells were harvested and washed twice with 50 ml ice-cold distilled H_2O and finally with 50 ml ice-cold 10 % glycerol. Cells were taken up in 250 µl 10 % glycerol and 70 µl aliquots were used for directly for transformation (or stored at -80 °C.) Prior to transformation cells were collected by centrifugation (10 min at 9000 x g), taken up in 70 µl of 0.5 M sucrose, 10 % glycerol. 5 µg *E. coli*-derived DNA or 1 µg *S. lugdunensis*-derived DNA was added (in up to 5 µl H_2O) and the mixture was incubated for 10 min at room temperature. The electroporation was carried out in 0.1 cm cuvettes (Bio-Rad) at 2.1 V, 100 Ω and 25 µF. Immediately after electroporation 930 µl TSB with 0.5 M sucrose was added and the cells were incubated with shaking for 1.5 h followed by incubation on ice for 30 min. Aliquots were plated on TSA containing chloramphenicol (10 µg/ml).

Allelic exchange in *S. lugdunensis*. Construction of cassettes for generating deletion mutations was carried out as described previously (Monk *et al.*, 2012). In brief, A and B primer combinations (Table 2) were used for each construct to amplify a 500 bp upstream sequence (up to the start codon) and a 500 bp sequence downstream of stop codon (C and D primers). The PCR products were used as templates for the spliced overlap extension (SOE) PCR using primers A and D. The resulting 1 kb fragment was gel-purified, cleaved at

123 endonuclease cleavage sites introduced in forward and reverse primers (A and D) and cloned
124 into pIMAY treated with the same endonucleases.

125 In order to reverte the Δ*srtA* mutation, the program [emboss.bioinformatics.nl/cgi-
126 bin/emboss/silent] was used to identify single nucleotides within *srtA* that can be mutated to
127 create novel restriction sites without causing changes in the amino acid sequence of the
128 protein. Primers E and F were synthesised exchanging nucleotide 252 of *srtA* (T to G)
129 thereby creating a novel SmaI restriction site. Primers A/E were used to amplify the upstream
130 sequence and the 5'-*srtA* fragment with nucleotide exchange and primers F/D were used to
131 amplify the downstream region together with 3'-*srtA* fragment introducing the nucleotide
132 exchange. PCR products were gel purified and used for the SOE PCR using primers A and D.
133 The reversion cassette was gel-purified, cleaved at endonuclease cleavage sites introduced in
134 primers A and D and cloned into pIMAY treated with the same endonucleases**.** After
135 reversion of the Δ*srtA* mutant, the presence of the novel restriction site was confirmed by
136 PCR amplification and subsequent SmaI digestion. A schematic diagram of the deletion and
137 reversion constructs is given in Fig. S2.

138 The protocol for allelic exchange described by Monk *et. al.* (Monk *et al.*, 2012) was
139 used successfully in *S. lugdunensis*. Strain N920143 was transformed with recombinant
140 plasmids and plated at 28 °C in the presence of 10 μg/ml chloramphenicol (Cm10). Clones
141 with integrated plasmids were selected by growth on TSA Cm10 at 37 °C and loss of
142 replicating plasmid and was tested. Protein secretion is an essential capability and bacteria
143 without a functional Sec-system are not viable. Thus *secY* antisense RNA has been used
144 previously for the positive selection of *S. aureus* clones that have lost the integrating plasmid
145 (Bae & Schneewind, 2006). In pIMAY the *S .aureus secY* antisense RNA is under control of
146 the TetR promoter and tightly repressed. After induction with unhydrotetracycline, only cells
147 without integrated or replicating plasmid are able to grow. Although the *secY* genes of *S.*

aureus and *S. lugdunensis* are similar (the 561 nucleotide *secY* antisense fragment shares 81.3% identity with the *S. lugdunensis* N920143 *secY*), induction of *secY* antisense RNA did not result in selection of colonies lacking the plasmid in *S. lugdunensis* (data not shown). In order to detect plasmid loss, clones with an integrated plasmid were grown for 18 h in TSB at 28 °C, sub-cultured (1 : 1000) in fresh TSB and grown at 28 °C for 18 h. The culture was plated out on TSA and incubated at 37 °C. Colonies were patched on TSA and TSA with Cm10 and Cm sensitive derivatives were screened by colony PCR for the presence of the mutant allele. To prepare template DNA, one colony was resuspended in 20 µl TE buffer (10 mM Tris, 1 mM EDTA), boiled for 10 min and centrifuged at 6000 x g for 3 min. 1 µl of the supernatant was used as template for a 20 µl PCR reaction.

Adherence assay. Adherence assays were performed as described previously (Hartford *et al.*, 1997). Microtiter plates were coated with 100 µl fibrinogen (Calbiochem; 10 µg/ml in PBS) or vWF (Haemolytic Technologies) (10 µg/ml in sodium carbonate buffer (100 mM $NaHCO_3$, 33 mM Na_2CO_3, pH 9.6)) overnight at 4 °C. Wells were washed three times with 200 µl PBS, blocked with 5 % (w/v) milk powder in PBS for 2 h at 37 °C and then washed three times with 200 µl PBS. Bacterial strains were grown to stationary phase, harvested by centrifugation, washed once with PBS and adjusted to an OD_{578} of 1. 100 µl of cells were added to each well and the plate was incubated for 2 h at 37 °C. The wells were washed 3 times with 200 µl PBS and bound cells were fixed with 100 µl formaldehyde 25 % (v/v). Wells were washed 3 times with 200 µl PBS, stained with crystal violet for 1 min and washed 5 times with PBS. 100 µl of acetic acid (5 % v/v) was added to the wells and the absorbance read with an ELISA plate reader at 570 nm.

Cell Fractionation. The assay was carried out as described earlier (Monk *et al.*, 2004) with minor modifications. Cells were grown in TSB or RPMI to stationary phase and washed once with buffer WB (10 mM Tris-HCl pH7, 10 mM MgCl). A 1 ml aliquot OD_{600} = 5 was centrifuged (18000 x g) and resuspended in 100 µl buffer DB (10 mM Tris-HCl pH7, 10 mM MgCl, 500 mM sucrose, 0.3 mg/ml lysostaphin, 250 U/ml (Ambi) mutanolysin, 30 µl protease inhibitor cocktail (Roche - 1 complete mini tablet dissolved in 200 µl H_20), 1 mM PSMF. The digestion of the cell wall was carried out at 37 °C for 1.5 h followed by centrifugation (18,000 x g, 10 min, 4 °C). The supernatant was designated the cell wall fraction. The pellet containing the protoplasts was washed with 1 ml WB (with 500 mM sucrose) and centrifuged again as above. The protoplasts were resuspended in 200 µl buffer LB (100 mM Tris-HCl pH7, 10 mM MgCl, 100 mM NaCl, 10 µg/ml DNaseI, 100 µg/ml RNaseA). The suspension was frozen and thawed three times to ensure protoplast lysis and centrifuged for 30 min (4 °C, 18,000 x g). The pellet (designated the membrane fraction) was washed with 1 ml of buffer LB, centrifuged and resuspended in 100 µl TE buffer (100 mM Tris-HCl pH 8, 1 mM EDTA).

Western Immunoblotting. Western Immunoblotting was carried out using standard procedures with a 7.5 % acrylamide gel. Prestained protein ladders were obtained from Fermentas. Rabbit anti-IsdB antibodies were described previously (Zapotoczna *et al.*, 2012a) and anti-SrtA antibodies (directed against the *S. aureus* protein) were obtained from Abcam. Bound antibodies were detected with goat anti-rabbit IgG conjugated to horse radish peroxidase. Immunoreactive bands were detected with the “ImageQuant Las 4000” system and the corresponding “ImageQuant TL” Software.

Animal model. Animal experiments were approved by the local animal protection committee at the University of Regensburg and the responsible state authorities. Male Sprague-Dawley rats (~200 g) were obtained from Charles River Laboratories, Sulzfeld, Germany. Rats were maintained under standard housing conditions and given food and water ad libitum. A model of catheter-induced staphylococcal endocarditis was described previously (Lee *et al.*, 1997). Rats were anesthetized with a mixture of ketamine and xylazine, and a polyethylene catheter (Becton Dickinson, Heidelberg, Germany) was passed through the right carotid artery and the aortic valve into the left ventricle. Vigorous pulsation of blood within the catheter indicated correct positioning of the device. The catheter was sealed and tied in place with sterile suturing material, and the incision was closed. The rats were challenged intravenously with $3x10^4$ to $1x10^5$ *S. lugdunensis* or *S. aureus* as indicated 48 h after surgery. Heparinized blood was collected from each animal by tail vein puncture 24, 48, 72 h and 96 h (for *S. lugdunensis* infections) after inoculation and plated on agar. Surviving rats were euthanized 72-96 h after challenge, and their hearts and kidneys were removed. The position of the catheter within the heart and the presence or absence of vegetations was noted. The kidneys and aortic valve vegetations were weighed and homogenized in PBS or TSB, respectively. Quantitative plate counts were performed on serial dilutions of the homogenates, and the CFU per g of tissue was calculated.

RESULTS

Virulence of *S. lugdunensis* N920143 in a rat endocarditis model compared to *S. aureus*. Clinical reports suggest that *S. lugdunensis* causes both native and prosthetic valve endocarditis (Frank *et al.*, 2008; Liu *et al.*, 2010). An animal model of *S. lugdunensis* endocarditis has not previously been reported. We used a rat model of a catheter-induced endocarditis that is well established for *S. aureus* to compare the virulence of *S. lugdunensis*

strain N920143 to *S. aureus* strains Newman and COL. *S. lugdunensis* N920143 was chosen because the complete genome sequence is available (Heilbronner *et al.*, 2011) and it expresses Fbl (Geoghegan *et al.*, 2010), a fibrinogen binding protein that is related to ClfA of *S. aureus*, a known virulence factor in rat endocarditis (Moreillon *et al.*, 1995). Infection caused by *S. lugdunensis* seemed to be less severe than that caused by both *S. aureus* strains. Rats infected with *S. lugdunensis* displayed weaker symptoms than *S. aureus*-infected rats and most *S. lugdunensis*-infected animals were still alive at day 4 post infection. *S. aureus*-infected rats were either dead or had to be euthanized at day 3 post infection. Animals infected with *S. lugdunensis* consistently showed lower levels of bacteraemia compared to either *S. aureus* strain (Fig. 1(a)). This might explain the milder course of disease and could be due to a more effective clearance of *S. lugdunensis* from the bloodstream as well as to less severe endocarditis.

Rats infected with *S. lugdunensis* had significantly fewer viable bacteria in their kidneys than animals infected with *S. aureus* strain Newman (Fig. 1(b)). Furthermore, the cardiac vegetations were smaller and bacterial densities in vegetations were lower (Fig. 1(c)). Interestingly, vegetations caused by *S. lugdunensis* N920143 and *S. aureus* strain COL were similar in size and bacterial density. Furthermore spreading of the bacteria to the kidneys was comparable between *S. lugdunensis* N920143 and *S. aureus* COL. In summary, *S. lugdunensis* was less virulent then either *S. aureus* strain with regard to the levels of bacteremia but formed comparable vegetations to *S. aureus* strain COL.

Genetic manipulation of *S. lugdunensis* N920143. In order to investigate the molecular basis of pathogenesis of endocarditis caused by *S. lugdunensis* it was necessary to be able to manipulate the organism genetically. For this a plasmid DNA transformation system and a plasmid vector that promotes allelic exchange were required. Several different protocols to render strain N920143 electrocompetent were attempted using the shuttle plasmid pRMC2

(Corrigan & Foster, 2009) isolated from *E. coli* XL-1 blue. The protocol of Augustin and Götz (Augustin & Götz, 1990) did not yield any transformants. However, a modified procedure based on the protocol originally optimized for the *S. carnosus* (Löfblom *et al.*, 2007) did allow the transformation of *S. lugdunensis* N920143 with a low frequency of ca 0.6 x 10^1 (see Fig. 2). The low efficiency of transformation was sufficient to allow genetic manipulation to be attempted. The thermosensitive plasmid pIMAY was developed to facilitate the creation of allelic exchange mutations in *S. aureus* (Monk *et al.*, 2012) and the procedure has now been optimized for use in *S. lugdunensis*.

By-passing the restriction barrier in *S. lugdunensis* N920143. *S. lugdunensis* N920143 could be transformed with *E. coli* XL1-blue-derived plasmid DNA at a low frequency. The transformation frequency increased ca. 100-fold when plasmid DNA isolated from *S. lugdunensis* (Fig. 2) was used suggesting that a restriction-modification (R-M) system prevented efficient uptake of *E. coli* DNA. Therefore the *S. lugdunensis* N920143 genome sequence (Heilbronner *et al.*, 2011) was searched for the presence of orthologous genes to those responsible for restriction and modification in *S. aureus*. We could not identify a gene similar to *sauUSI* or *mcrR* that encode the primary barriers against the transfer of plasmid DNA from *E. coli* into *S. aureus* and *S. epidermidis*, respectively (Corvaglia *et al.*, 2010; Monk *et al.*, 2012). The absence of a SauUSI homologue in *S. lugdunensis* suggested that the organism does not recognize and degrade cytosine methylated DNA. To test this hypothesis, plasmid DNA was isolated from XL-1 blue (dcm^+) and DC10B (dcm^-) and used for transformation of N920143 (Fig. 2). No difference in transformation of *S. lugdunensis* was seen, indicating that cytosine methylation is not recognized.

S. lugdunensis N920143 and HKU09-01 (Tse *et al.*, 2010) possess closely linked *hsdR, hsdM* and *hsdS* genes with the potential to encode a type I R-M (here named SluI). In order to investigate whether this is responsible for the reduced transfer of DNA from *E. coli*,

an *hsdR* mutant was constructed by allelic exchange. This improved the transformation frequency achieved with DNA isolated from *E. coli* XL-1 blue and DC10B to the same level as that achieved with DNA isolated from wild-type *S. lugdunensis*, suggesting that SluI is the only RM system present. Plasmid isolated from the *ΔhsdR* strain transformed the wild-type strain with the same efficiency as DNA isolated from the wild-type strain itself (data not shown). Thus, *ΔhsdR* can be used as an intermediate host for the easy transfer of DNA from *E. coli* into *S. lugdunensis*.

Transformation of strains from different *S. lugdunensis* clonal complexes. In *S. aureus* strains from different lineages show differences in transformation frequency (Monk *et al*., 2012). We investigated whether the same is true for *S. lugdunensis*. Recently, multilocus sequence typing (MLST) was established for *S. lugdunensis* (Chassaîn *et al.*, 2012) and showed a clonal population structure with five clonal complexes (CCs). We chose the CC1 strain HKU09-01 and two isolates from each clonal complex for comparative transformation experiments (Fig. 3). All strains from CC1, CC2 and CC5 were transformable with XL1-blue-derived plasmid DNA with a low efficiency ($1x10^1$ – $1x10^2$) similar to N920143 (CC1). The use of *S. lugdunensis* N920143-derived plasmid DNA increased the transformation frequency 10-50 fold in strains from CC1 and CC2 indicating that the same restriction system occurs in these strains. However, strain SL13 from CC1 was transformed with *E. coli*-derived DNA at $>10^3$ per μg, a frequency that was not improved when DNA from *S. lugdunensis* was compared. This suggested a defect in the *hsdR* gene.

Interestingly N920143-derived DNA did not improve the transformation frequency of CC5 isolates above the level achieved with XL1-blue-derived DNA suggesting that a different methylation pattern is recognized. CC3 and CC4 strains were very difficult to

transform (frequency 0.1 x 10^1 to 1 x 10^1) which indicates that induction of competence might also be a problem. DNA from N920143 improved the transformation frequency slightly suggesting that *E. coli* XL1-Blue derived DNA was also being restricted. However, using DNA from *E. coli* DC10B did not improve the transformation frequency (data not shown) showing recognition of cytosine methylation is not involved.

***S. lugdunensis* sortaseA mutant.** In *S. aureus* cell wall-anchored proteins play a crucial role in the development of experimental endocarditis (Moreillon *et al.*, 1995; Que *et al.*, 2001; Que *et al.*, 2005). Mutants defective in sortase A are severely attenuated in several animal models since the cell wall-anchored proteins are mislocalized (Weiss *et al.*, 2004). It was anticipated that cell wall-anchored proteins would play a major role in *S. lugdunensis* endocarditis. To investigate this, the *srtA* gene was deleted by allelic exchange. In addition, the *ΔsrtA* mutation was reverted to wild-type by the same procedure (Fig. S2).

The phenotype associated with the *srtA* mutation was investigated by studying the localization of IsdB, a protein that is known to be anchored to the cell wall and which contains a SrtA cleavage motif (Zapotoczna *et al.*, 2012a). In the wild-type strain an immunoreactive band of 70 kDa corresponding to IsdB was found exclusively in the wall fraction consistent with proper sorting. Furthermore, a protein of 27 kDa corresponding to SrtA was detected in the membrane fraction (Fig. 4). This band was missing in the Δ*srtA* mutant and the IsdB protein was mislocalised to the membrane fraction. The IsdB protein in the Δ*srtA* mutant was slightly larger, most likely due to retention of residues C-terminal to the LPXTG sorting signal. In the SrtA-R strain with the sortase mutation reversed IsdB and SrtA expression was the same as in the wild-type (Fig. 4).

***S. lugdunensis srtA* mutant in the endocarditis model.** To assess the contribution of wall-anchored surface proteins to the pathogenesis of *S. lugdunensis* endocarditis, we tested the *srtA* mutant in the rat endocarditis model. A statistically significant reduction in the number of bacteria in the blood stream (Fig. 5(a)), kidneys (Fig. 5 (b)) and cardiac vegetations (Figure 5(c)) was observed in rats infected with the Δ*srtA* mutant compared to those infected with the wild-type strain. It is important to mention that infected animals could be divided into two groups: (i) those where vegetations were of similar size and had similar bacterial densities as the wild-type, or (ii) those where vegetations were not infected. Six out of nine rats (67%) infected with the Δ*srtA* mutant did not develop vegetations. In contrast, all rats infected with the *S. lugdunensis* wild-type strain developed endocarditis. These results demonstrate the requirement for LPXTG-anchored proteins in the pathogenesis of *S. lugdunensis* endocarditis.

Two proteins that might act as virulence factors in the IE model are Fbl and vWbl. The former is an MSCRAMM that is closely related to ClfA while the later binds to a von Willbrand factor which might indicate a role in IE. Deletion mutations were isolated and tested in the IE model. The absence of Fbl in the Δ*fbl* mutant was verified by dot immunoblotting and by bacterial adherence to immobilized fibrinogen (Fig. S3). Neither mutant exhibited a significant reduction in virulence as measured by viable counts of bacteria in the blood, kidneys or vegetations although a trend towards a reduction was noted (Fig. S4). The number of sterile vegetations was intermediate between the wild-type and the *srtA* mutant which is also indicative of a role in disease.

Discussion

Several case studies have described *S. lugdunensis* as an important opportunistic pathogen that is associated with severe cases of IE in humans (Carpenter *et al.*, 2012;

Cevasco & Haime, 2012; Chung *et al.*, 2012; Sibal *et al.*, 2011; Stair *et al.*, 2012). Although infections are infrequent, it is remarkable that *S. lugdunensis* is associated with native valve endocarditis showing fulminant and a highly destructive clinical course. This distinguishes *S. lugdunensis* from the other CoNS which are normally primarily associated with prosthetic valve endocarditis (Frank *et al.*, 2008; Huebner & Goldmann, 1999). Although *S. lugdunensis* is recognized as an important pathogen, hypothesis-driven research to identify virulence factors has been neglected.

The development of a quick and effective method for the electroporation of the *S. lugdunensis* clinical isolate N920143 described in this study will simplify any future experiments regarding this organism. The eletroporation protocol allowed the convenient transformation of *S. lugdunensis* strains from CC1 / CC2 and CC5 with DNA derived from commonly used *E. coli* strains. CC3 and CC4 isolates were much harder to transform. Nevertheless sporadic transformants could be isolated in all strains. Interestingly, none of the *S. lugdunensis* strains recognizes cytosine methylated DNA as indicated by the observation that DNA isolated from *E. coli* DC10B did not improve transformation efficiency. This distinguishes *S. lugdunensis* from *S. aureus* and *S. epidermidis* (Monk *et al.*, 2012)

The only restriction barrier in *S. lugdunensis* appears to be the type I system SluI that was identified here. Deletion of *hsdR* from the chromosome of N920143 improved the efficiency of transformation with *E. coli* XL1-blue derived DNA 100-fold to reach the same level achieved with *S. lugdunensis* derived DNA. This suggests that SluI represents the only R-M system in N920143. We found that DNA from *S. lugdunensis* N920143 wild-type or the *ΔhsdR* mutant allowed 10-100 fold higher transformation of strains from CC1 and CC2. It can be assumed that DNA isolated from the *ΔhsdR* mutant N920143 will allow efficient transformation of the CC1 and CC2 strains. CC5 strains could be transformed using *E. coli* derived DNA with low efficiency. However, DNA isolated from CC1 *S. lugdunensis* did not

improve the transformation efficiency, suggesting that a different methylation pattern is recognized. Strains from CC3 and CC4 were very difficult to transform. An additional restriction systems might be present and the cell envelope might impede electroporation.

We adapted the protocol for allelic exchange developed in *S. aureus* using pIMAY to *S. lugdunensis*. A particular advantage of pIMAY is that plasmid integration is selected at the optimal growth temperature of 37 °C rather than the stress-inducing 42-44 °C needed for earlier vectors (Bae & Schneewind, 2006), with the additional risk of selecting secondary mutations. We were also able to reverse a deletion mutation in *srtA* by allelic exchange. This has several advantages over plasmid-based complementation. The revertant is stable in the absence of antibiotic selection needed to maintain a plasmid and the gene is likely to be regulated in the same way as the wild-type.

For the first time, we have investigated the virulence of a clinical isolate of *S. lugdunensis* in a rat endocarditis model and compared it to two strains of *S. aureus*. Interestingly, *S. lugdunensis* did not display an elevated degree of virulence in our experiments. In contrast, rats infected with *S. lugdunensis* survived longer and displayed lower levels of bacteraemia than *S. aureus*-infected rats. In general, the level of bacteremia is regarded to be a good indication for the severity of endocarditis, since bacteria are constantly released from the vegetation into the blood stream. However, reduced bacteraemia and mortality in *S. lugdunensis*-infected rats could reflect differences in the clearance of bacteria from the blood stream rather than differences in the endocardial infections. Regarding immune evasion, a more effective clearance of *S. lugdunensis* seems a likely possibility. The analysis of two genome sequences has given some insight into the virulence potential of this species (Heilbronner *et al.*, 2011) and genes encoding orthologues of *S. aureus* toxins and immune evasion factors were not detected. Altogether the virulence potential of *S. lugdunensis* compared to *S. aureus* seems to be limited.

Regarding size and density of the vegetations formed, *S. lugdunensis* showed similar characteristics to *S. aureus* COL. However, *S. aureus* Newman caused significantly bigger vegetations. It would be interesting to compare several other clinical isolates of *S. lugdunensis* with *S. aureus* to draw conclusions about virulence of *S. lugdunensis* in this model. In addition, it has to be considered that the rat endocarditis model might not be ideally suited for this organism. *S. lugdunensis* is the only CoNS with an iron responsive surface determinant locus (Isd), encoding wall-anchored heme-binding proteins, membrane transporters and a heme degrading monoxygenase (Heilbronner *et al.*, 2011). In *S. aureus*, this locus permits the utilization of haemoglobin as a source of nutrient iron. Recent studies by Haley *et. al*. (Haley *et al.*, 2011) and Zapotoczna *et al*. (Zapotoczna *et al.*, 2012b) showed that the Isd system in *S. lugdunensis* is active and functions in a similar fashion to that of *S. aureus*. However, the *S. lugdunensis* IsdB protein specifically binds human haemoglobin and has a low affinity for rodent version (Zapotoczna *et al*., 2012a). As a result, *S. lugdunensis* cannot use mouse haemoglobin as a source of nutrient iron (Pishchany *et al.*, 2010; Zapotoczna *et al.*, 2012b). This might also be true for rat haemoglobin as well, since the α- and β-chains of mouse and rat haemoglobin show >84% sequence identity. In contrast the *S. aureus* Isd system is capable of using mouse haemoglobin, although not as efficiently as the human variant (Pishchany *et al.*, 2010). This adaption to the human host might help to explain the rather low virulence of *S. lugdunensis* in the rat infection model.

The importance of LPXTG-anchored proteins as virulence factors has been widely recognized (Que *et al.*, 2001; Que *et al.*, 2005). The expression of the *S. aureus* MSCRAMM ClfA by *Lactococcus lactis* leads to increased virulence in an experimental rat endocarditis model (Moreillon *et al.*, 1995). Mutants of *S. aureus* deficient in sortase A displayed strongly reduced virulence in various infection models including experimental sepsis and infectious endocarditis, highlighting the importance of properly displayed MSCRAMMs during

infection (Jonsson *et al.*, 2003; Weiss *et al.*, 2004). *srtA* mutants were recently discussed as vaccine candidates due to the attenuated but still immune-stimulating phenotype (Kim *et al.*, 2011). Here the *srtA* mutant of *S. lugdunensis* showed significant defects in virulence including reduced bacteraemia, reduced bacterial spreading to the kidneys and reduced size/density of endocardial vegetations. This highlights the importance of LPXTG-anchored proteins in pathogenesis. Mutants defective in individual surface proteins Fbl and vWbl were not significantly less virulent than the wild-type. However trends towards reduced virulence were observed for both mutants. An interesting observation is that the development of endocardial vegetations appears to be an all-or-nothing phenomenon. Either the infected rats developed vegetations of similar size and density to the wild-type, or they did not infect the vegetation detectably. This was particularly noticeable with the Δ*srtA* mutant where 66% of rats did not develop endocardial vegetations. 33% of rats infected with Δ*vWbl* and Δ*fbl* failed to establish a thrombus on the heart valve while not a single rat maintained sterile vegetations ($>10^{-3}$ CFU) when infected with the wild-type strain. This suggests that several surface proteins act in concert to promote adhesion to the thrombus and possibly survival in the bloodstream. However, as soon as the thrombus on the heart valve is formed, surface proteins might only play a minor role and size and density develops independently.

Knowledge of the function of *S. lugdunensis* surface proteins is limited. Apart from fibrinogen for Fbl, von Willebrand factor for vWbl and haem/haemoglobin for Isd proteins, no ligands have been identified for the remaining proteins. More experiments are needed to identify their ligands and to understand their roles in the colonization of the host or in the development of disease.

Acknowlegements

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Figures and tables:

Fig. 1: Virulence of *S. lugdunensis* compared to *S. aureus* in the catheter-induced rat endocarditis model. After placement of the catheter, rats were challenged with *S. aureus* or *S. lugdunensis*. The level of bacteraemia was monitored every day. Rats were sacrificed after 3 days (*S. aureus*) or 4 days (*S. lugdunensis*) and CFU in endocardial vegetation and kidneys were determined.

(a) CFU per ml blood up to day 4 after infection. (b) CFU in kidneys, (c) CFU in endocardial vegetations. Total CFU counts per vegetation (CFU/vegetation) and bacterial densities (CFU/g) were compared to *S. aureus* Newman and to *S. aureus* COL.

Fig. 2: Transformation frequency of *S. lugdunensis*. Strain N920143 and the isogenic Δ*hsdR* mutant were transformed with plasmid pRMC2 isolated from *E. coli* XL1-blue Dcm^+, *E. coli* DC10B Dcm^- *or S. lugdunensis* N920143. The number of transformants per µg plasmid DNA is recorded.

Fig. 3: Transformation frequency of *S. lugdunensis* strains from different clonal complexes. Strains were transformed with plasmid pRMC2 from *E. coli* XL1-blue or *S. lugdunensis* N920143. The number of transformants per µg plasmid DNA is recorded.

Fig. 4: Western immunoblotting. (a) Detection of IsdB using rabbit anti-IsdB IgG in cell fractions of *S. lugdunensis* grown in RPMI. (b) Detection of SrtA using rabbit anti-SrtA in cell fractions of *S. lugdunensis* grown in TSB. Binding of primary antibodies was detected using goat anti-rabbit IgG-HRP. CW – cell wall fraction, mem – membrane fraction.

Figure 5: Virulence of the *S. lugdunensis* wild-type and *ΔsrtA* in the rat endocarditis model. After placement of the catheter, rats were challenged with the *S. lugdunensis* strains. The level of bacteremia was monitored over 4 days. Rats were sacrificed after 4 days and CFU in endocardial vegetation and kidneys were determined. (a) CFU per ml blood up to day 4 after infection. (b) CFU in kidneys after 4 days. (c) CFU in *S. lugdunensis* endocardial vegetations after 4 days

Supplementary Figures:

Fig. S1: Schematic diagrams of the fibrinogen binding protein Fbl and the von Willebrand Factor binding protein vWbl. Domains are indicated as grey boxes. S – Signal sequence, SDSDSA- Serine-Aspartate-Serine-Aspartate-Alanine, RGD-Arginine-Glycine-Aspartate. See text for functions of the domains. The horizontal arrow indicates the region expressed as recombinant protein used for antibody production.

Fig. S2: Deletion and reversion constructs: (a) schematic diagrams of the constructed deletion and reversion cassettes in pIMAY. Integration and subsequent excision of the thermosensitive plasmid over the homologous sites AB and CD, respectively, allows the exchange of the chromosomal and plasmid DNA (Monk *et al.*, 2012). Arrows indicate the location of screening primers to confirm the successful exchange of cassettes after excision. The novel restriction site in the reversion constructs allows discrimination of wild-type and reversion strains when a gene was deleted and subsequently restored.

(b) Confirmation of *srtA* wild-type, deletion mutant and reversion strains.

PCR analysis of wild-type, Δ*srtA* and *srtA*-reversion (srtA-R) strains using screening primers as indicated in Fig. S2 (a). Amplimers were digested with SmaI as indicated. Only the *srtA-R*

amplimer was cleaved by SmaI due to the introduction of the recognition sequence during the reversion process.

Fig. S3: Expression of Fbl. (a) Whole cell immuno dot blot for Fbl expression. *S. lugdunensis* stationary phase cells were adjusted to OD_{600} = 1 and 5 µl of the suspension was dotted on a nitrocellulose membrane. Fbl was detected with rabbit anti-Fbl IgG followed by goat anti-rabbit-IgG conjugated to HRP.
(b) Adherence of *S. lugdunensis* to immobilized fibrinogen. *S. lugdunensis* cells (100 µl of OD_{600} = 1) were added to wells of microtiter plates coated with human fibrinogen. Adhering cells were stained with crystal violet and the absorbance was measured in an ELISA plate reader at 570 nm. Values represent the mean of triplicate wells. The experiment was carried out three times with similar results.

Fig. S4: Virulence of *S. lugdunensis Δfbl* and *Δvwbl* mutants in the rat endocarditis model. After placement of the catheter, rats were challenged with the *S. lugdunensis* strains. The level of bacteraemia was monitored over 4 days. Rats were sacrificed after 4 days and CFU in endocardial vegetation and kidneys were determined. (a) CFU per ml blood up to day 4 after infection. (b) CFU in kidneys after 4 days. (c) CFU in *S. lugdunensis* endocardial vegetations after 4 days

707 **Table 1**: Bacterial strains and plasmids

Bacterial Strain / Plasmid	Description	Reference / Source
Staphylococci		
S. lugdunensis N920143	Human clinical isolate, genome sequenced, CC1	(Heilbronner *et al.*, 2011)
S. lugdunensis Δ*fbl*	Deletion of *fbl*	This study
S. lugdunensis Δ*vWbl*	Deletion of *vWbl*	This study
S. lugdunensis Δ*srtA*	Deletion of *srtA*	This study
S. lugdunensis Δ*srtA-R*	Reverted *srtA* deletion with novel SmaI site	This study
S. lugdunensis HKU09-01	Clinical isolate genome sequenced CC1	(Tse *et al.*, 2010)
S. lugdunensis SL2	Clinical isolate CC2	(Chassaîn *et al.*, 2012)
S. lugdunensis SL9	Clinical isolate CC5	(Chassaîn *et al.*, 2012)
S. lugdunensis SL13	Clinical isolate CC1	(Chassaîn *et al.*, 2012)
S. lugdunensis SL27	Clinical isolate CC4	(Chassaîn *et al.*, 2012)
S. lugdunensis SL37	Clinical isolate CC3	(Chassaîn *et al.*, 2012)
S. lugdunensis SL57	Clinical isolate CC3	(Chassaîn *et al.*, 2012)
S. lugdunensis SL62	Clinical isolate CC4	(Chassaîn *et al.*, 2012)
S. lugdunensis SL71	Clinical isolate CC2	(Chassaîn *et al.*, 2012)
S. lugdunensis SL72	Clinical isolate CC1	(Chassaîn *et al.*, 2012)
S. lugdunensis SL81	Clinical isolate CC5	(Chassaîn *et al.*, 2012)
S. aureus Newman	Human clinical isolate, MSSA, genome sequenced	(Baba *et al.*, 2008)
S. aureus COL	Human clinical isolate, MRSA, genome sequenced	(Gill *et al.*, 2005)
E.coli		
DH10B	*dam* $^{+}$ *dcm* $^{+}$ Δ*hsdRMS endA1 recA1* high efficiency cloning strain	Invitrogen
Plasmids		
pIMAY	Thermosensitive vector for allelic exchange	(Monk *et al.*, 2012)
pIMAY-Δ*hsdR*	A deletion encompassing the entire *fbl* gene (form ATG to TAA codons) amplified from N920143	This study
pIMAY-Δ*fbl*	A deletion encompassing the entire *fbl* gene (form ATG to TAA codons) amplified from N920143	This study
pIMAY-Δ*vwbl*	A deletion encompassing the entire *vwbl* gene (form ATG to TAA codons) amplified from N920143	This study
pIMAY-Δ*srtA*	A deletion encompassing the entire *srtA* gene (form ATG to TAA codons) amplified from N920143	This study
pIMAY-*srtA-R*	A reversion fragment containing the entire *srtA* gene with a novel SmaI restriction site amplified from	This study

pRMC2	N920143 *E. coli-S. aureus* shuttle vector	(Corrigan & Foster, 2009)

Table 2: Oligonucleotides used in this study

Primer	5'-3' Sequence	Restriction site
HsdR-A	TGTTGAGCTCTTATTAAAGATCAAAAATTATGAAATTCCG	SacI
HsdR-B	CATCAAATCACCCAAAAATTAGTAGTTTCTTTAAATATAGCAC	
HsdR-C	CTAATTTTTGGGTGATTTGATGTAAATAAGTTAGGCGGCATACC	
HsdR-D	GAATGAATTCATCTTCACTGTCATGGCCTCGGG	EcoRI
HsdR-Sc. F	GAACTTGTCGTAAAGATATAGAAGATTTGAATAG	
HsdR-Sc. R	ATTAAATATTCATACGCATCGCCTAACATATC	
Fbl – A	CAATTGAAGGAGCTCTTGGAGGATTATTTAGC	SacI
Fbl – B	CATTTAATCTCTCCTTTGATTGATATGATTATGCCC	
Fbl – C	CAATCAAAGGAGAGATTAAATGTAAAAGATAGTAAGATGGAAATGTTC	
Fbl – D	CACCTCTATAATTTATTTGAATTCATGCTGAAAATC	EcoRI
Fbl – OutF	TACAGATACAGGTGCATATATTTTTGGG	
Fbl – OutR	CTCCAAATACGATAGCAAATGATACAACTG	
vWbl – A	CTGTAATGAGCTCATTAAGAAAATTAGCACC	SacI
vWbl – B	CAATGGGTTCTCTCTCCTTAATTGAAATTATTAAG	
vWbl – C	AAGGAGAGAGAACCCATTGTAATATAGCAATACACGTCGAG	
vWbl – D	GTAAAATGAATTCAATAGCAAATTGATTATATACTAAAACC	EcoRI
vWbl – OutF	AATACATATCTCTATGTTCATGAATTGAGG	
vWbl – OutR	CAAAATCTATCTCAACTAATTCAACAATACC	
SrtA – A	TGTACGAGAGCTCTCATCTTTAGCAATTTG	SacI
SrtA – B	CATGCAGTATTTCTCCTTTAAACCGTAAAA	
SrtA – C	AAAGGAGAAATACTGCATGTAATTGTAGAACACTTTGATCCG	
SrtA – D	CCTCTGTAGTAGGGAATTCTTTATCTTGCT	EcoRI
SrtA – E	GGTGTTGCTGGTCCCGGGTAAACAGGTTC	SmaI
SrtA – F	GAACCTGTTTACCCGGGACCAGCAACACC	SmaI
SrtA – OutF	CCTGCATGAATAAAACCAATTTTTTCGTG	
SrtA – OutR	GATTTTGCTCTTTCTGTGGTGCTACGTGC	
vWbl t1200-F	CTAATTCTAGATCTCATACTGCAGAGATA	BglII
vWbl t1200-R	ATTAAGTCGACCTATTTAGTTTGACCTTT	SalI
HsdR-F	TTAAAATTATAGAACTTTCCTTCTAAATATTGTGC	
HsdR-seqF	ATCGTTGTCGCATTGCTAAGAT	
HsdR-R	TATCCTCCTCTGTTCATCGTTTGTAT	

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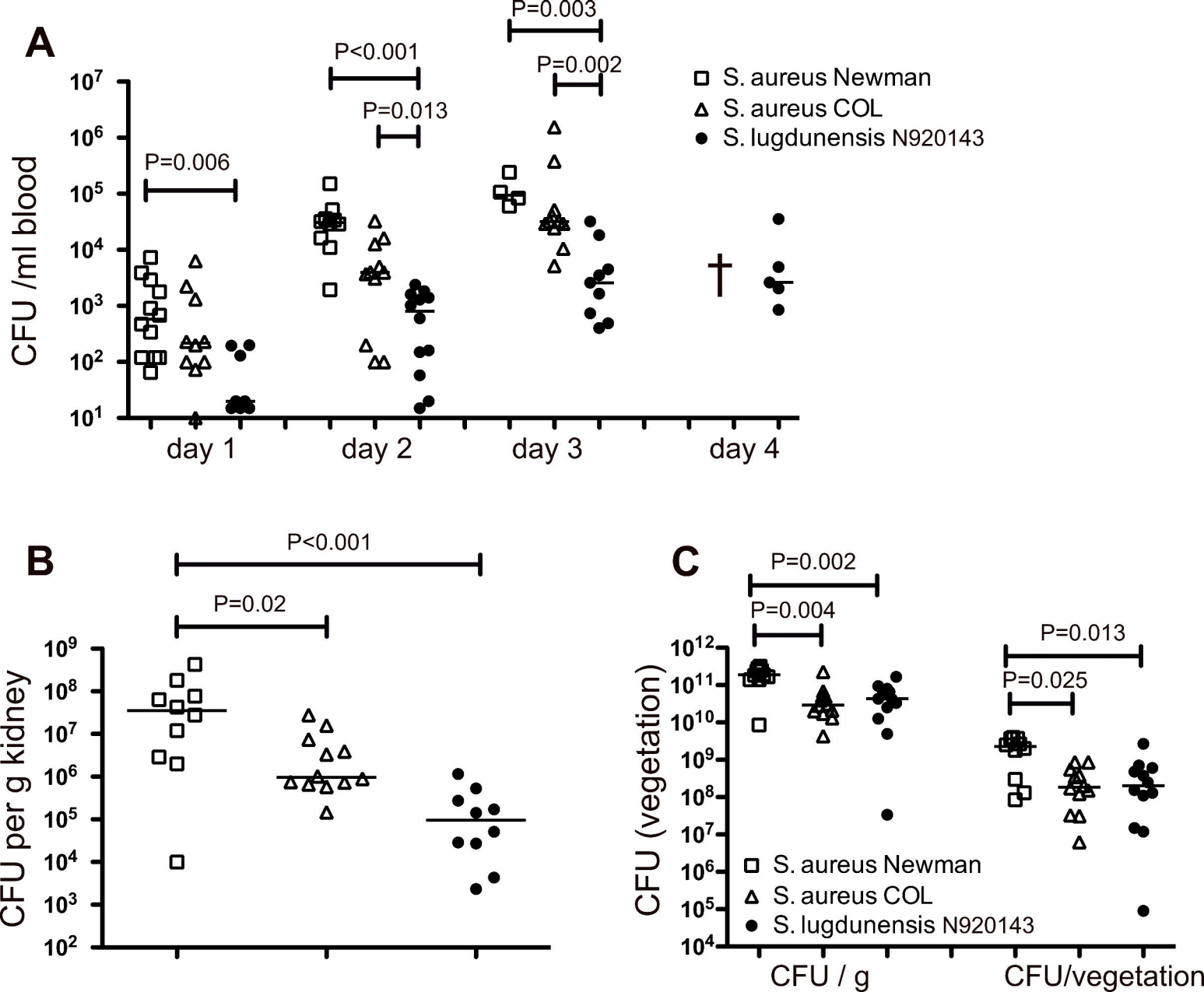
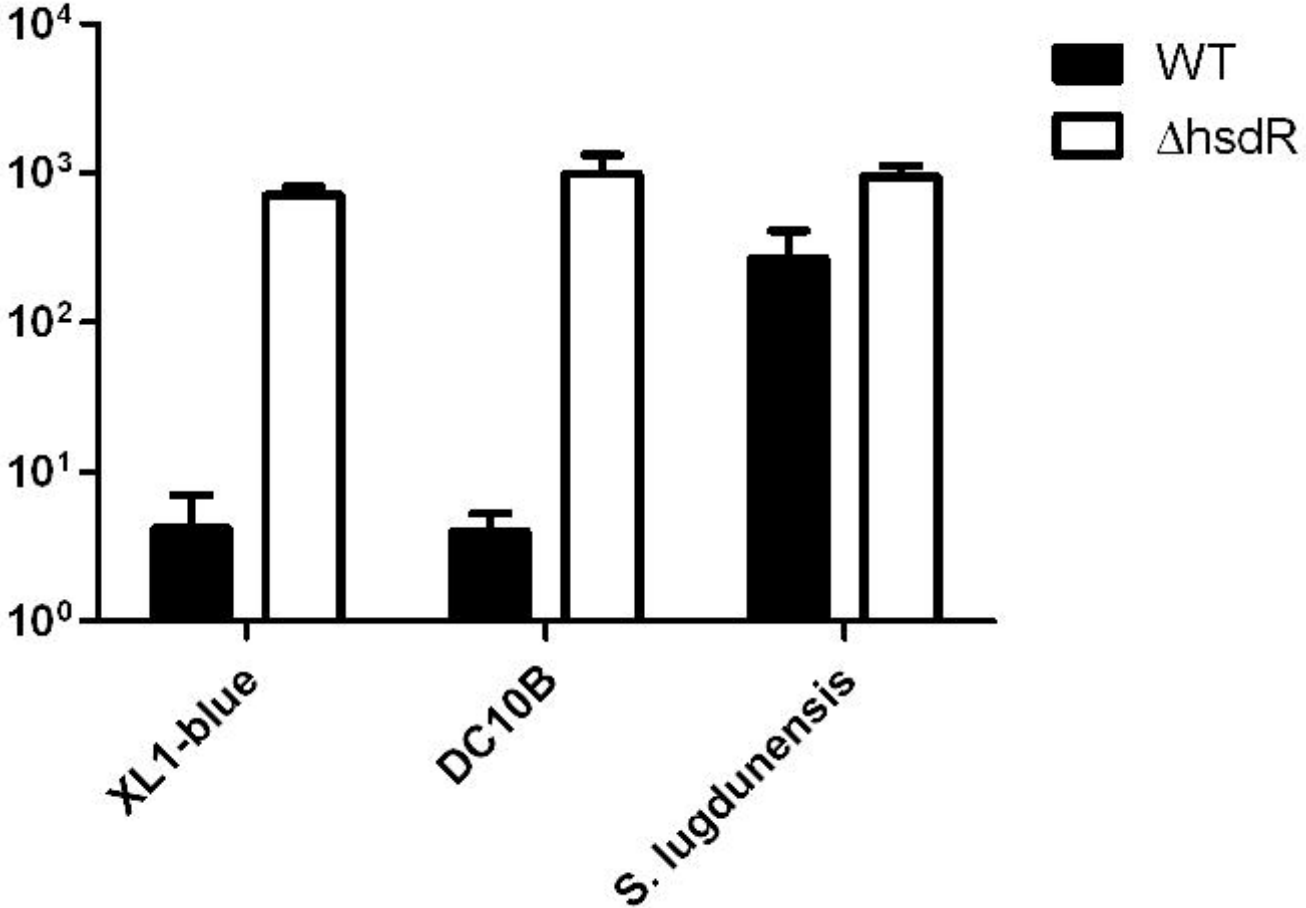
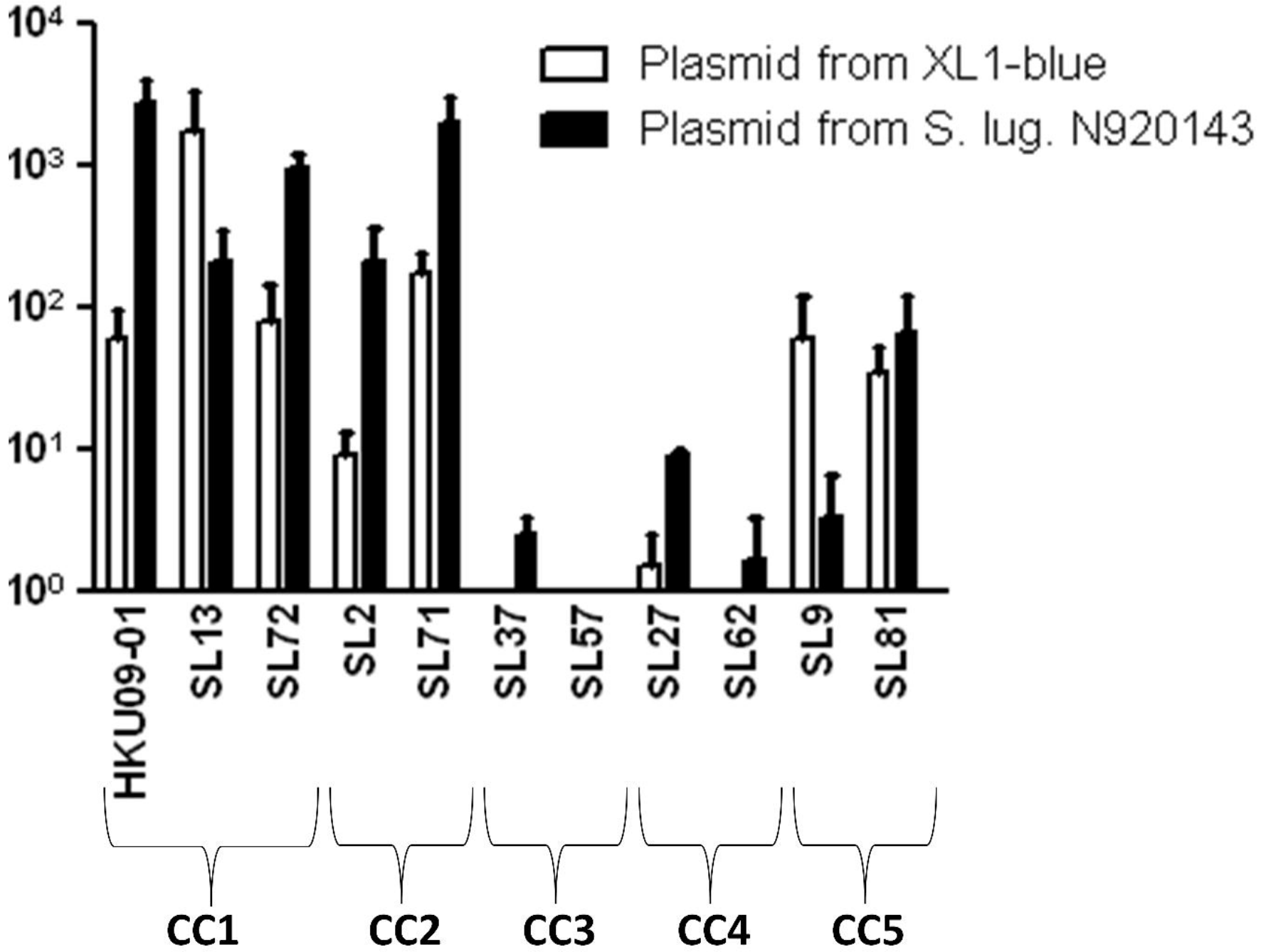
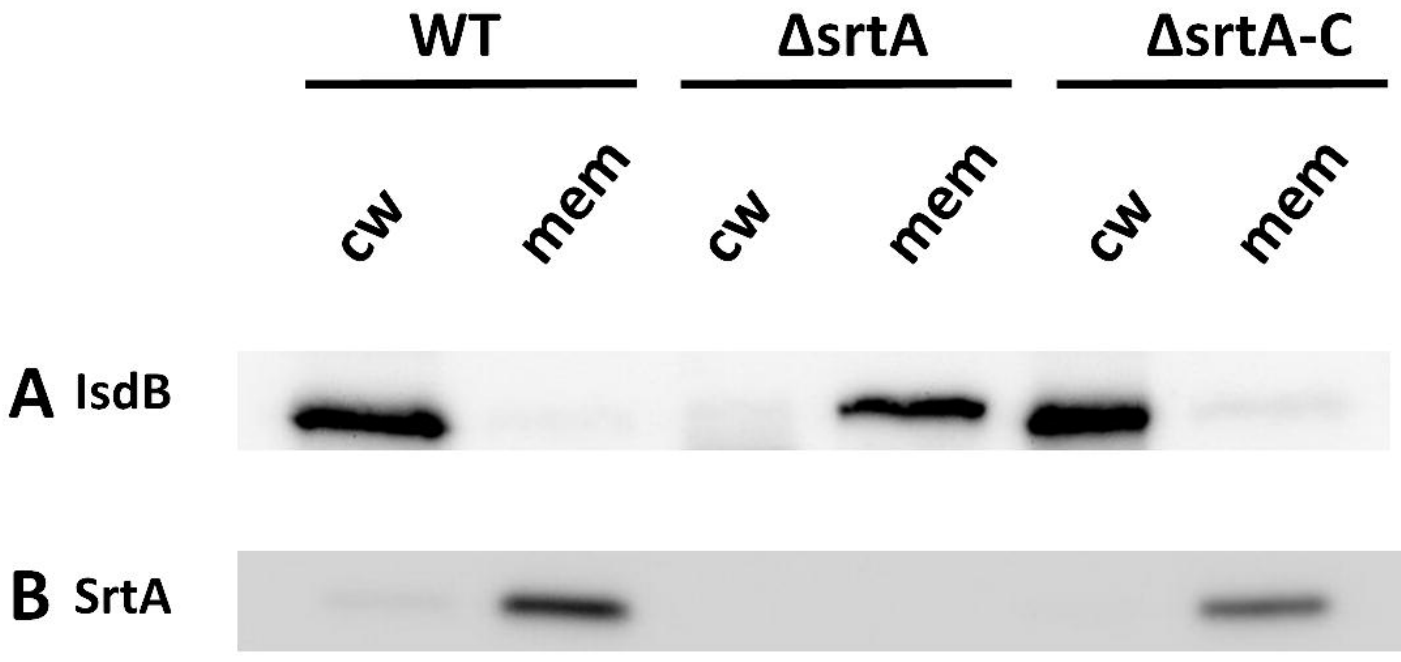
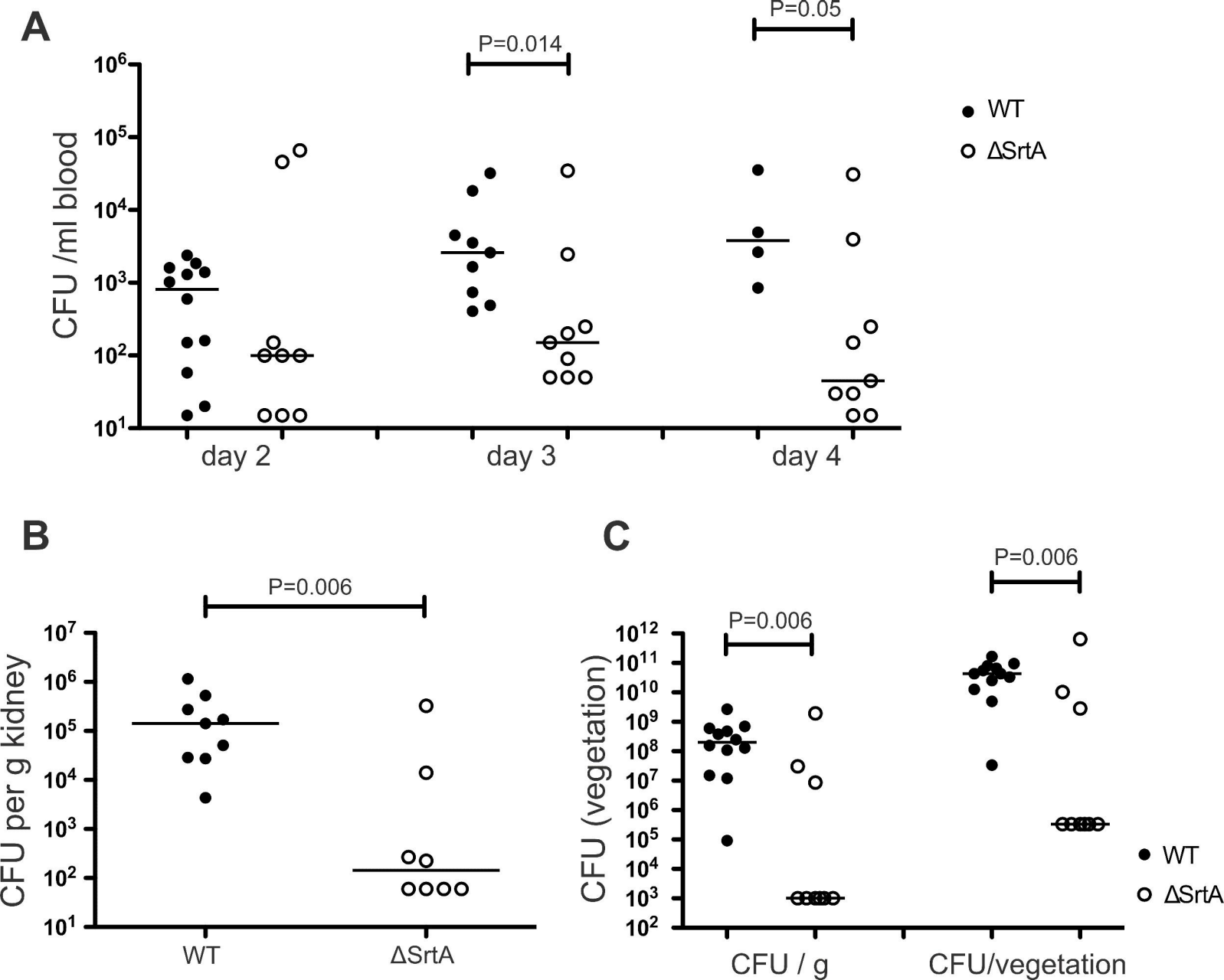

A
CFU /ml blood
10^7
10^6
10^5
10^4
10^3
10^2
10^1
P=0.006
P<0.001
P=0.013
P=0.003
P=0.002
day 1
day 2
day 3
day 4
S. aureus Newman
S. aureus COL
S. lugdunensis N920143
B
CFU per g kidney
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
P<0.001
P=0.02
C
CFU (vegetation)
10^{12}
10^{11}
10^{10}
10^9
10^8
10^7
10^6
10^5
10^4
P=0.002
P=0.004
P=0.013
P=0.025
S. aureus Newman
S. aureus COL
S. lugdunensis N920143
CFU / g
CFU/vegetation


10^4
10^3
10^2
10^1
10^0
WT
ΔhsdR
XL1-blue
DC10B
S. lugdunensis


Plasmid from XL1-blue
Plasmid from S. lug. N920143
10⁴
10³
10²
10¹
10⁰
HKU09-01
SL13
SL72
SL2
SL71
SL37
SL57
SL27
SL62
SL9
SL81
CC1
CC2
CC3
CC4
CC5


WT
ΔsrtA
ΔsrtA-C
cw
mem
cw
mem
cw
mem
A IsdB
B SrtA


A
P=0.014
P=0.05
CFU /ml blood
10^6
10^5
10^4
10^3
10^2
10^1
day 2
day 3
day 4
WT
ΔSrtA
B
P=0.006
CFU per g kidney
10^7
10^6
10^5
10^4
10^3
10^2
10^1
WT
ΔSrtA
C
P=0.006
P=0.006
CFU (vegetation)
10^{12}
10^{11}
10^{10}
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
CFU / g
CFU/vegetation
WT
ΔSrtA