Associations between enterotoxin gene cluster types *egc1*, *egc2* and *egc3*, *agr* types, enterotoxin and enterotoxin-like gene profiles, and molecular typing characteristics of human nasal carriage and animal isolates of *Staphylococcus aureus*

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Twenty genes encoding enterotoxin and enterotoxin-like proteins have been described in *Staphylococcus aureus* strains. Five of these occur commonly in the enterotoxin gene cluster (*egc*: *selo*, *selm*, *sei*, *seln* and *seg*). In the *sei*–*seln* intergenic region, two pseudogenes, *ψent1* and *ψent2*, can be present or an additional gene designated *selu* or a variant *selu_v*. Whilst frequencies of loci bearing pseudogenes (*egc1*) or the *selu* gene (*egc2*) have been reported, the distinction between *selu*-bearing and *selu_v*-bearing (*egc3*) loci has rarely been made. A PCR-RFLP procedure involving cleavage of the *sei*–*seln* intergenic region by restriction endonuclease *BbvI* or *TseI* was developed that allowed differentiation of *selu*⁺ and *selu_v*⁺ loci. In addition, PCR primers were designed to yield a 203 bp amplicon for sequencing of a *selu* or *selu_v* intragenic region, which encompassed ten signature nucleotide differences. A total of 43 *egc*⁺ human nasal isolates and 53 *egc*⁺ bovine, ovine, caprine, leporine and gallinaceous isolates were *egc* typed and *agr* typed. None of the animal isolates was of *agr* type III. A total of 12 out of 17 *egc3*⁺ human nasal isolates were of *agr* type III, the other 5 being *agr* type I. On the basis of representative multilocus sequence typing, *agr* type III/*egc3*⁺ strains belonged to CC30. Human nasal isolates bearing an *egc1* locus were distributed evenly across *agr* types I, II and III. Only two nasal isolates had an *egc2* locus. All 14 *agr* type IV isolates, only 1 of which was of human origin, possessed an *egc2* locus. The *agr* IV nasal isolate was fusidic acid sensitive and was found to be ST123 (CC121). There were strong associations between bovine, leporine and gallinaceous *S. aureus* clonal types and *egc* locus types. The PCR-RFLP procedure was used to screen an additional 45 *S. aureus* isolates from dogs, cats, rats, pigs and horses for *egc* locus types. Of these, 33 were *egc*⁺. Six equine isolates were *selu*⁺. One canine and three porcine isolates possessed pseudogenes *ψent1* and *ψent2*. One porcine and one canine isolate each had the *selu_v* gene. Putative relationships between disease-causing propensity and *egc* type need (re-)evaluation.

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INTRODUCTION

Following the identification of the staphylococcal enterotoxins SEG and SEI by Munson *et al.* (1998), Monday & Bohach (2001) and Jarraud *et al.* (2001) revealed that the genes encoding these enterotoxins were part of a chromosomal operon, termed the enterotoxin gene cluster

(*egc*), comprising five genes and two pseudogenes now designated *selo*, *selm*, *sei*, *ψent1*, *ψent2*, *seln* and *seg*. Subsequently, Letertre *et al.* (2003) demonstrated that some *egc* clusters possess an additional gene designated *selu* instead of the two pseudogenes, arising from a 15 bp insertion in pseudogene *ψent1* and a single adenine deletion that abolishes the pseudogene *ψent1* stop codon.

Letertre *et al.* (2003) further reported that one isolate, 382F, harboured variant alleles of four genes, namely, *seg_v*, *sei_v*, *seln_v* and *selu_v*. The available *egc* sequence of strain 382F (GenBank accession no. AY158703) shows 100 % identity with that of the *egc* locus of the genome-sequenced strain MRSA252 (GenBank accession no. NC_002952;

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Abbreviations: MLEE, multilocus enzyme electrophoresis; MLST, multilocus sequence typing; RAPD, randomly amplified polymorphic DNA.

The GenBank/EMBL/DDBJ accession numbers for the sequences of the *egc* loci genes of *S. aureus* are EU885488–EU885496.

Holden *et al.*, 2004). Furthermore, the recently described *selm_v* gene (strain BK087, GenBank accession no. EF551341; Holtfreter *et al.*, 2007) shows 100 % identity with the corresponding gene in strain MRSA252. The *selo* gene of strain MRSA252 shows only 92 % identity to that of the archetypal *egc* locus of strain A900322 (GenBank accession no. AF285760; Jarraud *et al.*, 2001), confirming its status as a *selo_v* gene. Thus, three generally occurring *egc* loci have been described (genes/pseudogenes given in transcriptional order): *egc1* (*selo*, *selm*, *sei*, ψ *ent1*, ψ *ent2*, *seln*, *seg*), *egc2* (*selo*, *selm*, *sei*, *selu*, *seln*, *seg*) and *egc3* (*selo_v*, *selm_v*, *sei_v*, *selu_v*, *seln_v*, *seg_v*) (Fig. 1).

Thomas *et al.* (2006) observed further genetic variation within the *egc* locus. A recombination event between the *selm* and *sei* genes in strain A900624 produced a new gene designated *selv* (GenBank accession no. EF030427). In addition, a limited deletion event, namely, one adenine between the overlapping 5' end of the ψ *ent2* pseudogene and 3' end of the ψ *ent1* pseudogene, generated a novel gene, designated *selu2*, which differs from the *selu* and *selu_v* genes by the absence of the 15 bp insert (GenBank accession no. EF030428). Based on the 3303 nt *egc* locus sequence of strain A900624 (kindly provided by Dr Damien Y. Thomas, AbAg, Technopolis, Île-de-France, France), this *egc* locus, herein designated *egc4*, has the genes *selo*, *selv*, *selu2*, *seln* and *seg* (Fig. 1).

Whilst there has been a plethora of reports on the occurrence of *egc* genes in strains of *Staphylococcus aureus* of human and animal origin (e.g. Becker *et al.*, 2004; Chen *et al.*, 2004; Omoe *et al.*, 2005; Smyth *et al.*, 2005; Boerema *et al.*, 2006), few data exist on the relative occurrences of the *selu* or *selu_v* gene versus that of the ψ *ent1* and ψ *ent2* pseudogenes within the *egc* locus, let alone distinction of the *selu_v* gene from the *selu* gene (Letertre *et al.*, 2003; Blaiotta *et al.*, 2004, 2006; Bania *et al.*, 2006a, b; Chini *et al.*, 2006; Thomas *et al.*, 2006; Holtfreter *et al.*, 2007; Nashev *et al.*, 2007; Chiang *et al.*, 2008). Moreover, whereas the SEG and SEI enterotoxins are emetic in monkeys (Munson *et al.*, 1998), no comparative information is available on the emetic or superantigenic properties of variant enterotoxins. Accordingly, in assessing the potential role(s) of enterotoxins, enterotoxin-like proteins and their variants encoded by the *egc* locus in *S. aureus*-associated diseases and infections, distinction between the *egc* genotypes could potentially be important. The aims of the present study were to analyse the *sei*–*seln* intergenic regions of the *egc* loci of *S. aureus* of human nasal carriage and animal origins to determine the relative frequencies of occurrence of the pseudogenes ψ *ent1* and ψ *ent2*, and of the *selu* and *selu_v* genes, and to assess whether there were any associations between *agr* type, *egc* locus type, enterotoxin and enterotoxin-like genotypes and molecular typing features.

METHODS

Bacterial strains and growth conditions. The fully genome-sequenced *S. aureus* strains RF122 (*egc2*, ET3, ST151, CC151, Ridom

Fig. 1. Structure of the *egc* loci in *S. aureus*. Further details on the origins of strains, their years of isolation and literature references have been published previously (Collery & Smyth, 2007). The *egc1* sequence reported by Jarraud *et al.* (2001) is missing 69 nt, which was corrected by resequencing (Collery & Smyth, 2007). The database sequences for strains 383F, 352E and FRI137 were incomplete with respect to the PSE1/PSE4-amplified region, and were completed as described previously based on the complete sequence for strain R122 (Collery & Smyth, 2007). The sequences and their GenBank accession nos are: A900322 (AF285760 corrected by DQ993159), FRI157 (AF156894), Mu50 (BA000017), N315 (BA000018), RF122 (AJ938182), 383F (AY205306), 352E (AY205305), FRI137 (AY205307), 382F (AY158703), MRSA252 (BX571856) and A900624 (EF030427, EF030428; the 3303 nt *egc* locus sequence of strain A900624 kindly provided by Dr Damien Y. Thomas). Collery & Smyth (2007) have proposed that the *selu2* gene of the *egc4* locus be renamed the *selv* gene.

spa type t529, *agr* type II; Fitzgerald *et al.*, 2001; Smyth, 2006; Herron-Olson *et al.*, 2007), Mu50 (*egc1*, ST5, CC5, Ridom *spa* type t002, *agr* type II, SCCmec type II; Kuroda *et al.*, 2001; Kuhn *et al.*, 2006), MRSA252 (Sanger 252, *egc3*, ST36, CC30, *agr* type III, EMRSA16, SCCmec type II; Holden *et al.*, 2004; Kuhn *et al.*, 2006) and NCTC8325-4 (*egc*⁺), a derivative of strain NCTC8325 (ST8, CC8, *agr* type I, Ridom *spa* type t211), which is cured of prophages ϕ 11, ϕ 12 and ϕ 13 (Novick, 1967; Iandolo *et al.*, 2002), were used as control strains of known *egc* locus status. The latter three strains were kindly provided by T. J. Foster (Department of Microbiology, Trinity College Dublin). In addition, strain A900322 (*egc1*, *selp*, Ridom *spa* type t002), an isolate from a case of tampon-associated toxic shock syndrome, which yielded the archetypal *egc* locus sequence, was kindly provided by S. Jarraud (Université Lyon 1, Centre National de Référence des Staphylocoques, Lyon, France) (Jarraud *et al.*, 2001; Blaiotta *et al.*, 2006). Strain AB-8802 (*seg_v*, *sei_v*, Ridom *spa* type t021), originally isolated from raw poultry meat, was kindly supplied by G. Blaiotta (Dipartimento di Scienza degli Alimenti, Università degli Studi di Napoli 'Federico II', Portici, Italy) (Blaiotta *et al.*, 2004, 2006). *S. aureus* NCTC 6571 (Oxford strain) was used as a control strain in disc sensitivity tests with 10 μ g fusidic acid discs (Oxoid).

A total of 43 *egc*⁺ human nasal isolates, 41 of which have been shown previously to harbour the five common *egc* genes (*selo*, *selm*, *sei*, *seln*

Table 1. Known characteristics of human nasal and animal isolates of *S. aureus*

Animal origin/strain no.	No. of isolates	<i>agr</i> type	Classical <i>se</i> gene*	<i>egc</i> locus†	Other <i>se</i> and <i>sel</i> genes‡	<i>tst</i> gene
Human nasal						
MC29	1	I	<i>sea, seb</i>	+	–	–
MC48	1	I	<i>sea, seb</i>	–§	–	–
MC49	1	I	<i>sea, seb</i>	–§	<i>selk, selq</i>	–
MC20	1	I	<i>seb</i>	+	–	–
MC35, MC37	2	I	<i>seb</i>	+	<i>selp</i>	–
MC08, MC33	2	I	<i>seb</i>	+	<i>selk, selq</i>	–
MC09, MC10	2	I	<i>sec</i>	+	<i>sell</i>	–
MC04, MC42, MC45, MC50	4	I	–	+	–	–
MC40	1	I		+	<i>seh</i>	–
MC43	1	I	–	+	<i>seh, selp</i>	–
MC06	1	I	–	+	<i>sell</i>	–
MC23, MC28	2	II	<i>seb</i>	–§	–	–
MC18	1	II	<i>seb</i>	–§	<i>selp</i>	–
MC31, MC34, MC41	3	II	<i>seb</i>	+	<i>selp</i>	–
MC47	1	II	<i>seb</i>	+	–	–
MC30, MC39	2	II	–	+	<i>selp</i>	–
MC38	1	II	–	+	–	–
MC13	1	II	–	+ II	–	–
MC44	1	II	–	+ ¶	–	–
MC01, MC12	2	III	<i>sea</i>	+	–	+
MC03, MC07	2	III	<i>sea</i>	+	<i>seh</i>	–
MC15	1	III	<i>sea</i>	+	–	–
MC22	1	III	<i>seb</i>	+	<i>sell</i>	+
MC24	1	III	<i>seb</i>	+	–	+
MC14, MC17, MC19, MC36	4	III	<i>seb</i>	+	–	–
MC32	1	III	<i>seb</i>	+	<i>selp</i>	–
MC16	1	III	<i>seb, sec</i>	+	<i>sell</i>	+
MC02	1	III	<i>sec</i>	+	<i>sell</i>	+
MC27	1	III	–	+	<i>sell</i>	–
MC21, MC26, MC46	3	III	–	+	–	–
MC25	1	IV	<i>seb</i>	+	–	–
Cow#						
DS42	1	I	<i>sed</i>	+	<i>selj</i>	–
DS37, MSA915, MSA1363	3	I	–	+	–	–
RF80, RF102, RF108, RF110, RF113, RF122	6	II	<i>sec</i>	+	<i>sell</i>	+
K2DB9	1	II	<i>sec</i>	+	–	+
MSA1007	1	II	<i>sed</i>	+	<i>selj</i>	–
MSA10.6, MSA72.3, MSA103.14, MSA1460, RF114, RF120, RF121	7	II	–	+	–	–
MSA930, MSA1005, MSA1047	3	II	–	+ **	–	–
PSA5, PSA6.1, MSA12.1, MSA26.1, MSA31, MSA73.2, MSA148	7	IV	–	+ **	–	–
MSA1014	1	ND	<i>sec, sed</i>	–§	–	–
Chicken						
DS55 – DS67	13	II	–	+	–	–

Table 1. cont.

Animal origin/strain no.	No. of isolates	agr type	Classical <i>se</i> gene*	<i>egc</i> locus†	Other <i>se</i> and <i>sel</i> genes‡	<i>tst</i> gene
Rabbit						
DS31, DS44, DS45, DS46, DS53	5	IV	–	+	–	–
Sheep						
DS97, DS95	2	II	–	+	–	–
DS103	1	IV	–	+**	–	–
Goat						
DS26, DS27, DS30	3	I	–	+	–	–

ND, Not determined.

*Screened for the *sea*, *seb*, *sec*, *sed* and *see* genes.

†Screened for the *egc* locus genes *seg*, *sei*, *selm*, *seln* and *selo*.

‡Screened for the *seh*, *selj*, *selk*, *sell* and *selq* genes.

§*egc*[–] control isolates.

||*egc* locus missing the *sei* gene, i.e. gene not detected by PCR.

¶*egc* locus missing the *seln* and *selo* genes, i.e. gene not detected by PCR.

#RF isolates are from Ireland, MSA and PSA isolates are from the USA, DS42 is from Argentina, K2DB9 is from Denmark and DS37 is from Spain.

***egc* locus missing the *seg* gene, i.e. not detected by PCR.

and *seg*; referred to herein as a ‘complete’ locus) and 2 which were ‘missing’ (i.e. the genes were not detected by PCR) the *sei* gene, and the *seln* and *selo* genes, respectively (referred to herein as an ‘incomplete’ locus), were included (Collery *et al.*, 2008) (Table 1). In addition, five human nasal isolates lacking the *egc* locus were included as negative controls (Table 1). All isolates had been confirmed as *S. aureus* as described previously (Collery *et al.*, 2008).

A total of 53 *egc*⁺ isolates of *S. aureus* from cows (*n*=29), goats (*n*=3), chickens (*n*=13), rabbits (*n*=5) and sheep (*n*=3) from the study of Smyth *et al.* (2005) were used (Table 1). Of these animal isolates, 42 had a complete *egc* locus (33 of these possessed only the *egc* locus) and 11 (10 from cows and 1 from a sheep) had an incomplete *egc* locus. Of the bovine isolates, 9 were from the Republic of Ireland, 17 were from the USA and 1 each was from Argentina, Denmark and Spain. All of the chicken isolates were from Northern Ireland. The three goat isolates were from Italy. Two of the sheep isolates were from Denmark and one was from Sweden. Of the rabbit isolates, four were from Belgium and one was from Spain. Bovine isolate MSA1014 from the USA (Table 1) was used as an *egc*[–] control animal strain. All isolates had been confirmed as *S. aureus* as described previously (Smyth *et al.*, 2005).

A further 44 fresh *S. aureus* isolates were obtained from animals courtesy of N. Leonard (Veterinary School, University College Dublin, National University of Ireland, Dublin, Republic of Ireland). A total of 3 isolates were from cats (1 nasal isolate and 2 from wounds), 6 from dogs (2 from skin, 2 nasal isolates and 2 from wounds), 28 from horses (26 nasal isolates, 1 from a wound and 1 from a joint), 3 from rats (nasal isolates) and 4 from pigs (3 nasal isolates and 1 from a joint). The identification of these isolates was confirmed by Gram staining, a tube coagulase test, the API ID32 Staph system (bioMérieux) and the detection of clumping factor, protein A and capsular polysaccharide using a Pastorex Staph Plus latex agglutination kit (Bio-Rad Laboratories). In addition, an *S. aureus* isolate from a cat was obtained from E. Olsson (Swedish Zoonosis Centre, National Veterinary Laboratory, Uppsala, Sweden).

DNA extraction. Preparation of genomic DNA from *S. aureus* was performed as described previously (Fitzgerald *et al.*, 1997). The procedure involved lysostaphin lysis, proteinase K treatment, addition of EDTA, sarkosyl and cetyltrimethylammonium bromide, extraction with chloroform:isoamyl alcohol and phenol:chloroform:isoamyl alcohol, and precipitation with ethanol. The quantity and quality of the extracted DNA were checked by running 1 µl DNA preparation on a 1% (w/v) agarose gel alongside DNA standards of known concentration (2 log DNA ladder; Promega).

PCR. The sets of primers used for detection of the *selu* or *selu_v* gene and the *ψent1* and *ψent2* pseudogenes, namely, PSE1/PSE4, PSE2/PSE4 and PSE2/PSE6, have been described by Letertre *et al.* (2003) and were used according to Collery & Smyth (2007). The primer set PSE1/PSE4 was designed to amplify the *egc* locus region encoding the pseudogenes *ψent1* and *ψent2*, and either the *selu* or *selu_v* gene from the stop codon of the *sei* gene to the first 172 nt of the 5′ end of the *seln* gene, yielding amplicons of 1134 and 1148 bp, respectively. Nucleotides 13–27 of the 27 nt primer PSE2 matched the specific 15 nt insert in the *selu* and *selu_v* genes. PCR assays with the primer sets PSE2/PSE4 and PSE2/PSE6 yielded amplicons of 790 and 142 bp, respectively, when either the *selu* or the *selu_v* gene was present. DNA preparations from strains A900322, Mu50, RF122, MRSA252 and NCTC8325-4 were run as controls as appropriate.

The primers *Sei*_rev-RC (5′-CAGGAGATGGACTGCCTG-3′) and *Seln*_for-RC (5′-GCAGCTATGTAGAACAATCTCATAAGC-3′) were designed to amplify the region between the *sei* and *seln* genes when the primers PSE1 and PSE4 were unable to generate a PCR product. These primers were the reverse complements of the two primers originally described by Monday & Bohach (1999) and Smyth *et al.* (2005) for amplification of the *sei* and *seln* genes, respectively. By taking the reverse complements of the 3′ *sei* primer and the 5′ *seln* primer, these primers annealed outwards from the genes they were originally designed to amplify and were orientated towards each other to amplify the *sei*–*seln* intergenic region, yielding an amplicon of 1095 bp. The PCR conditions used for these primers were 45 cycles of

94 °C for 30 s, 62 °C for 30 s and 72 °C for 30 s. In addition, the SEU-1 and SEU-2 primers described by Nashev *et al.* (2007) were tested for amplification within the *egc* loci using the PCR conditions of these authors.

Based on gene alignments for strains RF122, 352E, FRI137 and 383F (*selu*⁺), and strains MRSA252 and 382F (*selu*_v⁺), a region with ten conserved nucleotide differences between the *selu* and *selu*_v genes was identified between nt 440 and 520 (Table 2). However, it is important to note that the only difference between the *selu*_v sequences in this discriminatory intragenic region and the pseudogene ψ ent1 and ψ ent2 sequences of strains Mu3, Mu50, N315 and A900322 is a T479→A465 transversion (corresponding nucleotide positions in the alignment). To distinguish isolates bearing either the *selu* or *selu*_v gene, universal primers that amplified between nt 382 and 584 of the *selu* and *selu*_v genes were designed: *selu*(v)-for (5'-AAAACGTGTATGTATGGCG-3') and *selu*(v)-rev (5'-TTATGCTTAAGTAAATAGTTTCTAACTT-TATC-3').

An *agr* group-specific multiplex PCR was performed using the primers and thermal cycling conditions described by Gilot *et al.* (2002). With the exception of strain RF122 (*agr* type II) (Fitzgerald

et al., 2000), the following *agr* control strains were provided by T. J. Foster and A. Rossney (National MRSA Laboratory, St James's Hospital, Dublin, Republic of Ireland): strains Newman, COL and NCTC8325-4 (*agr* type I); strains MRSA252 and MW2 (*agr* type III); and strain RN4850 (*agr* type IV).

The standard nomenclature for SE and SEI gene designations recommended by Lina *et al.* (2004) has been used throughout. All of the previously characterized human and animal isolates have been screened for the 15 SE and SEI genes *sea-see*, *seg-sei*, *selj-selo* and *selq*, and for the *tst* gene using multiplex and singleplex PCR protocols (Smyth *et al.*, 2005; Collery *et al.*, 2008). The human nasal isolates and isolates from chickens, goats, sheep and rabbits were further screened for the *seI* gene using primers and PCR conditions described by van Wamel *et al.* (2006).

In silico analyses of the *sei-seI* intergenic regions of *egc* loci bearing *selu* and *selu*_v genes. FASTA files of DNA sequences of *egc* loci containing *selu* and *selu*_v genes were retrieved from the National Center for Biotechnology Information nucleotide database. PSE1/PSE4-amplified sequences were analysed using RestrictionMapper version 3 (<http://www.restrictionmapper.org>) to identify restriction

Table 2. Nucleotides differences between *selu* and *selu*_v genes and pseudogenes ψ ent1 and ψ ent2 of reference *S. aureus* strains

Nt position*	Strain and nucleotide difference			Nt position*	Nucleotide difference for strains Mu50, N315, FRI572 and A900322 (ψ ent1, ψ ent2)
	RF122, 352E (<i>selu</i>)	FRI137†, 383F (<i>selu</i>)	MRSA252, 382F (<i>selu</i> _v)		
74	G	G	A‡	74	A§
106	A	T	A	106	A
112	C	T	C	112	C
141	C	C	T‡	141	T§
161	C	C	T‡	161	C
173	C	C/T†	C	173	C
267	A	A	T‡	252	A
298	G	G	A‡	283	G
325	A	A	T‡	310	A
442	C	C	T‡	428	T§
443	T	T	C‡	429	C§
477	A	A	T‡	463	T§
479	A	A	T‡	465	A
480	C	C	T‡	466	T§
489	A	A	G‡	475	G§
495	C	C	T‡	481	T§
496	A	A	T‡	482	T§
514	T	T	C‡	500	C§
519	C	C	T‡	505	T§
524	A	T	A	510	A
619	A	T	A	605	A
649	A	G	G	635	G
657	A	T	T	643	T

*Nucleotides numbered with respect to the start codons of the *selu* and *selu*_v genes and pseudogene ψ ent1; the *selu* and *selu*_v gene sequences differ from pseudogene ψ ent1 and ψ ent2 sequences by the insertion of 15 nt after nt 218 of pseudogene ψ ent1 and a single adenine deletion corresponding to nt 371 of pseudogene ψ ent1 that abolishes the pseudogene ψ ent1 stop codon.

†Strain FRI137 differs at nt 173 from all other reference strains by a C→T transition.

‡Nucleotide sequence differences of *selu*_v⁺ strains MRSA252 and 382F from *selu*⁺ strains RF122, 352E, FRI137 and 383F.

§Shared nucleotide sequence differences of pseudogene ψ ent1 and ψ ent2 strains and *selu*_v⁺ strains from the four *selu*⁺ strains RF122, 352E, FRI137 and 383F.

||Nucleotide sequence differences between *selu*⁺ strains RF122 and 352E and *selu*_v⁺ strains FRI137 and 383F.

enzyme cleavage sites. The CLC Free Workbench software version 4.5.1 (<http://www.clcbio.com>) was used to align DNA sequences of the PSE1/PSE4 amplimers of *egc* loci.

PCR-RFLP analysis of PSE1/PSE4-amplified PCR products of the *egc* locus. The PCR-RFLP procedure of Collery & Smyth (2007) was used. Endonuclease *Hind*III cleaves PSE1/PSE4 amplimers containing pseudogenes ψ ent1 and ψ ent2, but not those containing either the *selu* or *selu_v* gene. In contrast, endonuclease *Hph*I cuts PSE1/PSE4 amplimers containing either the *selu* or *selu_v* gene, but not those with pseudogenes ψ ent1 and ψ ent2 (Collery & Smyth, 2007).

For PCR-RFLP analysis using endonuclease *Bbv*I cleavage of PSE1/PSE4 amplimers containing *selu* and *selu_v* genes, the standard reaction mixture was modified to contain 3 μ l 10 $\times$ NEBuffer 2 [50 mM NaCl, 10 mM Tris/HCl (pH 7.9), 10 mM MgCl₂, 1 mM DTT; NEB] and 1 μ l *Bbv*I restriction endonuclease (2 U μ l⁻¹; NEB). The *Bbv*I digestion mixture was incubated overnight at 37 °C. For *Tse*I digestion of PSE1/PSE4 amplimers containing *selu* and *selu_v* genes, the incubation was performed at 65 °C. The standard reaction mixture contained 3 μ l NEBuffer 3 [100 mM NaCl, 50 mM Tris/HCl (pH 7.9), 10 mM MgCl₂, 1 mM DTT; NEB] and 1 μ l *Tse*I restriction endonuclease (3 U μ l⁻¹; NEB).

Sequencing of *selu_v* genes and intragenic *selu* or *selu_v* amplimers. Two *selu_v*⁺ isolates identified in this study by PCR-RFLP analysis of PSE1/PSE4 amplimers of the *egc* locus, comprising human nasal isolate MC15 (ST30, *agr* type III) belonging to MLVA population IIa (Collery *et al.*, 2008) and strain AB-8802 (Blaiotta *et al.*, 2004), were selected for gene sequencing to confirm their status. PCR was carried out using the PSE1/PSE4 primer pair of Letertre *et al.* (2003) with *Taq* DNA polymerase. Intragenic 203 bp PCR products using primers *selu*(v)-for/*selu*(v)-rev were obtained for strains MC15, MC20, MC40 and MSA1047. These were purified using a High Pure PCR product purification kit (Roche) and sequenced by GATC Biotech.

The derived sequences were aligned with *selu* gene sequences from GenBank of strains RF122 (bovine mastitis; GenBank accession no. NC_007622), 352E (farm fromage frais; GenBank accession no. AY205305), 383F (rice salad; GenBank accession no. AY205307) and FRI137 (thigh abscess, Ridom *spa* type t352; GenBank accession no. AY205306), and with *selu_v* gene sequences of strains MRSA252 (post-operative infection; GenBank accession no. NC_002952) and 382F (water, Ridom *spa* type t166; GenBank accession no. AY158703), and with ψ ent1 and ψ ent2 pseudogene sequences of strains Mu50 (surgical wound; GenBank accession no. NC_002758), N315 (pharyngeal smear; GenBank accession no. NC_002745), FRI572 (nasal isolate, Easter Island; GenBank accession no. AF156894) and A900322 (tampon isolate; GenBank accession no. AF285760 as corrected by DQ993159) using the CLC Free Workbench software version 4.5.1.

RESULTS AND DISCUSSION

Distribution of pseudogenes ψ ent1 and ψ ent2 and the *selu* or *selu_v* gene among *egc*⁺ *S. aureus* isolates of human and animal origin

Using the PCR primer sets PSE1/PSE4, PSE2/PSE4 and PSE2/PSE6 (Letertre *et al.*, 2003), the genome-sequenced control strains Mu50 (*egc*1), RF122 (*egc*2) and MRSA252 (*egc*3), and the archetypal *egc*⁺ strain A900322 (*egc*1) yielded amplimers consistent with their known *egc* locus status. Neither DNA from control strain NCTC8325-4 (*egc*⁻) nor DNA from the five human nasal isolates (MC18,

MC23, MC28, MC48 and MC49) and the one bovine isolate (MSA1014) lacking an *egc* locus gave amplimers with any of these primer pairs.

Of the 43 *egc*⁺ human nasal isolates, 18 had either the *selu* or the *selu_v* gene, and 16 had the pseudogenes ψ ent1 and ψ ent2. The nine remaining isolates, seven with a complete *egc* locus and two with incomplete *egc* loci (MC13 missing the *sei* gene and MC44 missing the *seln* and *selo* genes) either failed to yield amplimers or did not yield amplimers of the expected size of ~1150 bp with the PSE1/PSE4 primer pair, despite repeated attempts, even after adjustments to the PCR conditions. Anomalously sized PSE1/PSE4 amplimers of ~500 bp (three isolates), ~1100 bp (two isolates) and ~3000 bp (four isolates) were occasionally but not reproducibly obtained. Blaiotta *et al.* (2006) also reported obtaining odd-sized amplimers using the primers of Letertre *et al.* (2003) with both human and food isolates of *S. aureus* belonging to their restriction endonuclease analysis *egc* groups 1 and 4, both of which possess pseudogenes ψ ent1 and ψ ent2.

The nine isolates that were refractory to yielding PSE1/PSE4 amplimers were rechecked for the presence of the *sei* and *seln* genes with individual primer pairs (Smyth *et al.*, 2005), with positive findings in all expected instances. Primers *Sei*-rev-RC and *Seln*-for-RC were used as an alternative strategy to amplify the *sei*-*seln* intergenic regions of these PSE1/PSE4-refractory isolates. Control strains MRSA252, Mu50 and RF122 yielded amplimers of ~1100 bp. This approach, followed by sequencing of the resulting amplimers, resolved the nature of the *egc* loci of a further two nasal isolates – MC14 (595 nt alignment; GenBank accession no. EU885495) possessing pseudogenes ψ ent1 and ψ ent2, and MC33 (618 nt alignment; GenBank accession no. EU885494) harbouring the *selu_v* gene. The SEU-1 and SEU-2 primers of Nashev *et al.* (2007) were utilized with the remaining seven isolates in a further attempt to obtain amplimers suitable for sequencing. Strain MRSA252, used as a positive control, yielded an amplimer of the expected size of ~500 bp, but the seven test nasal carriage strains did not. Of these refractory isolates, two were ST6 and ST932, both belonging to CC6 and of *agr* type I, one was ST937, a singleton and *agr* type II, and one was ST933, belonging to CC30 and of *agr* type III. Of the remaining three isolates, one was of *agr* type I and two were of *agr* type II, none of which had been typed by multilocus sequence typing (MLST).

Thus, 19/43 (44.2%) *egc*⁺ human nasal isolates were confirmed as possessing either the *selu* or the *selu_v* gene, and 17 (39.5%) had the pseudogenes ψ ent1 and ψ ent2. The recalcitrant isolates may have mutations or insertion sequences (Rve-like 1-transposase 8 and Rve-like 2-*IstB*-like; Thomas *et al.*, 2006) in their *sei*-*seln* intergenic sequences, or they may have deletions in their *sei*-*seln* intergenic regions or may genuinely lack the *selu* or *selu_v* gene or the pseudogenes ψ ent1 and ψ ent2.

A total of 28 of the 53 *egc*⁺ bovine, ovine, caprine, leporine and gallinaceous isolates possessed either the *selu* or *selu_v*

gene, and 25 isolates possessed the pseudogenes ψ ent1 and ψ ent2. The frequencies of either the *selu* or *selu_v* gene for isolates from the different animal species were: cows (21/29, 72.4 %), goats (0/3, 0 %), rabbits (5/5, 100 %), sheep (2/3, 67 %) and chickens (0/13, 0 %), whilst the remainder possessed pseudogenes ψ ent1 and ψ ent2. The ten bovine isolates and one ovine isolate that had previously been shown to have an *egc* locus with a missing *seg* gene by PCR tests (Table 1) (Smyth *et al.*, 2005) all had either the *selu* or *selu_v* gene. Strain AB-8802 from raw poultry meat was found to be either *selu*⁺ or *selu_v*⁺.

To verify the nature of the *egc* locus in the human and animal isolates, the PCR-RFLP differential endonuclease susceptibility assay developed by Collery & Smyth (2007) was employed. Control strains A900322, Mu50, RF122 and MRSA252 gave the expected differential endonuclease fragment profiles (Collery & Smyth, 2007). PSE1/PSE4 amplimers from the animal isolates and human nasal isolates that were pseudogene-positive were cleaved by restriction enzyme *Hind*III, but not by endonuclease *Hph*I. In contrast, all PSE1/PSE4 amplimers from the animal and human nasal isolates bearing either the *selu* or *selu_v* gene were cleaved by endonuclease *Hph*I, but not by endonuclease *Hind*III. Strain AB-8802 was confirmed as either *selu*⁺ or *selu_v*⁺ on the basis of PCR-RFLP analysis.

Few studies have reported the relative frequencies of *egc* genotypes among *S. aureus* isolates (Bania *et al.*, 2006a; Chini *et al.*, 2006; Vancraeynest *et al.*, 2006a). However, none of these studies differentiated their '*selu*'-containing loci as either *egc2* or *egc3*, and accordingly reference to an *egc2* locus in these publications should be taken to infer an *egc2* or *egc3* locus (*selu*⁺ or *selu_v*⁺). Thomas *et al.* (2006) reported the *egc1* locus in 409/421 isolates (97.1 %) of nasal and infection origins having at least one *egc*-associated gene. However, the forward (yent1: 5'-ACGTAGATTTGTTGGGACAAACT-3') and reverse (yent2: 5'-GTGCTGTTATGTTTTCTTATTAGTAGG-3') primers used by these authors have 100 % sequence matches for *egc1*, *egc3* and *egc4* loci sequences of strains Mu50 (ψ ent1⁺ ψ ent2⁺), MRSA252 (*selu_v*⁺) and A900624 [*selu*2⁺ (*selu*⁺)], respectively. In the case of *selu* sequences, whilst primer yent1 matches those in the National Center for Biotechnology Information database for *egc2* loci, the 28 nt yent2 reverse primer has a 3'-end G/T mismatch with the sequences for strains RF122, 352E, 383F and FRI137. For primers with T, C or G as the 3' nucleotide, *Taq* DNA polymerase is highly specific for template complementarity to this base and, because it lacks 3'→5' exonuclease activity, this enzyme cannot correct mismatches at the primer 3' terminus (Ayyadevara *et al.*, 2000). Nonetheless, some primers with 3'-end G/T mismatches have been demonstrated to be as efficient in PCR as the normal primer with a 3'-end G/C match (Simsek & Adnan, 2000). Thus, it seems entirely possible that many of the 409 isolates of Thomas *et al.* (2006) purported to possess an *egc1* locus may in fact have an *egc3* locus, or possibly even an *egc2* locus.

Occurrence of *selu* and *selu_v* genes among human nasal and animal *S. aureus* isolates

At the time of this investigation, the only method for distinguishing the *selu* and *selu_v* genes was DNA sequencing. The DNA sequences of the *selu* genes of strains RF122 and 352E are identical, and differ from the *selu* gene sequences of strains FRI137 and 383F at six nucleotide positions (Table 2). In contrast, whilst the sequences of the *selu_v* genes of strains MRSA252 and 382F are identical, they differ from those of the 4 annotated *selu* genes at 16 conserved nucleotide positions (Table 2). Whilst 11 of these *selu_v* nucleotide differences are shared with pseudogenes ψ ent1 and ψ ent2, it seemed that it might be possible to distinguish the *selu* gene from the *selu_v* gene on the basis of differential endonuclease susceptibility, as has been achieved for pseudogenes ψ ent1 and ψ ent2, and either a *selu* or *selu_v* gene (Chini *et al.*, 2006; Collery & Smyth, 2007).

The PSE1/PSE4-amplified sequences bearing the above-mentioned six reference *selu* and *selu_v* genes were analysed using the RestrictionMapper 3 program in comparison with known PSE1/PSE4-amplified sequences bearing the pseudogenes ψ ent1 and ψ ent2 (Fig. 2, Table 3). Two endonucleases yielded promising *in silico* findings, namely, *Bbv*I and *Tse*I. Whilst these endonucleases do not distinguish strains with *egc* loci bearing pseudogenes ψ ent1 and ψ ent2 from those bearing the *selu_v* gene, both enzymes

Fig. 2. Schematic diagram of PSE1/PSE4-amplified PCR amplimers bearing the *selu* or *selu_v* gene, showing the cleavage sites for restriction endonucleases *Bbv*I and *Tse*I (indicated by arrows) above and below each PCR product diagram, respectively, with the sizes of the fragments in bp. The black bars shown within the *selu* and *selu_v* genes correspond to the signature 15 bp insert in the ψ ent1 pseudogene. The 5'-cleavage site for *Tse*I is between nucleotides G-41 and C-42 of the *selu* and *selu_v* genes yielding a 194 bp fragment, and that for *Bbv*I is between T-53 and T-54 of the *selu* and *selu_v* genes yielding a 206 bp fragment. An additional *Bbv*I cleavage site lies between T-7 and T-8 of the *selu_v* gene. For *Tse*I, this additional site lies between G-19 and C-20 of the *selu_v* gene. Thus, the distinction of *selu*-bearing from *selu_v*-bearing PSE1/PSE4 sequences is made possible by the co-occurrence of variant genes in the *egc3* cluster (Letertre *et al.*, 2003; Blaiotta *et al.*, 2004; Holden *et al.*, 2004; Holtfreter *et al.*, 2007).

Table 3. *In silico* analysis of restriction endonuclease cleavage of the *sei*–*seln* intergenic sequences of the *egc* locus between primers PSE1 and PSE4 in strains known to possess *egc1*, *egc2* and *egc3* loci

Restriction endonuclease	Size (bp) of restriction fragments of PSE1/PSE4 regions for the indicated strains*		
	A900322/Mu50/N315/FRI572 (1135 nt <i>egc1</i>)	RF122/352E/FRI137/383F (1149/1150 nt <i>egc2</i>)†	382F/MRSA252 (1149 nt <i>selu_v</i>)
<i>BbvI</i>	763	942	777
	206	206/207†	206
	165		165
<i>TseI</i>	788	954	802
	194	194/195†	194
	152		152

*For GenBank accession numbers see Fig. 1.

†The second value is that for restriction fragments of strain 383F, the PSE1/PSE4 amplicon of which is 1 nt longer due to the insertion of a thymine in the *sei*–*selu* intergenic region between T-68 and T-70.

exhibit differential cleavage of PSE1/PSE4-amplified sequences from *egc2*⁺ and *egc3*⁺ strains, the *selu*-bearing sequence yielding two fragments and the *selu_v*-bearing sequence yielding three fragments with both enzymes (Table 3). The presence of a 942 or 954 bp fragment in endonuclease-cleaved PSE1/PSE4-amplified sequences from *selu*⁺ isolates in contrast to 777 and 165 bp or 802 and 152 bp fragments in endonuclease-cleaved PSE1/PSE4-amplified sequences from *selu_v*⁺ isolates can be used to distinguish the *egc2* and *egc3* loci (Fig. 2, Table 3), provided that a 'selu'-type gene has been shown to be present using the primers of Letertre *et al.* (2003).

To test the *in silico* findings, PSE1/PSE4 amplicons from the genome-sequenced strains RF122 (*selu*) and MRSA252 (*selu_v*) were digested with endonucleases *BbvI* and *TseI*. In both cases, the endonucleases allowed distinction according to the *in silico* criteria. The PSE1/PSE4 amplicons for the 28 animal and 18 human isolates possessing either a *selu* or *selu_v* gene were cut using *BbvI*. Of the 18 human nasal isolates, only two had a *selu* gene (*egc2* locus).

Regarding the 16 *selu_v*⁺ (*egc3*⁺) human nasal isolates identified by PCR-RFLP and the single *selu_v*⁺ isolate identified by PCR with reverse complement primers, 5 were of *agr* type I (MC08, MC33, MC35, MC37 and MC42 – none of these had been MLST typed), 4 of which were *seb*⁺ (2 of these having in addition the *selk* and *selq* genes and 1 the *selp* gene), and 12 were of *agr* type III (MC01, MC03, MC07, MC12, MC15, MC17, MC19, MC24, MC26, MC27, MC36 and MC46), 5 of which had the *sea* gene (2 in addition to the *seh* gene and 2 with the *tst* gene) and 4 of which had the *seb* gene (1 in addition to the *tst* gene). Of the three *egc3*⁺ isolates of *agr* type III that lacked a classical enterotoxin-encoding gene, one possessed the *sell* gene.

All 28 of the bovine, ovine and leporine isolates had the *selu* gene. Of these, 20 (13 from cows, 5 from rabbits and 2 from sheep) had only an *egc* locus, 11 of which (bovine and ovine) were missing the *seg* gene (Smyth *et al.*, 2005). Of

the remaining eight bovine isolates, six also possessed the pathogenicity island SaPI_{bov} (*sec*, *sell*, *tst*), one had the *sec* and *tst* genes, and one had the *sed* and *selj* genes (Fitzgerald *et al.*, 2001; Smyth *et al.*, 2005). Strain AB-8802 was found to possess the *selu_v* gene.

To confirm the identity of the *selu_v* genes, PSE1/PSE4 amplicons of two *selu_v*⁺ isolates, human nasal isolate MC15 (Coltery *et al.*, 2008) and strain AB-8802 (Blaiotta *et al.*, 2004), were selected for single-strand sequencing. When these sequences were compared with the *selu* gene sequences of strains RF122, 352E, 383F and FRI137, and with the *selu_v* gene sequences of strains MRSA252 and 382F, strain AB-8802 (680 nt alignment; GenBank accession no. EU885493) and isolate MC15 (668 nt alignment; GenBank accession no. EU885496) gave 100 % matches to those of the *selu_v* genes.

Based on gene alignments for strains RF122, 352E, FRI137 and 383F (*selu*⁺), and strains MRSA252 and 382F (*selu_v*⁺), 10 of the 16 conserved nucleotide differences between the *selu* and *selu_v* genes occurred between nt 440 and 520 (Table 2). Universal primers designed to amplify a 203 bp fragment encompassing the 80 bp *selu*/*selu_v* discriminatory intragenic region were used to obtain PCR products for control strains MRSA252, RF122 and Mu50, for four nasal isolates (MC20 – *ψent1* and *ψent2*, GenBank accession no. EU885488; MC40 – *selu*, GenBank accession no. EU885489; and MC15 and MC36 – *selu_v*, GenBank accession nos EU885490 and EU885492, respectively) and for one bovine strain (MSA1047 – *selu*, GenBank accession no. EU885491). The sequences obtained were aligned with those of the reference *selu* strains RF122 and FRI137, reference *selu_v* strain MRSA252, and reference *ψent1* and *ψent2* pseudogene sequences of strains Mu50, N315, FRI572 and A900322. Although the obtained sequences varied in length (127–145 nt), all included eight of the ten target differential nucleotides from nt 477 to 519. These alignments confirmed the identity of the test strains as

Table 4. Distribution of the pseudogenes $\psi ent1$ and $\psi ent2$ (*egc1*), the *selu* gene (*egc2*) and the *selu_v* gene (*egc3*) among animal and human nasal isolates of *S. aureus*

<i>agr</i> type	No. in human nasal isolates				No. in animal isolates		
	<i>egc1</i> *	<i>egc2</i> †	<i>egc3</i> ‡	<i>egc</i> not determined	<i>egc1</i> §	<i>egc2</i>	<i>egc3</i>
I	6	1	5	3	6	1	0
II	6	0	0	3	19	14	0
III	5	0	12	1	0	0	0
IV	0	1	0	0	0	13	0
Total	17	2	17	7	25	28	0

*Of the *agr* type I isolates, three had the *sell* gene (two of which also had the *sec* gene), one had the *seb* gene and two were *egc* only; of the *agr* type II isolates, three possessed the *seb* gene and three were *egc* only; of the *agr* type III isolates, three had the *seb* gene (one in addition to the *sell* and *tst* genes), one the *sec*, *sell* and *tst* genes, and one was *egc* only.

†The *agr* type I isolate also had the *seh* gene, whilst the *agr* type IV isolate also possessed the *seb* gene.

‡Of the *agr* type I isolates, one was *egc* only and four possessed the *seb* gene (two of which had the *sell* and *selq* genes in addition); of the *agr* type III isolates, two were *egc* only, one had the *egc* locus plus the *sell* gene, five possessed the *sea* gene (two with the *seh* gene and two with the *tst* gene) and four had the *seb* gene, one with the *tst* gene.

§This group comprised 13 chicken (all of *agr* type II), 1 sheep (*agr* type II), 3 goat (all of *agr* type I) and 8 bovine isolates (3 of *agr* type I and 5 of *agr* type II).

||This group comprised 2 sheep (1 each of *agr* types II and IV), 5 rabbit (all *agr* type IV) and 21 bovine isolates (1 of *agr* type I, 13 of *agr* type II and 7 of *agr* type IV), including all 10 bovine isolates with an *egc* locus missing the *seg* gene, 3 of which were of *agr* type II and 7 of *agr* type IV.

possessing the pseudogenes $\psi ent1$ and $\psi ent2$, the *selu* gene or the *selu_v* gene.

To date, the *egc3* locus has only been reported in isolates from food (Letertre *et al.*, 2003; Blaiotta *et al.*, 2004), although genome sequencing has identified strain MRSA252 as being *egc3*⁺ (Holden *et al.*, 2004). Chini *et al.* (2006) and Collery & Smyth (2007) have demonstrated the usefulness of PCR-RFLP analysis with restriction endonucleases *Hind*III, *Hph*I and *Bcl*I for distinction of *egc* loci containing pseudogenes $\psi ent1$ and $\psi ent2$ from those harbouring either the *selu* or the *selu_v* gene. The present investigation extended this PCR-RFLP analysis to allow distinction of *egc* loci harbouring the *selu* and *selu_v* genes using restriction enzymes *Bbv*I and *Tse*I. Alternatively, Blaiotta *et al.* (2004, 2006) demonstrated that PCR in conjunction with restriction endonuclease analysis of a 3375 bp region encompassing 47 nt upstream of the 3' end of the *selm* gene to 610 nt downstream of the 5' end of the *seg* gene could be used to distinguish *egc* loci. However, the PCR-RFLP procedures developed by Collery & Smyth (2007) and in this study have the advantage of specifically examining differences in the *sei*–*seln* intergenic region of interest.

Clonality of *S. aureus* isolates and *egc* locus types

The distribution of *egc* locus types according to the *agr* genotypes of the 43 human and the 53 bovine, ovine, caprine, leporine and gallinaceous isolates are shown in Table 4. None of the animal isolates was of *agr* type III. All

14 *agr* type IV isolates, only one of which was of human origin, possessed an *egc2* locus.

The human nasal isolates that possessed *egc1* loci were evenly distributed between *agr* types I, II and III. These strains were also evenly distributed between the previously described MLVA (sub)populations: four in cluster I, three in subcluster IIa, two in subcluster IIb, three in subcluster IIc and five subcluster IId, with the *agr* type III isolates being confined to MLVA subpopulations IIa and IIb (Collery *et al.*, 2008). The distribution of the *egc3*⁺ isolates among the MLVA clusters and subclusters was: two in cluster I, ten in subcluster IIa, two in subcluster IIb, two in subcluster IIc and one in subcluster IId (Collery *et al.*, 2008). Four of the ten isolates in MLVA subcluster IIa had been MLST typed (MC15 and MC26 as ST30, MC19 as ST484 and MC24 as ST938, all of which belong to CC30). The two *egc2*⁺ isolates belonged to MLVA cluster I (isolate MC40 – ST934, singleton, *agr* type I) and MLVA subcluster IIa (isolate MC25 – ST123, CC121, *agr* type IV). The latter isolate was tested for fusidic acid resistance using a 10 µg disc in comparison with *S. aureus* strain Oxford. Both gave identical zones of growth inhibition, indicating that strain MC25 was fusidic acid sensitive. Thus, the latter isolate is a related clonal variant of the epidemic European fusidic acid-resistant impetigo strain (O'Neill *et al.*, 2007; Larsen *et al.*, 2008).

The *egc* type distributions of the animal isolates were evaluated on the basis of evidence of clonality from the present and previous studies (Table 5). The 13 gallinaceous isolates, which were all from broiler chickens in Northern Ireland (McCullagh *et al.*, 1998), share several molecular

Table 5. Clonality of bovine, leporine and gallinaceous *S. aureus* strains based on molecular typing characteristics and *egc* locus type

Animal/country of origin	<i>n</i>	Strain	Molecular typing characteristic*	<i>egc</i> locus type
Chicken/Northern Ireland	13	DS55–DS67	RAPD type 2, PFGE type I.24 and variants I.24B-E, I.24G and I.24K†, <i>agr</i> type II	<i>egc1</i>
Rabbit/Belgium and Spain	3	DS31, DS46, DS53	RAPD type 18, <i>agr</i> type IV	<i>egc2</i>
Bovine/Ireland	9	RF80, RF102, RF108, RF113, RF122, RF110, RF114, RF120, RF121	ET3‡, RAPD type 7, <i>hlg</i> RFLP type 3, ribotype 4, <i>agr</i> type II	<i>egc2</i>
Bovine/USA	3	MSA930, MSA1005, MSA1047	ET1, RAPD type 10, <i>hlg</i> RFLP type 5, ribotype 6, PFGE type H, <i>agr</i> type II	<i>egc2</i>
Bovine/USA	7	PSA5, PSA6.1, MSA12.1, MSA26.1, MSA31, MSA73.2, MSA148	ET36, RAPD type 9, <i>hlg</i> RFLP type 7, ribotype 1, <i>agr</i> type IV	<i>egc2</i>
Bovine/USA	4	MSA10.6, MSA72.3, MSA103.14, MSA1460	ET2, RAPD type 2, <i>hlg</i> RFLP type 4§, ribotype 9§, <i>agr</i> type III	<i>egc1</i>

*MLEE typing (ET) data from Fitzgerald *et al.* (1997); RAPD typing data for bovine strains from Fitzgerald *et al.* (1997) and Smyth (2006); *hlg* RFLP typing data and ribotyping data from Fitzgerald *et al.* (2000); PFGE data from Fitzgerald (1997).

†PFGE data from McCullagh *et al.* (1998).

‡Strain RF114 is ET40, a single enzyme MLEE variant of ET3 for shikimic acid dehydrogenase; strain RF80 was not typed by MLEE.

§Strain MSA72.3 was not typed by *hlg* RFLP; strain MSA1460 is ribotype 2.

typing characteristics and possessed the pseudogenes *ψent1* and *ψent2* (Table 5). Seven of these isolates were of PFGE type I.24, which was the predominant PFGE type associated with skeletal infections (McCullagh *et al.*, 1998). The other six strains had PFGE types with slightly different banding patterns from PFGE I.24, although related to it.

Three rabbit isolates, one from Spain and two from Belgium, were of the same randomly amplified polymorphic DNA (RAPD) type and *agr* type, and possessed an *egc2* locus. Previous molecular typing characteristics were not available for the other two Belgian isolates. All of these isolates belonged to the so-called 'high virulence rabbit *S. aureus* clone' (Vancraeynest *et al.*, 2006a, b).

Among the bovine isolates (Table 5), nine from Ireland were of multilocus enzyme electrophoresis (MLEE) type ET3 and RAPD type 7 (Selander *et al.*, 1986; Kapur *et al.*, 1995; Fitzgerald *et al.*, 1997, 2000; Herron-Olson *et al.*, 2007). All possessed an *egc2* locus and were of *agr* type II. However, they could be subdivided into two groups based on the presence (six isolates: RF80, RF102, RF108, RF110, RF113 and RF122) or absence (three isolates: RF114, RF120 and RF121) of the *SaPI*bov pathogenicity island (i.e. the presence or absence of the *sec*, *sell* and *tst* genes), and belonged to the different but related PFGE types B1 and B2, respectively (Fitzgerald, 1997; Fitzgerald *et al.*, 2001; Smyth *et al.*, 2005).

Three bovine isolates of MLEE type ET1 and RAPD type 10, and seven strains of MLEE type ET36 and RAPD type 9 (Kapur *et al.*, 1995; Fitzgerald *et al.*, 1997, 2000), all from the USA, were of *agr* types II and IV, respectively. All possessed *egc2* loci (Table 5). A further group of four

isolates, all from the USA, of MLEE type ET2 and RAPD type 2 (Kapur *et al.*, 1995; Fitzgerald *et al.*, 1997, 2000) were of *agr* type II and had an *egc1* locus.

The remaining six bovine isolates had individual molecular typing characteristics. Four strains, three from the USA and one from Spain, had *egc1* loci (MSA1363 – MLEE type ET7, RAPD type 4, *agr* type I; MSA1007 and DS37 – *agr* type II; and MSA915 – RAPD type 4, *agr* type I) (Selander *et al.*, 1986; Kapur *et al.*, 1995; Fitzgerald *et al.*, 1997, 2000; Smyth, 2006). The other two isolates had *egc2* loci – one strain from Argentina (DS42) was *agr* type I and the other from Denmark was *agr* type II.

The three Italian goat isolates were all of *agr* type I and had *egc1* loci. Two of the three sheep isolates were of *agr* type II, one of Danish origin with an *egc1* locus, the other of Swedish origin with an *egc2* locus, whilst the remaining Danish isolate was of *agr* type IV and had an *egc2* locus that was missing the *seg* gene.

In a study of hospital- and community-acquired methicillin-resistant *S. aureus*, the *egc* locus was found to be restricted to isolates of clonal complex CC5 (ST5:USA100) and CC30 (ST30:USA1100 and ST36:USA200) genomic backgrounds (Diep *et al.*, 2006). However, using microarray genotyping with *S. aureus* representative of all major clonal complexes, Monecke *et al.* (2008) found the *egc* locus additionally in CC10, CC20 and CC25, and in MLST types ST22, ST39, ST45, ST151, ST228 and ST254. The human nasal isolates examined by Collery *et al.* (2008) and included in the present study that were MLST typed as CC30 (ST30 and ST938) were *selu_v*⁺, and the one strain that was MLST typed as CC5 (ST935) possessed pseudogenes *ψent1* and *ψent2* in its *egc* locus. Holtfreter *et al.* (2007) found that

87.9 % of 33 nasal isolates of CC30 possessed a *selm_v* gene and a 'selu' gene. On the basis of the present and the aforementioned findings, and the data from genome-sequenced isolates Mu50, N315 and MRSA252, the evidence substantiates that the *egc1* locus occurs in CC5 and the *egc3* locus in CC30. However, isolates belonging to other ST types (ST6, ST109, ST932, ST934, ST937 and ST singletons) and clonal complexes (CC6, CC9, CC10, CC20, CC22, CC25 and CC45) have also been shown to possess the *egc1* or *egc3* locus (Holtfreter *et al.*, 2007; Colliery *et al.*, 2008).

Screening of *S. aureus* from infections in companion animals, pigs and horses for *egc* loci

Of the 45 isolates from cats, dogs, rats, pigs and horses screened by the procedure of Letertre *et al.* (2003) for *egc* loci, 33 were *egc*⁻. The isolates that yielded PSE1/PSE4 amplimers were examined by PCR-RFLP to confirm their pseudogene *ψent1* and *ψent2* status, and their *selu* and *selu_v* status. Of the six canine isolates, two had *egc* loci, one with pseudogenes *ψent1* and *ψent2*, and one with the *selu_v* gene. Three of the four porcine isolates possessed pseudogenes *ψent1* and *ψent2*, whilst the remaining isolate was *selu_v*⁺. A total of 6 of the 28 equine isolates, all nasal in origin, had the *selu* gene. None of the four feline or three rodent isolates had an *egc* locus.

Few data are available on the prevalence of enterotoxin or enterotoxin-like genes in *S. aureus* isolates from cats, dogs, rats, pigs and horses. Nitzsche *et al.* (2007) reported the *seg* or *sei* gene or both in 38.7 % of 142 *S. aureus* isolates from pig carcasses sampled at abattoirs. In a study of *S. aureus* isolates from 20 pork meat samples and 36 pig carcasses, Hwang *et al.* (2007) detected *egc* genes in 22 strains (39.3 %), 27.3 % of which had a 'selu' gene.

Conclusions

The present findings demonstrate that *S. aureus* isolates can be screened directly using the PCR primers of Letertre *et al.* (2003) and PCR-RFLP to detect the presence of and to characterize the *egc* loci, although some human nasal isolates were refractory to these procedures. The PCR primers for the 'hypervariable' region designed in the present study with subsequent sequencing of amplimers would offer a useful alternative or adjunct procedure for distinguishing *selu* and *selu_v* genes or the pseudogenes *ψent1* and *ψent2*. Using PCR amplification of the *sei-seg* region, Jarraud *et al.* (2001) showed that the *egc* locus was highly prevalent in isolates from toxic shock syndrome (59 % of 63 isolates), staphylococcal scalded skin syndrome (92 % of 39 isolates), suppurative infections (67 % of 58 isolates) and nasal carriage (57 % of 70 isolates). Jarraud *et al.* (2002) also found a strong association between the presence of the *egc* locus and amplified fragment length polymorphism group AF1, which was highly represented by isolates of *agr* type IV from bullous impetigo and

scalded skin syndrome. Whilst it remains to be established whether or not there is a correlation between such infections and the type of *egc* locus present, it is noteworthy that all of the *agr* type IV isolates examined herein possessed a *selu* gene. Further detailed study of *egc* polymorphisms in *S. aureus* may provide phylogenetic associations with other actual or potential infection markers (Peacock *et al.*, 2002).

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