Occurrence of *ssl* genes in isolates of *Staphylococcus aureus* from animal infection

Davida S. Smyth,^{1†} William J. Meaney,² Patrick J. Hartigan³ and Cyril J. Smyth¹

Correspondence

Cyril J. Smyth
csmyth@tcd.ie

¹Department of Microbiology, Moyne Institute of Preventive Medicine, Trinity College, University of Dublin, Dublin 2, Ireland

²Teagasc, Dairy Production Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

³Department of Physiology, School of Medicine, Trinity College, University of Dublin, Dublin 2, Ireland

The occurrence of 7 of the 11 known *ssl* genes that are found within the *vSax* genomic island of *Staphylococcus aureus* and encode the novel Ssl family of exoproteins was examined in isolates from cows (42 isolates), goats (4 isolates), sheep (1 isolate), rabbits (3 isolates) and chickens (2 isolates). Based on seven *S. aureus* genome sequences for human strains NCTC 8325, N315, Mu50, COL, MRSA 252, MW2 and MSSA-476, and bovine strain RF122, along with the *ssl* reference gene sequences from strains NCTC 6571, FRI326 and NCTC 8325, ClustalW-generated alignments were used to design PCR primers for unique regions of the *ssl* genes that are present in the allelic variants of each, except for the *ssl4* gene for which specific primers for the *set2* and *set9* allelic variants were designed individually. The genotypes of isolates were determined using random amplified polymorphic DNA (RAPD) typing. All of the animal-associated *S. aureus* isolates contained an *ssl* locus, but there were minor variations in the number of *ssl* genes present. Forty-nine of the animal isolates possessed a *vSax* genomic island containing the *ssl3* (*set8*), *ssl5* (*set3/set10*), *ssl7* (*set1/set11*), *ssl8* (*set12*), *ssl9* (*set5/set13*) and *ssl10* (*set4/set14*) genes. One bovine and one goat isolate lacked the *ssl3* gene. The *ssl9* gene was absent in one bovine isolate. The goat isolate lacking the *ssl3* gene was the only animal isolate that possessed the *set2* allele of the *ssl4* gene. PCR for the *set9* allele of the *ssl4* gene was inconclusive. Isolates that showed identical RAPD fingerprints had the same complement of *ssl* genes, but the *ssl* gene pattern was not RAPD-type specific. Southern blot hybridization showed similar *ssl* gene RFLPs in isolates of the same RAPD type.

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INTRODUCTION

Staphylococcus aureus causes a wide variety of clinical syndromes ranging from uncomplicated infections of the skin, such as boils and carbuncles, to life-threatening infections, such as endocarditis and toxic shock syndrome (Murray, 2005; Todd, 2005). *S. aureus* is also an important pathogen of animals including cows, goats, sheep, rabbits and chickens (Rich, 2005; Barkema *et al.*, 2006). Among the many known virulence factors of *S. aureus* are three groups of staphylococcal exoproteins: the enzymes such as hyaluronidase, proteases and nucleases; the non-enzymic activators of plasma clotting (coagulase) and fibrinolysis (staphylokinase); and the exotoxins, namely, cytolytic toxins, exfoliative toxins, leukocidins, enterotoxins, enterotoxin-like proteins

and toxic shock syndrome toxin no. 1 (TSST-1) (Ferry *et al.*, 2005). The enterotoxins, enterotoxin-like proteins and TSST-1 belong to the superantigen (SAG) family (Proft & Fraser, 2003). Nineteen staphylococcal enterotoxins (SEs) and staphylococcal enterotoxin-like (SEL) proteins, as well as a number of variants of some of these, have been described (Smyth *et al.*, 2004; Thomas *et al.*, 2006).

In addition to these superantigens, *S. aureus* also produces a family of exoproteins termed the staphylococcal exotoxin-like (Set) proteins (Williams *et al.*, 2000), which have since been renamed staphylococcal superantigen-like (Ssl) proteins (Lina *et al.*, 2004). The Ssl proteins are encoded by a cluster of genes (also termed genomic island *vSax*; Lindsay & Holden, 2004, 2006) that show sequence similarity of 36–67% (Williams *et al.*, 2000; Kuroda *et al.*, 2001). The *vSax* island is flanked upstream by a putative transposase gene, and downstream by an incomplete restriction and modification system (the *hsdS* and *hsdM* genes) forming

[†]Present address: New York Medical College, Department of Microbiology and Immunology, Valhalla, NY 10595, USA.

Abbreviation: RAPD, random amplified polymorphic DNA.

part of a three-component restriction/modification system (HsdS, HsdM and HsdR) that is believed to maintain the locus in the genome (Kuroda *et al.*, 2001). Although the crystal structures of the Ssl5 and Ssl7 proteins revealed that these had overall folding characteristics and the classical two-domain structure of the SAg family, the major histocompatibility complex-binding site of superantigens had been lost, accounting for the inability of Ssl proteins to induce T-cell proliferation (Arcus *et al.*, 2002; Al-Shangiti *et al.*, 2004).

Microarray analysis using 63 isolates from humans, sheep, cows and poultry (Fitzgerald *et al.*, 2003) showed that the *ssl* locus was present in all of the *S. aureus* isolates tested, but varied in size from 12 to 17 kb and displayed high levels of sequence variation. It was theorized that horizontal gene transfer, recombination events, and loss of *ssl* genes had led to this diversity. Recently Tsuru *et al.* (2006) compared whole genomic sequences of human strains of *S. aureus* and identified conserved sequences (45 and 61 bp repeats flanking indels) between the *ssl* genes and at identical relative positions, which could be functionally important in generating deletions by homologous recombination in the variable region between the *ssl3* and *ssl9* genes.

At the start of this work very limited data were available on the occurrence of individual *ssl* genes in *S. aureus* isolates from animals (Fitzgerald *et al.*, 2003). The aims of this study were to develop a PCR-based screening method for *ssl* genes within the variable region of the *vSax* genomic island and to investigate the occurrence of these genes in isolates of *S. aureus* of animal origin.

METHODS

Strains. Fifty-two isolates associated with animal *S. aureus* infections, namely, broiler chicken osteomyelitis ($n=2$), rabbit staphylococcosis ($n=3$) and mastitis (cows = 42, sheep = 1, goats = 4), which included animal-associated clonal types previously identified by molecular fingerprinting techniques (Rodgers *et al.*, 1999; Fitzgerald *et al.*, 1997, 2000; Hermans *et al.*, 2000; Foschino *et al.*, 2002), were examined. These were confirmed to belong to animal-associated clonal types using multiple locus sequence typing (Smyth, 2006). Six human control strains, MRSA 252, NCTC 8325-4, MSSA-476, COL and NCTC 6571, and one clinical human isolate (strain MSA 2335), were also used. *S. aureus* isolates were routinely grown on either trypticase soy agar (TSA) (Oxoid) or trypticase soy broth (Oxoid) with shaking at 37 °C. The identification of *S. aureus* isolates was verified by testing their ability to grow and produce acid on mannitol salt agar, a positive coagulase test, their ability to produce acetoin, their ability to grow on TSA supplemented with 7 µg acriflavin ml⁻¹ (Devriese, 1981), and a negative PCR for the *Staphylococcus intermedius* 16S rRNA gene.

Analysis of the *S. aureus* genome sequences using Artemis and BLASTN and translated amino acid sequences using BLASTX. Seven available *S. aureus* genome sequences for strains NCTC 8325, N315, Mu50, COL, MRSA 252, MW2 and MSSA-476, the *ssl* reference gene sequences from strains NCTC 6571, FRI326 and NCTC 8325, and the *ssl* locus sequences of bovine strain RF122 and of three human-associated strains JH1, JH9 and USA300 (Table 1) were downloaded and analysed by Artemis, a freeware

sequence analysis tool available at the Sanger Centre website (www.sanger.ac.uk) and BLASTN (<http://www.ncbi.nlm.nih.gov/blast/>) to determine the composition of the *ssl* locus in each strain. Once a putative identity had been assigned to each ORF of each genome sequence, the ORFs from each genome were aligned with each other using ClustalW (www.ebi.ac.uk/clustalW) and ClustalX ([ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/](http://ftp-igbmc.u-strasbg.fr/pub/ClustalX/)).

From the analysis it was observed that the *ssl* genes of each sequenced *S. aureus* strain varied in number and composition, and that there were a number of allelic variants of certain *ssl* genes, namely, *ssl3* (*set8*), *ssl5* (*set3/set10*), *ssl7* (*set1/set11*), *ssl8* (*set12*), *ssl9* (*set5/set13*) and *ssl10* (*set4/set14*). A schematic of the *ssl* loci from 11 human-associated *S. aureus* strains and the bovine strain RF122 is shown in Fig. 1. Using BLASTX it was possible to compare the translated amino-acid sequences of the *ssl* genes from the genome sequence of strain N315 with the amino-acid sequences of the *ssl* proteins of NCTC 6571, the archetypal *set* gene cluster strain, already in the NCBI protein database (Williams *et al.*, 2000). The output of BLASTX showed that the *ssl* genes from strains N315 and NCTC 6571 have translated amino-acid sequence similarities ranging from 29 to 66 % between individual pairs of genes, whereas the tandem paralogues within these strains have higher degrees of similarity (76–89 %). The translated amino-acid similarities of the *ssl3* and *ssl4* genes in strains N315 and NCTC 6571 were high, with the *set2* allelic variant of the *ssl3* gene (present in strain NCTC 6571) showing 87 % and 76 % translated amino-acid similarity to the *set8* and *set9* allelic variants of the *ssl4* gene (present in strain N315), respectively.

DNA isolation. Extraction of genomic DNA from *S. aureus* was performed as previously described with minor modifications (Fitzgerald *et al.*, 1997). The procedure involves lysostaphin lysis, proteinase K treatment, addition of EDTA, sarkosyl and cetyltrimethylammonium bromide (CTAB), extraction with chloroform/isoamyl alcohol and phenol/chloroform/isoamyl alcohol, and precipitation with ethanol.

Design of primers and PCR analysis. Using ClustalW/ClustalX-generated alignments it was possible to locate unique regions of each *ssl* gene that were present in their respective *set* allelic variants, except for the *ssl4* gene. These unique regions were used to design PCR primers for the *ssl* alleles, and to amplify PCR products for use as probes in Southern blot hybridization experiments (Table 2). Each set of primers used the same PCR mix comprising 10 pmol forward and reverse primers, 200 µM each of dATP, dTTP, dCTP and dGTP, 1 × magnesium-free buffer (Promega), 1.5 U *Taq* DNA polymerase (Promega), 3 mM MgCl₂, and 50–100 ng DNA. PCR tests also included appropriate *ssl* gene-positive and -negative controls (DNA from reference human strains NCTC 8325-4, MRSA 252, MSSA-476, COL and NCTC 6571).

The PCRs used the following cycling parameters: 95 °C for 10 min, 15 cycles of (95 °C for 1 min, 58 °C for 45 s and 72 °C for 1 min) and 16 cycles of (95 °C for 1 min, 54 °C for 45 s and 72 °C for 1 min). The reaction was terminated with a 10 min incubation at 72 °C. PCR products were resolved by electrophoresis in 1.5 % (w/v) agarose gel (0.5 × Tris/boric acid/EDTA buffer) (Sambrook & Russell, 2001) at 90 V (constant voltage), stained with ethidium bromide, and visualized using UV light. Product sizes were determined by comparison with a 100 bp ladder (Promega).

Southern blotting and *ssl* gene RFLP analysis. The presence of the *ssl* locus within isolates that were positive by PCR was confirmed using Southern blotting of genomic DNA from a number of representative Irish and American bovine isolates from the study of Fitzgerald *et al.* (1997) and human-associated control strains. Genomic DNA was digested with restriction endonuclease *Hind*III, which cuts within the *hdsM*, *hdsS* genes of control strain MRSA 252. The resulting fragments were resolved by electrophoresis in 0.8 % agarose. Southern blot

Table 1. *S. aureus* genomic sequences and *ssl* reference gene sequences examined using Artemis and BLASTN/BLASTX

Strain	Origin (year of isolation)*	GenBank accession no.	Reference
Mu50	Pus from surgical wound, VRSA, Juntendo University Hospital, Tokyo, Japan (1997)	NC_002758	Kuroda <i>et al.</i> (2001)
N315	Pharyngeal smear, MRSA, Nagasaki University Hospital, Japan (1982)	NC_002745	Kuroda <i>et al.</i> (2001)
RF122	Bovine mastitis, Ballymacoda, Ireland (1993)	NC_007622	Fitzgerald <i>et al.</i> (2001); Herron-Olson and others, GenBank database 2005
NCTC 8325	Corneal ulcer, Oxford, UK (1943)	NC_007795, AF188837	Richardson <i>et al.</i> (1994); Gillaspay <i>et al.</i> (2006)
COL	Operating theatre, London, UK (approximately 1960)	NC_002951	Gill <i>et al.</i> (2005)
MRSA 252	Postoperative infection, EMRSA-16 clone (1997)	NC_002923	Holden <i>et al.</i> (2004)
MW2	Fatal septicaemia and septic arthritis, CA-MRSA, North Dakota, USA (1998)	NC_003923	Baba <i>et al.</i> (2002)
MSSA-476	Tibial osteomyelitis and bacteraemia, invasive (1998)	NC_002953	Holden <i>et al.</i> (2004)
NCTC 6571	Pleural fluid (1935)	AF094826 AF188835	Williams <i>et al.</i> (2000) Williams <i>et al.</i> (2000)
FRI326	Chicken tetrazzini, food poisoning, enterotoxin E, Chicago, USA (1960)	AF188836	Williams <i>et al.</i> (2000)
JH1	Blood culture, MRSA, Baltimore, USA (2001)	NZ_AAPK00000000	Copeland and others, GenBank database 2006
JH9	Blood culture, VISA, Baltimore, USA (2001)	NZ_AAPL00000000	Copeland and others, GenBank database 2006
USA300 (FPR3757)	Wrist abscess, CA-MRSA, San Francisco, USA (2002)	NC_007793	Diep <i>et al.</i> (2006)

*CA-MRSA, community-acquired, methicillin-resistant *S. aureus*; EMRSA-16, epidemic methicillin-resistant *S. aureus* type 16; MRSA, methicillin-resistant *S. aureus*; VISA, vancomycin intermediate-resistant *S. aureus*; VRSA, vancomycin-resistant *S. aureus*.

hybridization analysis was performed using standard methods (Sambrook & Russell, 2001) and the DIG-labelling system (Roche). Probes were constructed using the PCR DIG-labelling mix (Roche) in PCRs that incorporated DIG-labelled dUTP into the synthesized DNA. Probes were designed to target genes that were present in all isolates tested and therefore would allow a comparison of RFLPs across all isolates analysed. Probes for the *ssl7* and *ssl5* genes were amplified using genomic DNA from human strain NCTC 6571, and a probe for the *ssl8* gene using genomic DNA from bovine strain RF122.

Random amplified polymorphic DNA (RAPD) typing. RAPD typing was carried out as previously described (Fitzgerald *et al.*, 1997; Smyth *et al.*, 2006). Briefly it was performed in a final volume of 25 µl consisting of 1 µl chromosomal DNA (approx. 100 ng), 2 µl 23-mer primer D11344 (20 pmol µl⁻¹) (Akopyanz *et al.*, 1992), 2.5 U *Taq* DNA polymerase (Promega), 2.5 µl 10× buffer (Promega), 3 µl 25 mM MgCl₂ buffer (Promega), and 0.2 mM dNTPs. The PCR program used was 4 cycles of (94 °C for 5 min, 40 °C for 5 min, 72 °C for 5 min) and then 30 cycles of (94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min). Of the PCR products 5 µl was run on a 2% (w/v) agarose gel. Strains that generated the same banding pattern were considered to be of the same RAPD type.

RESULTS

PCR for *ssl* genes

PCR was done on strains NCTC 8325-4, MSSA-476, MRSA 252, COL and NCTC 6571, each of known genome sequence

and *ssl* gene content, and on a clinical isolate (MSA 2335). The *ssl* genes tested for were all amplifiable by PCR and the results for these strains were consistent with their *ssl* genomic island sequences (Table 3, Fig. 1). PCR analysis of the animal-associated *S. aureus* isolates showed that, whereas all of them contained an *ssl* locus, there were minor variations in the number of *ssl* genes encoded (Table 3). The *ssl5*, *ssl7*, *ssl8* and *ssl10* genes were present in all of the animal-associated isolates. The *ssl9* gene was present in all isolates bar one bovine isolate, namely, isolate MSA1455. The *ssl3* gene was present in 50 of the 52 animal-associated isolates tested, except for one bovine (MSA1003) and one caprine isolate (St24).

The *set2* allelic variant of the *ssl4* gene was found to be present in 1 of the 52 animal-associated isolates (St24 from a goat). In contrast to PCR findings with strains NCTC 8325, MSSA-479 and COL PCR, for each of which the *set9* allelic variant of the *ssl4* gene was amplifiable using the designed primers (Fig. 1, Table 2), PCR for the *ssl4* (*set9*) allele with the animal isolates was inconclusive. The primers for the *set9* allelic variant of the *ssl4* gene generated multiple non-specific products with the animal isolates, including strain RF122, which is known to lack the *ssl4* gene based on the genomic sequence data (Fig. 1). This implies that the primers could anneal to other targets, possibly *ssl*-like

Fig. 1. Schematic of the *ssl* locus based on *S. aureus* genome and *ssl* locus sequences. The staphylococcal genomes show variation in the number and complement of *ssl* alleles. There is a central variable region that contains allelic variants of *ssl* genes, e.g. the *ssl5* gene (formerly *set3* and *set10*) and the *ssl7* gene (formerly *set1* and *set11*) have variants. Deletions of *ssl* genes also occur, e.g. the *ssl6* gene is only present in strains NCTC 8325, MSSA-476, USA300 and MW2. Strain RF122 is a bovine strain, while the rest are of human origin. The *ssl* genes are numbered above the diagram. T, putative transposase gene; M, *hdsM* gene; S, *hdsS* gene of the restriction and modification system; 8325, NCTC 8325; 6571, NCTC 6571; MSSA, MSSA-476; MRSA, MRSA 252.

sequences, in the animal isolates. While 5 other isolates (RF102, RF108, RF110, FR120 and RF121) included in this study are of the same RAPD type 33 as strain RF122 (see

below and Table 3), and thus might be surmised to lack the *ssl4* gene, it seems highly likely that a number of the other 45 animal isolates might test positive for the *set9* allelic variant

Table 2. Primers used in *ssl* (*set*) gene PCR

<i>ssl</i> gene*	<i>set</i> gene	Primer sequence (5'–3')	Product size (bp)	<i>T_m</i> (°C)
3	<i>set8</i> for	CAC AAG ACA AGA ACG CAC GCC T	820	62.1
	<i>set8</i> rev	CTG CCA TAC GAT GCT CTT GCA G		
4	<i>set2</i> for	ATG CAG AAG TAG CAT CAG CAC T	783	57.2
	<i>set2</i> rev	GAT ACT AGT GCC ATC TAT GAC		
4	<i>set9</i> for	CGC GAC AAC ACC ATC TTC CAC T	550	58.4
	<i>set9</i> rev	CTT TAG TAA TTC TTA CTC CTG C		
5	<i>set3/10</i> for	TGA AAA TGT GAC AAA AGA TAT CTT	103	53.9
	<i>set3/10</i> rev	TGA AAA TGT GAC AAA AGA TAT CTT		
7	<i>set1/11</i> for	CAA GAG AGA GTA CAA CAT TTA	242	52.4
	<i>set1/11</i> rev	CCG TTT GGA TCA ATT AAT TCT T		
8	<i>set12</i> for	GAA CCG TTT ATA TGA TAC AAA C	296	52.8
	<i>set12</i> rev	GTT CTT AGA GAC TCA AAT AAT G		
9	<i>set5/13</i> for	GGT ATA ACA AAG AAA AAT GTG AGA TC	193	55.0
	<i>set5/13</i> rev	CCT TTA TAC AAT CTA TAT TTT TCG A		
10	<i>set4/14</i> for	AAC AAC AGG AAC TTT AAC AAC AGA	397	57.6
	<i>set4/14</i> rev	ACC TTT CAC AAA AGC ATC TTC ACC		

*Not all *ssl* genes were tested for (*ssl1*, *ssl2*, *ssl6* and *ssl11* were omitted). It was not possible to design a primer to amplify both the *set9* and *set2* allelic variants of *ssl4* so individual primers were used. Primers would amplify all allelic variants of *ssl* genes, e.g. the *ssl10* primers would amplify both the *set4* allele of strain MRSA252 and the *set14* allele of strain NCTC 8325.

Table 3. Combinations of *ssl* genes in 52 isolates of *S. aureus* of animal origin and 6 control human strains

Strain/isolate*	Origin	Host	Known characteristic		RAPD type§	<i>ssl</i> genell						
			SAg and SAg-like genes†	Mitogenicity‡								
NCTC 8325-4	USA	Human	None	ND	ND	3	4	5	7	8	9	10
MSSA-476	UK	Human	<i>sea, seh</i>	ND	ND	3	4	5	7	8	9	10
MRSA 252	UK	Human	<i>egc</i>	ND	ND	3	4	5	7		9	10
COL	USA	Human	<i>seb, selk, selq</i>	ND	ND	3	4				9	10
NCTC 6571	USA	Human	<i>egc</i>	ND	ND	3	4	5	7		9	10
MSA 2335	USA	Human	ND	ND	ND		4	5	7	8	9	10
RF101	IRL	Cow	Negative	–	11	3		5	7	8	9	10
RF102	IRL	Cow	SaPIbov, <i>egc</i>	+	33	3		5	7	8	9	10
RF103	IRL	Cow	Negative	+	11	3		5	7	8	9	10
RF107	IRL	Cow	Negative	–	11	3		5	7	8	9	10
RF108	IRL	Cow	SaPIbov, <i>egc</i>	+	33	3		5	7	8	9	10
RF110	IRL	Cow	SaPIbov, <i>egc</i>	+	33	3		5	7	8	9	10
RF111	IRL	Cow	Negative	–	16	3		5	7	8	9	10
RF115	IRL	Cow	Negative	–	16	3		5	7	8	9	10
RF116	IRL	Cow	Negative	–	16	3		5	7	8	9	10
RF117	IRL	Cow	Negative	–	16	3		5	7	8	9	10
RF120	IRL	Cow	<i>egc</i>	+	33	3		5	7	8	9	10
RF121	IRL	Cow	<i>egc</i>	+	33	3		5	7	8	9	10
RF122	IRL	Cow	SaPIbov, <i>egc</i>	+	33	3		5	7	8	9	10
RF123	IRL	Cow	Negative	–	11	3		5	7	8	9	10
RF285	IRL	Cow	SaPIbov	+	23	3		5	7	8	9	10
RF286	IRL	Cow	SaPIbov	+	23	3		5	7	8	9	10
2 (2521-3rd)	IRL	Cow	<i>seh</i>	ND	ND	3		5	7	8	9	10
6 (2242-3rd)	IRL	Cow	<i>seh</i>	ND	ND	3		5	7	8	9	10
7 (2242-3rd)	IRL	Cow	<i>seh</i>	ND	ND	3		5	7	8	9	10
10 (2242-4th)	IRL	Cow	<i>seh</i>	ND	ND	3		5	7	8	9	10
12 (2242-4th)	IRL	Cow	<i>seh</i>	ND	ND	3		5	7	8	9	10
14 (2480-4th)	IRL	Cow	<i>egc</i>	ND	ND	3		5	7	8	9	10
21 (2487-3rd)	IRL	Cow	Negative	ND	ND	3		5	7	8	9	10
22 (2487-3rd)	IRL	Cow	Negative	ND	ND	3		5	7	8	9	10
MSA12.1	USA	Cow	<i>egc</i> without <i>seg</i>	+	ND	3		5	7	8	9	10
MSA13.1	USA	Cow	ND	ND	31	3		5	7	8	9	10
MSA17.1	USA	Cow	<i>egc</i>	±	35	3		5	7	8	9	10
MSA103.14	USA	Cow	<i>egc</i>	+	ND	3		5	7	8	9	10
MSA148	USA	Cow	<i>egc</i> without <i>seg</i>	+	ND	3		5	7	8	9	10
MSA915	USA	Cow	<i>egc</i>	ND	16	3		5	7	8	9	10
MSA916	USA	Cow	Negative	–	24	3		5	7	8	9	10
MSA927	USA	Cow	Negative	–	ND	3		5	7	8	9	10
MSA930	USA	Cow	<i>egc</i> without <i>seg</i>	+	ND	3		5	7	8	9	10
MSA948	USA	Cow	Negative	ND	19	3		5	7	8	9	10
MSA1003	USA	Cow	Negative	–	16			5	7	8	9	10
MSA1006	USA	Cow	<i>sed, selj</i>	ND	3	3		5	7	8	9	10
MSA1058	USA	Cow	Negative	–	ND	3		5	7	8	9	10
MSA1363	USA	Cow	<i>egc</i>	+	6	3		5	7	8	9	10
MSA1369	USA	Cow	ND	ND	11	3		5	7	8	9	10
MSA1455	USA	Cow	Negative	–	ND	3		5	7	8		10
MSA1460	USA	Cow	<i>egc</i>	+	ND	3		5	7	8	9	10
MSA1468	USA	Cow	Negative	±	21	3		5	7	8	9	10
St65	ITA	Goat	Negative	ND	42	3		5	7	8	9	10
St66	ITA	Goat	Negative	ND	42	3		5	7	8	9	10
St24	ITA	Goat	<i>sec, tst</i>	ND	7		4	5	7	8	9	10

Table 3. cont.

Strain/isolate*	Origin	Host	Known characteristic		RAPD type§		ssl genell					
			SAg and SAg-like genes†	Mitogenicity‡								
1563-4	NOR	Goat	ND	ND	26	3	5	7	8	9	10	
895-1	NOR	Sheep	SaPIbov, <i>egc</i>	ND	29	3	5	7	8	9	10	
KH454	BEL	Rabbit	<i>egc</i>	ND	18	3	5	7	8	9	10	
KH275	BEL	Rabbit	Negative	ND	3	3	5	7	8	9	10	
KH365	BEL	Rabbit	<i>seh</i>	ND	1	3	5	7	8	9	10	
39	NIR	Chicken	<i>egc</i>	ND	2	3	5	7	8	9	10	
72	NIR	Chicken	<i>egc</i>	ND	2	3	5	7	8	9	10	

ND, Not done.

*Strains 2, 6, 7, 10, 12, 14, 21 and 22 were isolated from milk samples from cows with recent mastitis and were not included in Smyth *et al.* (2005); the numbers in parentheses refer to the cow and the udder quarter from which the milk sample was obtained.

†*sea* (*seb*, *sec*, *sed*, *seg*, *seh*), staphylococcal enterotoxin a (b, c, d, g, h); *selj* (*selk*, *selq*), staphylococcal enterotoxin-like j (k, q); *egc*, enterotoxin gene cluster comprising the *seg*, *sei*, *selm*, *seln*, *selo* genes, with either the *selu* gene or two pseudogenes *ψent1* and *ψent2* located between the *sei* and *seln* genes; SaPIbov, *S. aureus* pathogenicity island bovine possessing the *sec*-bovine, *sell* and *tst* genes (Fitzgerald *et al.*, 2001; Jarraud *et al.*, 2001; Letertre *et al.*, 2003).

‡Mitogenicity assay (ability to induce proliferation of human T-lymphocytes) results for 29 strains (Smyth *et al.*, 2005) are indicated. +, positive, –, negative, ±, borderline mitogenicity.

§Although a number of the bovine strains included herein had been previously RAPD typed by Fitzgerald *et al.* (1997, 2000), for the purposes of the present study all RAPD typing was redone. RAPD types were numbered to provide a simple designation of RAPD genotype for in-house isolate comparison. Bovine RAPD types 4, 5 and 7 of Fitzgerald *et al.* (1997) are equivalent to our RAPD types 16, 11 and 33, respectively.

||Not all of the genes encoded by the *ssl* locus were investigated; only the seven genes that lie in the variable region of the *ssl* locus were tested for.

of the *ssl4* gene with redesigned primers as it occupies the same position as the *set2* variant. The PCR assay has as yet to be extended to screen animal isolates for the *ssl6* gene (Fig. 1, strains NCTC 8325, MSSA-476 and MW2).

RAPD typing

A total of 26 of the 42 bovine isolates (16 Irish and 10 American) together with the 10 isolates from rabbits, sheep, goats and chickens were RAPD typed. Although isolates that showed identical RAPD fingerprints had the same complement of *ssl* genes (Table 3), the *ssl* gene pattern was not RAPD-type specific due to the high commonality in the *ssl* locus in the tested animal isolates.

Southern blot hybridization for *ssl* genes

The *ssl5*, *ssl7* and *ssl8* probes appeared to be specific for their target *ssl* genes. Southern blot hybridization experiments were performed on representative bovine isolates – isolates RF103 and MSA1369 (RAPD type 11), isolates RF115, RF116 and RF117 (RAPD type 16), isolates RF108 and RF122 (RAPD type 33) and isolates MSA1058 and MSA1369 (RAPD type not determined) – and human isolates MSSA-476, MRSA 252, COL and NCTC 6571. The clonally related isolates by RAPD typing showed similar *ssl* gene RFLPs, whereas unrelated strains had dissimilar RFLPs (data not shown).

DISCUSSION

In the current study, the *ssl* locus was found to be present in every animal-associated isolate, but showed minor variations in gene content by PCR and polymorphisms in RFLP patterns in Southern blots. The *ssl* locus had been reported in 49 *S. aureus* isolates from humans, 8 isolates from cows, 2 isolates from sheep, 2 isolates from poultry and 2 isolates of unknown species origin (Fitzgerald *et al.*, 2003). However, no group had looked for the individual *ssl* genes with the exception of the *set1* allelic variant of the archetypal gene *ssl7* (Williams *et al.*, 2000). The combination of *ssl* genes and their RFLP patterns in isolates of animal origin seem to reflect clonal relationships as revealed by RAPD typing, supporting the view that the locus is stable in the genome.

Twenty-nine of the isolates tested herein for *ssl* genes had been previously analysed for mitogenicity (Smyth *et al.*, 2005). Of these 12 were non-mitogenic and negative for 16 SAg and SAg-like genes, and 14 were mitogenic and encoded SAg and SAg-like genes (Table 3). One isolate (RF103) was mitogenic and negative for SAg and SAg-like genes (the *selp*, *selr*, *selu*, *selu2* and *selv* genes had not been tested for). The remaining two isolates exhibited borderline mitogenicity, one of which (isolate MSA17.1) possessed SAg and SAg-like genes, whereas the other (isolate MSA1468) did not. SAg and SAg-like gene-negative isolates (Table 3) that were shown to encode the *ssl* locus were non-mitogenic. The ability to stimulate T-cells in a Vβ-restricted manner is a

hallmark of SAg activity. While species-restricted effects cannot be ruled out given that the animal isolates were tested on human T-lymphocytes, Ssl proteins associated with isolates of animal origin would appear not to be mitogenic, and thus not superantigenic, on the basis of this correlative analysis, which is in agreement with previous authors (Arcus *et al.*, 2002; Fitzgerald *et al.*, 2003; Al-Shangiti *et al.*, 2004, 2005).

Langley *et al.* (2005) demonstrated that immobilized Ssl7 protein bound human IgA H-chain and L-chain, and human complement component C5 α and C5 β chains. Allelic variants of the Ssl7 protein were not altered in immunoglobulin- or complement-binding activities. These studies suggested that Ssl proteins are members of a much larger family of molecules with varied activities designed to bind serum components involved in host immunity. Thus, the Ssl proteins are likely involved in the interaction with *S. aureus* within various environments in different animal species. Allelic variation of the *ssl* gene cluster between animal-associated strains may confer an adaptive advantage to diverse conditions during infection of different animal tissues.

It has been demonstrated that strain COL expresses its *ssl* genes concurrently and that multiple *ssl* genes are expressed during human infection, as evidenced by Western immunoblotting using sera from acute and convalescent patients (Arcus *et al.*, 2002; Fitzgerald *et al.*, 2003; Al-Shangiti *et al.*, 2005). However, not all of the Ssl proteins are immunogenic. Expression of the *ssl* genes *in vitro* has yet to be shown for isolates from animal infection, and *in vivo* expression has not been demonstrated. Preliminary data using bovine sera from cows with and without mastitis has shown that the Ssl proteins, unlike proteins encoded by the *egc* (enterotoxin gene cluster) locus, are expressed in the host (Smyth, 2006). If multiple *ssl* genes are concurrently expressed during animal infection, the *ssl* genes may play an important role in staphylococcal pathogenicity, a suggestion given credence by the fact that every isolate tested herein had an *ssl* locus. As has been described for staphylococcal enterotoxins, such as Sec-ovine and Sec-bovine, it is possible that animal-specific alleles of the *ssl* genes exist, the gene products of which might have particular functions in the infection of different animal hosts, e.g. recombinant Ssl7 protein from a bovine-associated strain might show higher affinity for bovine IgA. *In vitro* and *in vivo* expression studies of *ssl* genes of animal strains and affinity studies of Ssl proteins from animal-associated strains for IgA and complement factor C5 of the same animal origin are merited.

Aguiar-Alves *et al.* (2006) have newly described a genotyping method based on single nucleotide polymorphisms within sequenced PCR-amplified internal fragments of the *set2*, *set5* and *set7* genes (*ssl4*, *ssl9* and *ssl2* genes, respectively). This method was applied to clinical methicillin-resistant and methicillin-susceptible *S. aureus* isolates allowing differentiation of 61 isolates into 22 distinct subgroups, designated exotoxin sequence types. As indicated herein,

RFLP patterns of selected *ssl* genes may have useful applications in epidemiology and typing, especially for laboratories without sequencing capabilities.

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