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MICROARRAY BASED STUDY ON VIRULENCE-ASSOCIATED GENES AND RESISTANCE DETERMINANTS OF *STAPHYLOCOCCUS AUREUS* ISOLATES FROM CATTLE

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1 ABSTRACT

2

3 *Staphylococcus aureus* is a common pathogen which can colonise and infect not only man,
4 but also domestic animals. Especially, infection of cattle is of high economic relevance as *S.*
5 *aureus* is an important causal agent of bovine mastitis. In the present contribution, a DNA
6 microarray was applied for the study of 144 different gene targets, including resistance
7 genes and genes encoding exotoxins, in *S. aureus* isolated from cows. 128 isolates from
8 Germany and Switzerland were tested. These isolates were assigned to 20 different strains
9 and nine clonal complexes. The majority of isolates belonged either to apparently closely
10 related clonal complexes 8, 25, and 97 (together 34.4 %) or were related to the sequenced
11 bovine strain RF122 (48.4 %). Notable characteristics of *S. aureus* of bovine origin are the
12 carriage of intact haemolysin beta (in 82 % of isolates tested), the absence of staphylokinase
13 (in 89.1 %), the presence of allelic variants of several exotoxins such as toxic shock
14 syndrome toxin and enterotoxin N, and the occurrence of the leucocidin *lukF-P83/lukM* (in
15 53.1 %). Two isolates were methicillin-resistant *S. aureus* (MRSA). One of them was a
16 clonal complex 8 MRSA related to the epidemic MRSA strain Irish 01. The other one
17 belonged to ST398/*spa*-type 34 resembling a newly emerging MRSA strain which has been
18 described to occur in humans as well as in domestic animals. The presence of these two
19 strains highlights the possibility of transfers of *S. aureus* strains between different host
20 species.

1 **INTRODUCTION**

2

3 *Staphylococcus aureus* can cause a variety of pyogenic infections and toxin-mediated conditions.
4 It harbours a complex system of exotoxins which facilitates the killing of phagocytes, lysis of
5 erythrocytes, or manipulation of the T-cell response (Dinges et al., 2000). Many *S. aureus*
6 isolates carry multiple exotoxins, and there is a high redundancy within classes of exotoxins.
7 There are, at least, four distinct bicomponent leukocidins, approximately twenty different
8 superantigenic toxins (TST and enterotoxins), three exfoliative toxins, and six haemolysins. This
9 diversity could be a reason for the ability of *S. aureus* to colonise or to infect different host
10 species. Infection of cattle is of high economic relevance as *S. aureus* is an important agent of
11 bovine mastitis (Akineden et al., 2001; Nickerson et al., 1995; Rainard et al., 2003; Zschock et
12 al., 2005). The knowledge of possible host specificities of certain virulence factors could be
13 important for the understanding of the pathogenesis and for vaccine development.

14 Antimicrobial resistance of *S. aureus* is an increasingly important problem. The most important
15 issue is resistance towards methicillin and all beta-lactam antibiotics (methicillin resistant *S.*
16 *aureus*, or MRSA). There are reports of veterinary hospitals suffering from MRSA epidemics
17 (Seguin et al., 1999), and it has also been demonstrated that pets can become silent carriers of
18 MRSA (Manian, 2003). The emerging epidemic of virulent, Panton-Valentine leucocidin
19 carrying MRSA in humans appears to be mirrored by clinically similar infections in pets (Rankin
20 et al., 2005).

21 For these reasons, research on *S. aureus* in veterinary medicine should, like in human medicine,
22 focus on antimicrobial resistance and on the issue of exotoxins. In the present contribution, a
23 diagnostic DNA microarray based assay was applied for the study of resistance genes and genes
24 encoding exotoxins in *S. aureus* isolated from cows.

1 MATERIALS AND METHODS

2

3 **Isolates and DNA preparation**

4 128 bovine *S. aureus* isolates were tested. Fifty of them were obtained by the Institute of
5 Veterinary Bacteriology in Bern, Switzerland, in order to diagnose cases of bovine mastitis.
6 Another 77 were sampled as a part of routine surveillance activities by the Animal Health
7 Service of Thuringia and were sub-cultivated by the Friedrich Loeffler Institute (FLI), Germany.
8 One isolate was found by local authorities in a case of septic abort in Thuringia. All isolates were
9 cultured on Columbia blood broth (OXOID, Wesel, Germany) and incubated 12 to 48 hours at 37
10 °C. Single colonies were picked for further sub-culturing. Genomic DNA was prepared after
11 enzymatic lysis using 100 µl of a lysis solution containing 0.05 mg lysostaphin (AMBI Products,
12 Lawrence, USA), 2 mg lysozyme (Sigma, Steinheim, Germany), 2 mg ribonuclease A (Sigma), 2
13 µl TRIS/HCl (20 mM, pH 8.0), 2 µl EDTA (2 mM), and 1 µl Triton X-100. After incubation (45
14 min, 37 °C, 300 rpm), 10 µl proteinase K and 100 µl of buffer AL (both from Qiagen kit
15 DNeasy™, Qiagen, Hilden, Germany) were added. After a second incubation (45 min at 56 °C)
16 the sample was processed using the Qiagen device EZ1 according to the tissue lysis protocol
17 supplied by the manufacturer.

18

19 **Amplification, Labelling, and Array hybridisations**

20 Diagnostic DNA microarrays based on the array-tube platform (CLONDIAG chip technologies,
21 Jena, Germany) were used for genotyping of staphylococcal DNA. These microarrays were
22 manufactured and processed as described previously described (Korczak et al., 2005; Monecke
23 and Ehricht, 2005; Monecke et al., 2003). A set of probes covering species markers of *S. aureus*,

1 antibiotic resistance determinants and toxins, as well as probes for the discrimination of allelic
2 variants of the accessory gene regulator (*agr*) cluster has already been described and evaluated
3 (Monecke et al., 2007; Monecke and Ehricht, 2005). The array comprised the probes listed in
4 Table S1 (online supplement) in twofold redundancy. The set of probes included all sequences
5 which were available at the time of completion of bio-informatic work-up (early 2005). RF122
6 genome sequence data (GenBank NC_007622.1) were not covered as they were released only
7 after the completion of the array.

8 Genomic target DNA was amplified in a linear manner and labelled using a multiplex primer
9 elongation reaction incorporating biotin-16-dUTP (Roche, Penzberg, Germany), which has been
10 described previously in detail (Monecke et al., 2007; Monecke and Ehricht, 2005). Primers are
11 listed in Table S1 (online supplement). Biotinylated single stranded amplicons were subsequently
12 hybridised to the array and hybridisation was visualised using streptavidin-horseradish-
13 peroxidase and a precipitating chromogenic substrate (Seramun green, Seramun, Woizig,
14 Germany). The array tube reader ATR01 and the Iconoclust software package (CLONDIAG chip
15 technologies) were used combined with a defined script according to manufacturers
16 recommendations and to the procedure described in (Monecke et al., 2007; Monecke and Ehricht,
17 2005) in order to record and to analyse hybridisation patterns.

18

19 **Sequencing**

20 PCR amplification and sequencing were performed when ambiguous hybridisation results, the
21 presence of only one component of a bicomponent toxin, or the absence of a toxin from a
22 previously known pathogenicity island were observed.

23 For detection of the gene encoding toxic shock syndrome toxin (*tstI*) and enterotoxin gene N
24 (*sen*) following primers were used respectively: *tstI*-fw 5'-

1 ACGTTTACACATTTGAATGAAGG-3' and *tstI*-rv 5'-
 2 CGTTATAAAGATAAAAGGGAGAACG-3', *sen*-fw 5'-
 3 TCTATTAATGTAGAAGTTCATTTAACC-3' and *sen*-rv 5'-
 4 TCATGATTCAGTTGATAATCATCA-3'.

5 Primers for *lukE* were *lukE*-fw 5'-AAGTGAAATAATCTAGAATAAATTTGT-3' and *lukE*-rv
 6 5'- GTATTCGATAGCAAAAGCAG-3', and *splA* was amplified using primers *splA*-fw 5'-
 7 TGTATATAGTAAATGTGTATCTAGA-3' and *splA*-rv 5'-
 8 TGATATCTAGATACATAATTCAGT-3'.

9 PCR was performed using polymerase (1 µl per 100 µl reaction mixture) and 10x PCR-buffer by
 10 genaxxon Bioscience GmbH (Biberach, Germany) adding 1,5 mM MgCl₂ and 1 µM of each
 11 primer. PCRs were uniformly performed with a denaturation step of 2 min at 95 °C, followed by
 12 35 cycles á 30 sec at 95 °C, 20 sec at 55 °C, and 45 sec at 72 °C, and by a final extension period
 13 of 5 min at 72 °C.

14 The amplicons were inserted into a commercially available cloning vector (pCR2.1-topo,
 15 Invitrogen), and recombinants were subjected to DNA sequencing using the BigDye Terminator
 16 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Darmstadt, Germany), M13
 17 primers, and a ABI PRISM 310 Genetic Analyser (Applied Biosystems) according to
 18 manufacturers instructions.

1

2 ***Spa*-typing and MLST**

3 *Spa*-typing is based on sequence polymorphisms of repeating units within the staphylococcal
4 protein A gene (*spa*). It was performed according to previously described protocols (Harmsen et
5 al., 2003). Types were assigned using the code system described on Ridom SpaServer website
6 (www.spa.ridom.de), and SpatypeMapper software (CLONDIAG) freely downloadable at
7 <http://www.clondiag.com/technologies/download.php?file=spa>.

8 MLST for selected isolates was performed as described by Enright *et al.* (Enright et al., 2000).

9

10 **Split network tree construction**

11 The tree shown in Figure 5 was constructed using the SplitsTree software (Huson and Bryant,
12 2006) on default setting. Some sequenced or other reference strains were also included for
13 comparison. RF122 and the sequenced isolate of USA300 were not available for testing. For the
14 latter one, hybridisation results of clinical isolates (Monecke et al., 2007) were used instead.
15 Hybridisation results for every single gene (as shown in Figures 1 to 4) were converted into a
16 code (A = Gene present, T = Gene absent, G = Gene yielding variable results within one strain; C
17 = Gene appearing to be present in a deviant allelic variant, i.e., being detectable only in
18 experiments with high DNA load, or by PCR). The resulting “sequence”, which represented the
19 complete hybridisation pattern of a given strain, was used subsequently for tree construction.

1 RESULTS

2

3 Typing and strain characteristics

4 Using microarray analysis, it was possible to assign the 128 isolates included into the study to 20
5 distinct strains belonging to seven clusters or clonal lines. Clusters and strains primarily were
6 distinguishable by hybridisation profile (Figures 1 to 4), by *spa*-typing, MLST, and by
7 comparison of hybridisation profiles to strains of known affiliation.

8 Following strains were discernible.

9 Strain 1 and 2 were *agrI*/clonal complex (CC) 97 strains. One representative isolate was
10 subjected to MLST belonging to ST97. These isolates were very similar to the sequenced strain
11 NCTC8325 (CC8), but genes *ssII*, *ssI3*, and *ssIII*, or respectively, *ssII*, and *ssIII* were absent.

12 The hybridisation profile of Strain 3 (*agrI*/CC8) was practically identical to NCTC8325, its *spa*
13 repeats were similar. Strain 4 was related to Strain 3, carrying the same virulence factors, but it
14 was a MRSA harbouring *mecA*, the tobramycin resistance determinant *aadD*, and the
15 macrolides/clindamycin resistance gene *ermA*. Strains 5 and 6 differed in their carriage of
16 exotoxin genes harbouring respectively *sed+sej+ser* or *sea+sed+sej+ser*.

17 Strains 7 and 8 gave an overall similar hybridisation pattern, but contained the *egc*-enterotoxin
18 gene cluster (*seg+sei+sem+sen+seo+seu/y*), or that cluster plus the bovine leucocidin *lukF*-
19 *P83/lukM*. Strain 8 was subjected to MLST, yielding ST20.

20 Strain 9 belonged to *agr*-group I and *spa*-type t034, and Strain 10 was an otherwise similar
21 MRSA. It belonged to ST398 and *spa*-type t034.

1 Strain 11 belonged to *agr*-group I and *spa*-type t1403. According to MLST, it was
2 ST132/CC133.

3 Strain 12 was *agr*-group II and, according to hybridisation pattern and *spa*-type (t002), it
4 belonged to CC5. Strains 13 to 18 resembled RF122, a sequenced strain of bovine origin, with
5 regard to *spa*-types and overall virulence gene content, including the presence of leucocidin
6 *lukF-P83/lukM*. Some differences in exotoxin carriage allowed an assignment of isolates to
7 Strains 13 (*sed*), 14 (*ssl4*), 15 (*tst1*, *sec*, *sel*, and *ssl4*), 16 (*ssl7*), 17 (*tst1*, *sec*, *sel*, and *ssl7*), or 18
8 (*seb*, *tst1*, *sec*, and *sel*).

9 Strain 19 was *agr*-group II and *spa*-type t160. Strain 20 belonged to *agr*-group III and to CC1.

10 Representative isolates of every strain as defined by array hybridisation were *spa*-typed. Typing
11 results are summarised in Table 1. Figure 5 shows similarities between the strains in form of a
12 split network tree.

13

14 **Superantigen carriage**

15 Strains 15, 17, and 18 (23 isolates) harboured the pathogenicity island SaPI_{bov} (Fitzgerald et al.,
16 2001) comprised of enterotoxin genes C and L as well as gene for toxic shock syndrome toxin 1
17 (*tst1*). Enterotoxin genes *sec* and *sel* were detected by array hybridisation, but *tst1* was not
18 detectable. According to the description of the bovine pathogenicity island (Fitzgerald et al.,
19 2001), these three genes were expected to occur together. Therefore, a PCR for the detection of
20 *tst1* was developed (for primer sequences, see above), and *tst1* was indeed found in Strains 15,
21 17, and 18. Sequencing of PCR products from two Strain 18 isolates (GenBank entries EF531614
22 and EF531615) confirmed the presence of a variant of *tst1* which differed in three positions
23 within the probe binding site from the *tst1* sequences of human isolates (AB084255.1,

1 AY074881.1, BA000017.4[2137855:2137879], BA000018.3[2061422:2061446], J02615.1,
2 U93688.2). Regarding this region, both sequences were identical to previously published
3 sequences from bovine isolates (AF217235.1 and NC_007622.1[399750:400454]; see Figure 6).

4 The gene encoding enterotoxin A was found in six isolates, all of which were positive for *sak* and
5 negative for *hly*. Five isolates belonged to two *agr*-type I clusters. One isolate, which belonged to
6 *agr*-type II, carried a variant of *sea* (as in sequence strain N315; BA000018.3). The enterotoxin
7 B gene was found in Strain 18, which also harboured SaPI_{bov}.

8 Two CC8 strains (Strain 5 and 6; seven isolates) contained genes for enterotoxins D, J, and R.
9 These three genes always were found together.

10 The *egc*-cluster (comprising of enterotoxins genes G, I, M, N, O, and Y) was found in CC20
11 Strains 7 and 8, in Strain 12, and in the RF122-like strains 13 to 18. In the latter, the enterotoxin
12 N gene was not reliably detectable by probe hybridisation yielding positive results only in
13 samples with very high DNA content. PCR products were obtained for Strain 16, Strain 17, and
14 Strain 18 isolates. Sequencing of these products (GenBank entries EF531604, EF531605, and
15 EF531606) showed identity to the RF122 sequence (NC_007622.1[1839538:1840293]).
16 Compared to the Mu50 sequence (NC_002758.2[1955162:1955938]), two bases within the probe
17 binding site were exchanged (Figure 6).

18

19 ***Ssl*-gene carriage**

20 *Ssl*-genes were used for assignment of isolates to strains and clonal complexes. Results are
21 summarised in Figure 2.

1

2 **Leucocidin carriage**

3 The Panton-Valentine leucocidin (*lukS/F*-PVL) was not found, but the bicomponent leucocidin
4 *lukF-P83/lukM* was detected in 68 out of 128 isolates (53.1 %) belonging to Strains 8, 11, and 13
5 to 18. Notably, 61 of them (90 %) were *agr*-type II.

6 Leucocidin *lukD/E* was found in all but two related strains (Strain 9 and 10) although some
7 isolates gave variable, weak, or only PCR-positive results. Because weak or variable signal
8 intensity was regarded as an indicator for sequence variation, PCR primers were designed, and
9 sequencing of cloned PCR products was performed. PCR products of one Strain 16 isolate, two
10 Strain 17 isolates and one Strain 18 isolate were sequenced. GenBank entries are for Strain 16,
11 EF531607; for Strain 17, EF531609 and EF531610; and for Strain 18, EF531608. Compared to
12 COL and Mu50 sequences (NC_002951.2[1935656:1936591] and
13 NC_002758.2[1948025:1948960]), two base exchanges within the probe binding sites of these
14 isolates were found. Additionally, the primer binding sites of these isolates differed in one or two
15 (Strain 16) positions from RF122 (NC_007622.1[1831611:1832546]) and Mu50 sequences
16 (Figure 6).

17 A putative bi-component leucocidin (SAV2004 and 2005, SA1812 and 1813, MW1941 and
18 1942; “lukX/Y” in Figure 3) was also found in the majority of isolates.

19

20 **Haemolysin carriage and staphylokinase**

21 Genes for haemolysins alpha, delta, and gamma (*hla*, *hld* and the *hlg*-locus comprised of *lukS*,
22 *lukF*, and *hlgA*) were present in all isolates. All isolates also contained an unnamed putative
23 haemolysin (BA000017.4 [963137:963165], CP000046.1 [927983:928011], *hl* in Figure 3). The

1 haemolysin gene *hl III* was un-detectable in the RF122-like clonal complex (Strains 13 to 18) as
2 well as in Strain 11.

3 As staphylokinase (*sak*) and enterotoxin A carrying phages can integrate into the haemolysin beta
4 gene *hly* (Coleman et al., 1991; Coleman, 1989; Iandolo et al., 2002), the relations of these genes
5 were analysed. Primer and probe were designed to engulf that insertion site. Therefore, a positive
6 signal can be interpreted as indicator for the presence of untruncated *hly* and for the ability to
7 express haemolysin B (Monecke et al., 2007; Monecke and Ehrlich, 2005). 105 out of 128
8 isolates (82 %) were positive for *hly*. Staphylokinase (*sak*) was rather rare, being detectable in 14
9 isolates (10.9 %). Nine out of 23 *hly*-negative isolates were positive for the *sak*, while only five
10 out of 105 *hly*-positive isolates harboured that gene, all of these being Strain 20. Six out of 23
11 *hly*-negative isolates contained *sea*, and this gene was not found among *hly*-positive isolates.

12

13 **Other virulence genes**

14 Genes *coa* (although some isolates showed only a weak coagulase reaction), *kata*, *sbi*, and *spa*
15 always were present. Exfoliative toxin genes (*etA*, *etB*, *etD*) and epidermal cell differentiation
16 inhibitor genes (*edinA-C*) were not found.

17 Protease genes *splA* and *B* were present in all strains but Strain 9 and 10. Deviant sequence
18 variants of *splA* in RF122-like strains were detectable by PCR. Isolates from Strains 13, 16, and
19 17 were subjected to sequencing (GenBank entries EF531611, EF531612, and EF531613). All
20 isolates showed identical primer binding sites as the RF122 sequence
21 (AJ938182[1818275:1818967]), which differed from the Mu50 sequence
22 (NC_002758.2[1942203:1942910]) in two positions. Concerning the probe binding site, RF122
23 and Mu50 sequences were identical, but Strain 13 differed in one, and Strain 16 and 17 in
24 another position (Figure 6).

1

2 **Antibiotic resistance**

3 Two isolates, Strains 4 and 10 were *mecA* positive. Strain 4 belonged to *agr*-type I, CC8, and
4 *spa*-type t068. It also carried the tobramycin resistance determinant *aadD* and the macrolide
5 resistance gene *ermA*. Strain 10 was *agr*-type I, ST398, t034, and appeared to be closely related
6 to Strain 9 (see Figure 5). It carried two macrolide/clindamycin resistance genes (*ermA* and
7 *ermC*), as well as *tetM* which confers resistance to tetracyclines.

8 Among the other isolates, resistance genes were rather rare. Twenty-seven isolates carried the
9 beta-lactamase gene *blaZ*. Among the methicillin-susceptible isolates, one harboured the
10 macrolide/clindamycin resistance gene *ermA*, and another two contained *ermC*. The
11 clindamycin/lincomycin resistance gene *linA* was found twice. The tetracycline resistance
12 determinant *tetM* was present in one MRSA (Strain 10) and in six methicillin-susceptible isolates.
13 None of the other resistance genes represented on the array was detected (see Figure 4).

1 **DISCUSSION**

2

3 *S. aureus* is an important agent of infection in man as well as in cattle. The aim of the study was
4 to get insight into the virulence and antibiotic resistance traits of cattle isolates and to find
5 possible differences in virulence factors between *S. aureus* isolates of human and bovine
6 background. The geographic origin of the isolates from two rather distant areas, the Bern region,
7 Switzerland, and Thuringia, Germany, allowed to exclude the theoretical possibility of confusing
8 the features of predominant regional outbreak strains with host specific properties. The most
9 conspicuous regional difference was the presence of a CC1 strain in Thuringia, which was not
10 observed in Bern.

11 A remarkable difference between human and bovine *S. aureus* was the presence of leucocidin
12 *lukF-P83/lukM* in a great proportion of bovine isolates (53.1 %). This phage-borne bi-component
13 leucocidin (Kaneko and Kamio, 2004; Kaneko et al., 1997; Yamada et al., 2005; Zou et al., 2000)
14 has previously been found to be associated with mastitis in cows, ewes, and goats (Rainard et al.,
15 2003). There is apparently no publication describing that toxin from host organisms other than
16 ruminants, and the authors also were not able to detect its genes in a large number of human
17 isolates (Monecke et al., 2007). On the other hand, *S. aureus* sampled from humans carry related,
18 but discernible leucocidin genes (*lukS/F-PVL*). This allows to assume some host specificity
19 which, ultimately, might lead to the evolution of distinct human and bovine populations of *S.*
20 *aureus*.

21 Another striking difference between human and bovine isolates was observed regarding the
22 presence of the staphylokinase gene *sak*. In human strains, *sak* is highly prevalent (Monecke et
23 al., 2007) and haemolysin beta usually is inactivated by the insertion of phages carrying *sak* or
24 *sak* and *seA* (Coleman et al., 1991; Coleman, 1989; Iandolo et al., 2002). Some *sak*-positive

1 bovine isolates also carried *seA*, of which two distinguishable variants were detected. Therefore,
2 it can be assumed that at least three different phages were prevalent within the studied strains.
3 Some ST1 isolates contained both, *hly* and *sak* possibly indicating an integration of the phage at
4 an unusual site. In some RF122-like strains *hly* was disrupted although no *sak* was detectable.
5 This could either indicate a fourth phage, or a possible truncation of one of the phages which
6 integrate into *hly*. Generally, only a minority of bovine *S. aureus* carried *sak*, and these cases
7 could possibly indicate human-cow-transmissions. For instance, the CC8 MRSA (Strain 4)
8 resembles a strain which is known to be prevalent in humans. It can be speculated that the
9 presence of *sak* in staphylococci which colonise or infect humans gives a selective advantage
10 which is more pronounced than the disadvantage caused by the loss of haemolysin beta. For the
11 colonisation/infection of cows, staphylokinase could be less important than *hly*. Another
12 possibility is that *sak*-carrying phages emerged rather recently and simply had no time to spread
13 widely in a bovine environment. This could be in accordance to the observation that the
14 pandemic “phage pattern 80/81” strain from the 1940s did not carry *sak* (Monecke et al., 2007).

15 For two toxins, *tstI* and enterotoxin N (*sen*), divergent sequences were found which appear to be
16 specific for *S. aureus* of bovine origin as the recently released genome sequence of the bovine
17 strain RF122 also contained similar variants of *tstI* and *sen*. Human isolates of *S. aureus* should
18 be screened in order to find, or to rule out, the occurrence of these variants. Both, bovine *tstI* and
19 *sen*, could not be detected directly using the array as sequence variations affected probe binding
20 sites. Array hybridisation patterns, however showed signals for *sec* and *sel*, which form a
21 pathogenicity island with *tstI* or, respectively, for *sem* and *seo* which accompany *sen*. Because of
22 these reactions, the presence of variants of *tstI* and *sen* was suspected, and eventually confirmed
23 by PCR and sequencing. The detection of these genes shows an advantage of array-based
24 genotyping compared to other detection methods. Although some genes were not detectable
25 because of yet unrepresented allelic variants, the array facilitated “proofreading” by generating
26 enough information to check, e.g., for genes missed from known clusters or pathogenicity

1 islands. The putative pathogenicity island SaPI_{bov} comprising *tstI*, *sec*, and *sel* was found in
2 Strain 15, 17, and 18, but it was missed in closely related Strains 13, 14, and 16. This
3 underscores its mobility as already observed by Fitzgerald (Fitzgerald et al., 2001).

4 The haemolysin gene *hIII* was un-detectable in the RF122-like clonal complex. However,
5 RF122 sequence data show that it was not completely absent, but that this strain contained a
6 deviant allelic variant. Whether or not this was related to host specificity needs still to be
7 determined.

8 Antibiotic resistance determinants appeared to be rather rare. While some isolates contained beta-
9 lactamase, macrolide/clindamycin (*ermA*, *ermC*) or tetracycline resistance genes, only two *mecA*-
10 positive isolates were found. Two isolates contained the clindamycin/lincomycin resistance gene
11 *linA*, which could be related to a selective pressure by the occasional use of lincomycin in animal
12 husbandry. Another interesting result was the absence of the genes *aphA3* (neo- and kanamycin
13 resistance) and *sat* (streptothricin resistance). These genes together form a transposon (Boerlin et
14 al., 2001; Derbise et al., 1997; Werner et al., 2001) that was shown to be very common among
15 human MRSA isolates (Monecke and Ehrlich, 2005) from Dresden University Hospital (in
16 Saxony, the neighbouring Federal State to Thuringia). Its absence in veterinary isolates could
17 indicate that this high prevalence was rather a result of selective pressure due to the historical use
18 of streptomycin preparations contaminated with streptothricin in human medicine (W. Witte,
19 personal communication) than to the use of streptothricin in East German agriculture prior to
20 1989 (Teale, 2002; Werner et al., 2001).

21 Generally, we found some differences between *S. aureus* of human and bovine origin. These
22 include the presence of *sak*- and *seA* carrying phages which disrupt *hly*, the presence of allelic
23 variants of *tstI*, *sen* and, according to RF122 sequence data, also of *hIII*, as well as the
24 occurrence of the leucocidin *lukF-P83/lukM*. None of these differences allows to distinguish
25 bovine from human strains with certainty. Nevertheless, some clonal lines appear to be host-

1 specific. The RF122-like clonal complex apparently does not occur in humans, and CC30 which
2 is extremely important in men (including EMRSA-16 as well as the pandemic *lukS/F-PVL*-
3 positive strains Phage Pattern 80/81 and WSPP-MRSA) was not found in cows. Specialisation to
4 hosts appears not to be stringent enough to make transmissions of *S. aureus* from one host
5 species to another impossible. Some clonal lines, such as CC1 (represented by Sanger 476,
6 MW2, and Strain 20) and CC8 (COL, NCTC8325, USA300, Irish 01, Strains 3-6), seem to be
7 rather promiscuous although some specific properties (presence of *sak*, disruption of *hly*,
8 presence of *lukF-P83/lukM*) often indicate host adaptation of a given isolate. However, in some
9 cases a direct transmission can be assumed. The CC8 MRSA isolate (Strain 4) from this study
10 had an overall hybridisation pattern and a *spa*-type (t068) related to a human healthcare-
11 associated strain (Irish 01, (Monecke et al., 2007). The other MRSA (Strain 10) is related to an
12 emerging MRSA which was shown to occur in humans, pigs (de Neeling et al., 2007; Huijsdens
13 et al., 2006), dogs, and horses (Cuny et al., 2006), and one of the methicillin-susceptible bovine
14 isolates describe above (Strain 9: *agr I/spa* t034) could represent a methicillin-susceptible
15 ancestor.

16 Especially, the presence of the two MRSA strains among the isolates tested highlights the
17 possibility of transmissions between humans and domestic animals. This warrants close
18 surveillance and cooperation between clinical and veterinary microbiologists.

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2

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Table 1: Studied strains, strain definitions, *spa*-types and geographic origin.

Strain	Strain definition	Detected <i>spa</i> -types	Isolates from Switzerland	Isolates from Thuringia
Strain 1	<i>agr</i> I/clonal complex 97, <i>ssl</i> 1,3,11-neg.	04-17; 07-16-12-21-17-34-13-34-33-34; 07-16-12-21-17-34-34-34-33-34-13	2	14
Strain 2	<i>agr</i> I/clonal complex 97, <i>ssl</i> 3-pos.	t528 (-04-); (07-23-21-17-34-34-34-34-33-34-13)	3	4
Strain 3	<i>agr</i> I/clonal complex 8, <i>ssl</i> 1,3,11-pos.	04-20/21-17-34-22-25; 11-12-21-17-34-24-34-22-25-25	3	0
Strain 4	<i>agr</i> I/clonal complex 8 MRSA	t068 (11-19-19-12-21-17-34-24-34-22-25)	1	0
Strain 5	<i>agr</i> I/clonal complex 8, <i>sed, sej, ser</i> -pos.	11-12-21-17-34-24-34-22-25-25	2	1
Strain 6	<i>agr</i> I/clonal complex 8, <i>sea, sed, sej, ser</i> -pos.	11-12-21-17-34-24-34-22-25-25	4	0
Strain 7	<i>agr</i> I/clonal complex 20, <i>egc</i> -pos.	26-06-17-21-34-34-22-34-13	1	7
Strain 8	<i>agr</i> I/clonal complex 20, <i>egc</i> -pos., <i>lukF-P83/M</i> -pos.	07-06-17-21-34-34-22-34-13	1	0
Strain 9	<i>agr</i> I/ST398/ <i>spa</i> t034	t034 (08-16-02-25-02-25-34-24-25)	1	0
Strain 10	<i>agr</i> I/ST398/ <i>spa</i> t034, MRSA	t034 (08-16-02-25-02-25-34-24-25)	0	1
Strain 11	<i>agr</i> I/ clonal complex 133	2 isolates typed, both t1403 (03-23-24)	0	6
Strain 12	<i>agr</i> II/clonal complex 5	t002 (26-23-17-34-17-20-17-12-17-16)	1	1
Strain 13	<i>agr</i> II/RF122-like, <i>sed</i> -pos.	04-20-17-25-17	3	0
Strain 14	<i>agr</i> II/RF122-like, <i>ssl4</i> -pos.	2 isolates typed, both 04-20-17-31-24	1	10
Strain 15	<i>agr</i> II/RF122-like, <i>sec, sel, ssl4</i> -pos.	04-20-17-31-24	0	1
Strain 16	<i>agr</i> II/RF122-like, <i>ssl7</i> -pos.	4 isolates typed, all t529 (04-34)	11	13
Strain 17	<i>agr</i> II/RF122-like, <i>sec, sel, ssl7</i> -pos.	4 isolates typed, three times t529 (04-34) and once -34-	12	6
Strain 18	<i>agr</i> II/RF122-like, <i>seb, sec, sel</i> -pos	t529 (04-34)	4	0
Strain 19	<i>agr</i> II/ <i>spa</i> t160	t160 (07-23-21-24-33-22-17)	1	0
Strain 20	<i>agr</i> III/clonal complex 1	t127 (07-23-21-16-34-33-13) t174 (14-21-16-34-33-13)	0	13

1 **Figure 1:** Superantigenic toxins in studied strains (black spots: positive; black spots with
2 numbers: positive only in the given number of isolates; grey spots: ambiguous, positive only
3 in experiments with high DNA load, or in PCR; white spots: negative).

4 **Figure 2:** *Ssl*-genes in studied strains (black spots: positive; black spots with numbers:
5 positive only in the given number of isolates; grey spots: ambiguous, positive only in
6 experiments with high DNA load, or in PCR; white spots: negative).

7 **Figure 3:** Other virulence-associated genes in studied strains (black spots: positive; black
8 spots with numbers: positive only in the given number of isolates; grey spots: ambiguous,
9 positive only in experiments with high DNA load, or in PCR; white spots: negative).

10 **Figure 4:** Resistance genes in studied strains (black spots: positive; black spots with numbers:
11 positive only in the given number of isolates; white spots: negative).

12 **Figure 5:** A split network tree of studied strains and some reference strains based on
13 hybridisation results. Some reference strains isolated from humans (for hybridisation data see
14 (Monecke et al., 2007) are also shown for comparison, these strains are labelled in italics.

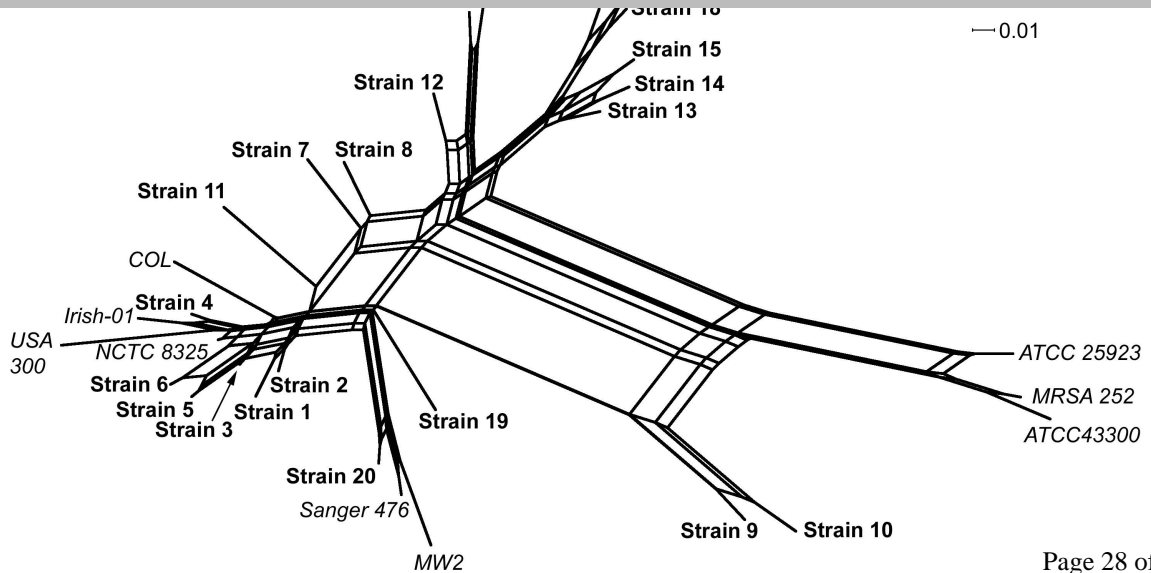
15 **Figure 6:** Alignments of sequenced primer/probe binding sites of *tstI*, *sen*, *lukE*, and *splA*.
16 Scales refer to the Mu50 sequence BA000017.4.

	Number of isolates	tst1	sea	sea (N315)	seb	sec	sed	see	seg	seh	sei	sej	sek	sel	sem	sen	seo	seq	ser	seuly	n homologue	ORF CM14
Strain 1	16	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	●	○
Strain 2	7	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	●	○
Strain 3	3	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	●	○
Strain 4 (MRSA)	1	○	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	●	○
Strain 5	3	○	○	○	○	○	●	○	○	○	○	●	○	○	○	○	○	○	○	○	●	○
Strain 6	4	○	●	○	○	○	●	○	○	○	○	●	○	○	○	○	○	○	○	○	●	○
Strain 7	8	○	○	○	○	○	○	○	●	○	●	○	○	○	●	●	●	○	○	○	●	●
Strain 8	1	○	○	○	○	○	○	○	●	○	●	○	○	○	●	●	●	○	○	○	●	○
Strain 9	1	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	●
Strain 10 (MRSA)	1	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	●
Strain 11	6	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	●
Strain 12	2	○	○	○	○	○	○	○	●	○	●	○	○	○	●	●	●	○	○	○	●	○
Strain 13	3	○	○	○	○	○	●	○	●	○	●	○	○	○	●	○	●	○	○	○	●	●
Strain 14	9	○	○	○	○	○	○	○	○	○	●	○	○	○	○	●	●	○	○	○	○	○
Strain 15	1	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Strain 16	26	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Strain 17	18	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Strain 18	4	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Strain 19	1	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Strain 20	13	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○

	31 (MRSA252) setB1	32 (MRSA252) setB2	33 (MRSA252) setB3	11 (MRSA252) ssl11 (MW2)	ssl11 (Mu50)	ssl11 (COL)	10 (MRSA252) ssl10	19 (MRSA252) ssl9	ssl8	ssl7 (FRI326)	17 (MRSA252) ssl7	ssl6	15 (MRSA252) ssl5	14 (MRSA252) ssl4	13 (MRSA252) ssl3	12 (MRSA252) ssl2	other variants	11 (MRSA252) ssl1 (MW2)	ssl1 (Mu50)	ssl1 (COL)	eIC (MW0345)	per of isolates
Strain 1	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	16
Strain 2	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	7
Strain 3	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	3
Strain 4 (MRSA)	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	1
Strain 5	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	3
Strain 6	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	4
Strain 7	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	8
Strain 8	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	1
Strain 9	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	1
Strain 10 (MRSA)	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	1
Strain 11	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	6
Strain 12	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	2
Strain 13	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	3
Strain 14	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	9
Strain 15	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	1
Strain 16	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	26
Strain 17	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	18
Strain 18	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	4
Strain 19	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	1
Strain 20	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	13

	Number of isolates	lukF	lukS	hlgA	lukF-PV	lukS-PV	lukF-P83	lukM	lukD	luke	"lukX"	"lukY"	γ (MRS252)"	hl	hla	hlb	sak	hld	hl III	kata	coa	spa	sbi	eA, B, D	spIA	spIB	ednA, B, C
Strain 1	16	●	●	●	○	○	○	○	●	●	○	●	○	●	●	●	○	●	●	●	●	●	○	○	●	●	○
Strain 2	7	●	●	●	○	○	○	○	●	●	○	●	○	●	●	●	○	●	●	●	●	●	○	○	●	●	○
Strain 3	3	●	●	●	○	○	○	○	●	●	●	○	○	●	●	●	○	●	●	●	●	●	○	○	●	●	○
Strain 4 (MRSA)	1	●	●	●	○	○	○	○	●	●	●	○	○	●	●	○	●	●	●	●	●	●	○	○	●	●	○
Strain 5	3	●	●	●	○	○	○	○	●	●	●	○	○	●	●	●	○	●	●	●	●	●	○	○	●	●	○
Strain 6	4	●	●	●	○	○	○	○	●	●	●	○	○	●	●	○	●	●	●	●	●	●	○	○	●	●	○
Strain 7	8	●	●	●	○	○	○	○	●	●	○	○	○	●	●	●	2	●	●	●	●	●	●	○	○	●	○
Strain 8	1	●	●	●	○	○	●	●	●	●	●	○	○	●	●	●	○	●	●	●	●	●	○	○	●	●	○
Strain 9	1	●	●	●	○	○	○	○	○	○	○	○	○	●	●	○	○	●	●	●	●	●	○	○	○	○	○
Strain 10 (MRSA)	1	●	●	●	○	○	○	○	○	○	○	○	○	●	●	○	○	●	●	●	●	●	○	○	○	○	○
Strain 11	6	●	●	●	○	○	●	●	●	●	○	○	○	●	●	○	○	●	○	●	●	●	○	○	●	●	○
Strain 12	2	●	●	●	○	○	○	○	●	●	○	○	○	●	●	●	○	●	●	●	●	●	○	○	●	●	○
Strain 13	3	●	●	●	○	○	●	●	●	●	○	○	○	●	●	●	○	●	○	●	●	●	○	○	●	●	○
Strain 14	9	●	●	●	○	○	●	●	●	●	○	○	○	●	●	●	○	●	○	●	●	●	○	○	●	●	○
Strain 15	1	●	●	●	○	○	●	●	●	●	○	○	○	●	●	●	○	●	○	●	●	●	○	○	●	●	○
Strain 16	26	●	●	●	○	○	●	●	●	●	○	○	○	●	●	21	○	●	○	●	●	●	○	○	●	●	○
Strain 17	18	●	●	●	○	○	●	●	●	●	○	○	○	●	●	17	○	●	○	●	●	●	○	○	●	●	○
Strain 18	4	●	●	●	○	○	●	●	●	●	○	○	○	●	●	●	○	●	○	●	●	●	○	○	●	●	○
Strain 19	1	●	●	●	○	○	○	○	●	●	○	○	○	●	●	○	●	●	○	●	●	●	○	○	●	●	○
Strain 20	13	●	●	●	○	○	○	○	●	●	○	○	○	●	●	●	5	○	●	○	●	●	○	○	●	●	○

	per of isolates	bla _Z	mecA	ermA	ermC	linA	msrA	vatA	vatB	vga	vgaA	vgb	aacA-aphD	aphA3	aadD	sat	dfrA	far1	mupR	tekK	temI	vanA	vanB	vanZ
Strain 1	16	③	○	○	①	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Strain 2	7	④	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	④	○	○	○
Strain 3	3	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Strain 4 (MRSA)	1	○	●	●	○	○	○	○	○	○	○	○	○	●	○	○	○	○	○	○	○	○	○	○
Strain 5	3	②	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Strain 6	4	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Strain 7	8	②	○	①	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Strain 8	1	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Strain 9	1	●	○	○	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	●	○	○
Strain 10 (MRSA)	1	○	●	●	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	●	○	○
Strain 11	6	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Strain 12	2	①	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Strain 13	3	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Strain 14	9	○	○	○	○	②	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	①	○	○
Strain 15	1	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Strain 16	26	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Strain 17	18	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Strain 18	4	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Strain 19	1	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Strain 20	13	⑧	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○



Mu50 probe primer	TAGCGAAGGA GCGAAGGA	ACTTATATCC ACTTATATCC	ATTTCCAAAT ATTTC	AAGTGCGGTT	ACAAATACTG	AAAAATTACC	TACTCCAATA	GAACTACCTT
RF122	TAGCGAAGGA	ACTTGGATTC	ATTTCCAAAT	AAGTGGTGTT	ACAAATACTG	AAAAATTACC	TACTCCAATA	GAACTACCTT
Strain 18	TAGCGAAGGA	ACTTGGATTC	ATTTCCAAAT	AAGTGGTGTT	ACAAATACTG	AAAAATTACC	TACTCCAATA	GAACTACCTT

seN	1955550							1955486
MU50, probe primer, r.c.	AAACATCATG TCATG	CTTATACGGA CTTATACGGA	GGAGTTACGA GGAGTTACGA	TACATGATGG	AAATCAATTA	GATGAAGAGA	AAGTT	
RF122, Strain 16	AAACATCGTG	CTTAAACGGA	GGAGTTACGA	TACATGATGG	AAATCAATTA	GATGAAGAGA	AAGTT	
Strain 17	AAACATCGTG	CTTAAACGGA	GGAGTTACGA	TACATGATGG	AAATCAATTA	GATGAAGAGA	AAGTT	
Strain 18	AAACATCGTG	CTTAAACGGA	GGAGTTACGA	TACATGATGG	AAATCAATTA	GATGAAGAGA	AAGTT	

luke	1948343							1948264
Mu50 probe primer, r.c.	TTTATTCGTA CGTA	CAAAGTCCAA CAAAGTCCAA	ATGGTCCAAC ATGGTCCAAC	AGGTTACGCA A	AGAGAATATT	TTGCTCCTGA TCCTGA	TAATCAATTG TAATCAATTG	CCACCTTTAG CCAC
RF122	TTTATTCGTA	CAAAGTCCAA	ATGGTCCGTC	AGGTTACGCA	AGAGAATATT	TTGCTCCTGA	TAATCAATTG	CCACCATTAG
Strain 16	TTTATTCGTA	CAAAGTCCAA	ATGGTCCGTC	AGGTTACGCA	AGGGAATATT	TTGCTTCTGA	TAATCAATTG	CCATCATTAG
Strain 17	TTTATTCGTA	CAAAGTCCAA	ATGGTCCGTC	AGGTTACGCA	AGAGAATATT	TTGCTTCTGA	TAATCAATTG	CCACCATTAG
Strain 18	TTTATTCGTA	CAAAGTCCAA	ATGGTCCGTC	AGGTTACGCA	AGAGAATATT	TTGCTTCTGA	TAATCAATTG	CCACCATTAG

splA	1942469							1942400
Mu50 probe primer, r.c.	ATTTCTGTTA TGTTA	TTGGTTATCC TTGGTTATCC	AAAGGGTGCA AAAGGGTGCA	CAAACAAAAT C	ATAAAATGTT	TGAATCGACA TGTT	GGAACGATTA GGAAC	
RF122	ATTTCTGTTA	TTGGTTATCC	AAAGGGTGCA	CAAACAAAAT	ATAAAATGTT	TGAATCAACT	GGAACGATTA	
Strain 13	ATTTCTGTTA	TTGGTTATCC	AAAGGGTCA	CAAACAAAAT	ATAAAATGTT	TGAATCAACT	GGAACGATTA	
Strain 16	ATTTCTATTA	TTGGTTATCC	AAAGGGTGCA	CAAACAAAAT	ATAAAATGTT	TGAATCAACT	GGAACGATTA	
Strain 17	ATTTCTATTA	TTGGTTATCC	AAAGGGTGCA	CAAACAAAAT	ATAAAATGTT	TGAATCAACT	GGAACGATTA	