Rapid differentiation of *Staphylococcus aureus* isolates harbouring *egc* loci with pseudogenes ψ ent1 and ψ ent2 and the *selu* or *selu_v* gene using PCR-RFLP

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The *egc* locus of *Staphylococcus aureus* harbours two enterotoxin genes (*seg* and *sei*) and three enterotoxin-like genes (*selm*, *seln* and *selo*). Between the *sei* and *seln* genes are located two pseudogenes, ψ ent1 and ψ ent2, or the *selu* or *selu_v* gene. While these two alternative *sei*–*seln* intergenic regions can be distinguished by PCR, to date, DNA sequencing has been the only confirmatory option because of the very high degree of sequence similarity between *egc* loci bearing the pseudogenes and the *selu* or *selu_v* gene. *In silico* restriction enzyme digestion of genomic regions encompassing the *egc* locus from the 3' end of the *sei* gene through the 5' first quarter of the *seln* gene allowed pseudogene- and *selu*- or *selu_v*-bearing *egc* loci to be distinguished by PCR-RFLP. Experimental application of these findings demonstrated that endonuclease *Hind*III cleaved PCR amplimers bearing pseudogenes but not those with a *selu* or *selu_v* gene, while *selu*- or *selu_v*-bearing amplimers were susceptible to cleavage by endonuclease *Hph*I, but not by endonuclease *Hind*III. The restriction enzyme *Bcl*I cleaved *selu*- or *selu_v*-harbouring amplimers at a unique restriction site created by their signature 15 bp insertion compared with pseudogene-bearing amplimers, thereby allowing distinction of these *egc* loci. PCR-RFLP analysis using these restriction enzymes provides a rapid, easy to interpret alternative to DNA sequencing for verification of PCR findings on the nature of an *egc* locus type, and can also be used for the primary identification of the intergenic *sei*–*seln* *egc* locus type.

Received 15 September 2006

Accepted 23 October 2006

INTRODUCTION

The staphylococcal enterotoxins (SEs) belong to the superantigen family of exotoxins and are the only superantigens to possess emetic activity (Le Loir *et al.*, 2003; Proft & Fraser, 2003). The International Nomenclature Committee for Staphylococcal Superantigens proposed that only staphylococcal superantigens that caused vomiting after oral administration in a primate model should be designated SEs, whereas other related toxins that either lacked emetic properties or had not been tested in a primate model should be designated staphylococcal enterotoxin-like toxins (SELTs) (Lina *et al.*, 2004). Fifteen years ago only five SEs were recognized, namely, SEA, SEB, SEC1/SEC2/SEC3, SED and SEE. Since then, through gene sequencing and partial and complete genome sequencing, 15 additional SEs and SELTs

have been described, namely, SEG–SEI, SEIJ–SEIR, SEIU, SEIU2 and SEIV, and three further variants of SEC (SEC-bovine, SEC-ovine and SEC-caprine) (reviewed by Le Loir *et al.*, 2003; Smyth *et al.*, 2004) (Thomas *et al.*, 2006).

Following the identification of enterotoxins SEG and SEI (Munson *et al.*, 1998), the genes encoding them were demonstrated to be part of a chromosomal operon, named the enterotoxin gene cluster (*egc*), comprising five genes, now designated *selo*, *selm*, *sei*, *seln* and *seg* in transcriptional order, and two pseudogenes, ψ ent1 and ψ ent2, between the *sei* and *seln* genes (Jarraud *et al.*, 2001a, b; Monday & Bohach, 2001). It was proposed that the *egc* cluster arose by gene duplication and variation from an ancestral gene through unequal crossing-over generated by recombination involving misalignment between non-allelic regions (Jarraud *et al.*, 2001a). Subsequently, Letertre *et al.* (2003) have demonstrated that some *egc* clusters possess a novel gene between the *sei* and *seln* genes, designated *selu*, arising from a 15 bp insertion (5'-CTCTAAAATTGATGG-3') in the ψ ent1 pseudogene sequence. Moreover, while the *selu* genes from three strains exhibit 99 % nucleotide sequence

Abbreviations: NCBI, National Centre for Biotechnology Information; SE, staphylococcal enterotoxin; SEI, staphylococcal enterotoxin-like toxin.

The GenBank/EMBL/DDBJ accession number for the sequence of the PSE1–PSE4 amplimer of *S. aureus* strain A900322 is DQ993159.

identity, one designated a variant (selu_v) yields a gene product with only 95–96 % amino acid sequence identity to the other three SEIU proteins (Letertre *et al.*, 2003).

Letertre *et al.* (2003) developed three sets of primers to allow distinction between strains possessing pseudogenes and the selu or selu_v gene in the egc locus. However, verification of PCR findings other than by DNA sequencing has not been described because of the very high sequence identity of the sei–seln intergenic region apart from the 15 bp insert. The development and application of a simple procedure for distinction of egc loci of *Staphylococcus aureus* bearing pseudogenes and the selu or selu_v gene is described herein. The method uses PCR-RFLP with either digestion by restriction endonucleases HindIII and HphI in tandem or endonuclease BclI digestion alone of PCR amplimers from the stop codon of the sei gene through the 5' first quarter of the seln gene obtained using the PSE1 and PSE4 primers of

Letertre *et al.* (2003). The differential susceptibility or resistance of pseudogene- and selu- or selu_v-bearing amplimers allows distinction of these two types of egc loci.

METHODS

Strains. The fully genome-sequenced *S. aureus* strains RF122, Mu50 (ATCC 700699), MRSA 252, MW2, COL and NCTC 8325-4 were used as control strains of known egc locus status (Table 1). Ten isolates from chickens, goats and cows, all of which were known to possess the egc locus, were also examined (Smyth *et al.*, 2005). Eight bovine isolates of RAPD type 7 that were known to possess or lack the sec and tst genes (Fitzgerald *et al.*, 2000), but which had not been included in the study of Smyth *et al.* (2005) and had not been further tested for the presence of SE and SEI genes, were also used (Table 1).

All isolates were confirmed as *S. aureus* on the basis of sugar fermentation tests (arabinose, maltose, mannitol, sucrose, trehalose,

Table 1. Strains of *S. aureus*

Strain/isolate	Intergenic sei–seln egc locus type*	Reference
RF122	selu	Fitzgerald <i>et al.</i> (2001)
Mu50 (ATCC 700699)	ψ ent1 ψ ent2	Kuroda <i>et al.</i> (2001)
MRSA 252†	selu _v	Holden <i>et al.</i> (2004)
MW2‡	egc [−]	Baba <i>et al.</i> (2002)
COL‡	egc [−]	Dyke <i>et al.</i> (1966)
NCTC 8325-4†‡	egc [−]	Novick (1967)
A900322§	ψ ent1 ψ ent2	Jarraud <i>et al.</i> (2001a)
5	egc ⁺	Smyth <i>et al.</i> (2005)
12.1	egc ⁺	Smyth <i>et al.</i> (2005)
148	egc ⁺	Smyth <i>et al.</i> (2005)
1007	egc ⁺	Smyth <i>et al.</i> (2005)
DS37	egc ⁺	Smyth <i>et al.</i> (2005)
DS56¶	egc ⁺	Smyth <i>et al.</i> (2005)
DS59¶	egc ⁺	Smyth <i>et al.</i> (2005)
DS63¶	egc ⁺	Smyth <i>et al.</i> (2005)
DS27#	egc ⁺	Smyth <i>et al.</i> (2005)
DS29#	egc ⁺	Smyth <i>et al.</i> (2005)
46**	ND	Fitzgerald <i>et al.</i> (2000)
83**	ND	Fitzgerald <i>et al.</i> (2000)
88**	ND	Fitzgerald <i>et al.</i> (2000)
92**	ND	Fitzgerald <i>et al.</i> (2000)
100**	ND	Fitzgerald <i>et al.</i> (2000)
101**	ND	Fitzgerald <i>et al.</i> (2000)
109**	ND	Fitzgerald <i>et al.</i> (2000)
112**	ND	Fitzgerald <i>et al.</i> (2000)

*egc⁺, possessing an egc locus; egc[−], lacking an egc locus; ND, not determined.

†Kindly provided by T. J. Foster, Department of Microbiology, Trinity College, Dublin.

‡A derivative of a human isolate NCTC 8325 that is cured of prophages ϕ 11, ϕ 12 and ϕ 13.

§Kindly provided by S. Jarraud, Université Lyon 1, Centre National de Référence des Staphylocoques, Lyon, France.

||Bovine isolates, possessing the selo, selm, sei, seln and seg genes.

¶Chicken isolates, possessing the selo, selm, sei, seln and seg genes.

#Goat isolates, possessing the selo, selm, sei, seln and seg genes.

**All isolates of bovine origin of RAPD type 7 (Fitzgerald *et al.*, 2000).

xylose), the Voges–Proskauer test, deoxyribonuclease production, and a tube coagulase test using standard methods, and by the Staphaurex rapid latex test kit (Murex Diagnostics), which detects protein A and clumping factor (fibrinogen-binding protein), used according to the manufacturer's instructions. Primers for the *femA* gene encoding the *S. aureus* FemA peptidyltransferase (Schneider *et al.*, 2004) were used for additional confirmation of species identity with animal isolates using the thermal cycling conditions described by Mehrotra *et al.* (2000). Strains and isolates were grown in Tryptic Soya broth (Oxoid) or on Tryptic Soya agar (Oxoid) at 37 °C overnight.

In silico analyses of *sei*–*seln* intergenic regions of *egc* loci.

FASTA files of DNA sequences of *egc* loci, *sei*–*seg* intergenic regions, and *selu* and *selu_v* genes were retrieved from the National Centre for Biotechnology Information (NCBI) nucleotide database (Table 2). For strains A900322, FRI572, RF122, 382F, Mu50, N315 and MRSA 252, the DNA sequences of the forward primer PSE1 and the reverse primer PSE4 of Letertre *et al.* (2003) were used to identify the *sei*–*seln* intergenic regions bearing pseudogene ψ ent1 and ψ ent2 and the *selu* or *selu_v* sequences. The ORF Finder (NCBI) was used to identify the pseudogenes ψ ent1 and ψ ent2 and the *selu* or *selu_v* genes (<http://www.ncbi.nlm.nih.gov/projects/gorf/>). Alignments performed using the CLC Free Workbench software version 3.0.2 (CLC bio; <http://www.clcbio.com>) on these PSE1–PSE4 sequences allowed identification of the PSE2 primer sequence of Letertre *et al.* (2003) in the *selu* and *selu_v* genes and of the 15 bp insertion that converts the pseudogene sequences to the *selu* or *selu_v* gene. Unless otherwise indicated, the nucleotide numbering system used herein refers to the first base of the PSE1 forward primer (T) as nt 1.

The database sequences for the *selu* genes of strains 352E, 383F and FRI137 were incomplete with respect to the PSE1–PSE4 region. Using the BLAST program, the available sequences for strains 352E (AY205305), 383E (AY205307) and FRI137 (AY205306) gave best matches of 1040/1052 (98.9%), 1041/1052 (99.0%) and 1024/1024 (100%), respectively, with that of strain RF122, in line with their own inter-identity values (Letertre *et al.*, 2003). Accordingly, the necessary sequence additions to the 5' and/or 3' ends to obtain complete *in silico* PSE1–PSE4 regions for these three strains were based on the complete PSE1–PSE4 sequence of strain RF122. All of the complete or completed

PSE1–PSE4 sequences were analysed using RestrictionMapper version 3 (<http://www.restrictionmapper.org>) to identify restriction enzyme cleavage sites.

DNA preparation. Extraction of genomic DNA from *S. aureus* was performed as previously described, with some 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.

PCR. All primers were synthesized by MWG Biotech. For amplification of the *sei*–*seln* intergenic region bearing the ψ ent1 and ψ ent2 pseudogenes and the *selu* or *selu_v* gene, the forward/reverse primer pairs PSE1/PSE4, PSE2/PSE4 and PSE2/PSE6 described by Letertre *et al.* (2003) were used. The primers PSE1 and PSE4 are designed to amplify the *egc* locus region encoding either the pseudogenes ψ ent1 and ψ ent2 or the *selu* or *selu_v* gene from the stop codon of the *sei* gene through the first 172 nt of the 5' end of the *seln* gene. The 27-mer forward primer PSE2 includes the specific 15 nt insertion of the *selu* or *selu_v* gene. PCR assays with the primer pairs PSE2/PSE4 and PSE2/PSE6 yield 790 and 142 bp amplicons, respectively, when the *selu* or *selu_v* gene is present (Letertre *et al.*, 2003).

PCR was performed in a final volume of 25 µl. The reaction mix contained 2–2.5 pmol each primer. Each reaction contained 15.2 µl H₂O, 1 µl each primer (forward/reverse combinations PSE1/PSE4, PSE2/PSE4, PSE2/PSE6), 1 µl dNTP mix, 0.3 µl *Taq* polymerase (5 U µl^{−1}), 2.5 µl 10 × Thermophilic DNA polymerase buffer (Mg²⁺ free), 3 µl MgCl₂ (15 mM) and 1 µl template DNA. All reagents were supplied by Promega. Thermal cycling conditions were: 94 °C for 4 min, 7 cycles of (94 °C for 30 s, 56 °C for 30 s, 72 °C for 4 min), 21 cycles of (94 °C for 30 s, 56 °C for 35 s, 72 °C for 4 min) and a final elongation step of 72 °C for 7 min. DNA preparations from strains A900322, Mu50, RF122, MRSA 252, MW2 and NCTC 8325-4 were used as controls.

To control for the presence of sufficient template DNA, the test samples were examined by PCR with a primer set that anneals to staphylococcal 16S rRNA genes, generating a 228 bp amplicon (Monday & Bohach, 1999), using the thermal cycling conditions of Smyth *et al.* (2005). All

Table 2. DNA sequences of *egc* loci, *sei*–*seg* intergenic regions, and *selu* and *selu_v* genes

Strain	Origin and <i>egc</i> locus type	Accession no.	Reference
A900322	Tampon – toxic shock syndrome; ψ ent1 ψ ent2	AF285760	Jarraud <i>et al.</i> (2001a)
FRI572	Nasal isolate, Easter Island*; ψ ent1 ψ ent2	AF156894	Monday & Bohach (2001)
Mu50†	Surgical wound, VRSA‡ (1997); ψ ent1 ψ ent2	BA000017	Kuroda <i>et al.</i> (2001)
N315†	Pharyngeal smear, MRSA‡ (1982); ψ ent1 ψ ent2	BA000018	Kuroda <i>et al.</i> (2001)
RF122	SaPIbov ⁺ bovine isolate (1993); <i>selu</i>	AJ938182	Fitzgerald <i>et al.</i> (2001)
383F	Isolate from rice salad (1987)§; <i>selu</i>	AY205306	Letertre <i>et al.</i> (2003)
352E	Isolate from fromage frais (1981)§; <i>selu</i>	AY205305	Letertre <i>et al.</i> (2003)
FRI137	Isolate from thigh abscess (1933)*; <i>selu</i>	AY205307	Bergdoll <i>et al.</i> (1965); Letertre <i>et al.</i> (2003)
382F	Isolate from water (1988)§; <i>selu_v</i>	AY158703	Letertre <i>et al.</i> (2003)
MRSA 252†	Postoperative infection EMRSA-16 clone‡ (1997); <i>selu_v</i>	BX571856	Holden <i>et al.</i> (2004)

*A. C. L. Wong, Food Research Institute, University of Wisconsin, Madison, personal communication.

†Genome-sequenced human isolates.

‡MRSA, methicillin-resistant *S. aureus*; VRSA, vancomycin-resistant *S. aureus*; EMRSA, epidemic MRSA.

§M. L. De Buyser, Laboratoire d'Etudes et de Recherches sur l'Hygiène et la Qualité des Aliments, Agence Française de Sécurité Sanitaire des Aliments (AFSSA), Maisons-Alfort, France, personal communication.

PCR products were analysed on 2 % (w/v) agarose gels alongside a 100 bp ladder (NEB), stained with ethidium bromide and visualized under UV light.

Restriction enzyme digestion of PSE1–PSE4 amplimers.

Using the above PCR protocol, forward/reverse primer pair PSE1/PSE4 was used to amplify the pseudogene- or *selu*/*selu_v*-containing region. PCR product (5 μ l) was run on a 1 % agarose gel to confirm that the reaction had been successful. Restriction endonuclease digestion was performed in a final volume of 30 μ l. For endonuclease *Hind*III cleavage, this contained 17 μ l H₂O, 6 μ l PCR product, 3 μ l 10 $\times$ Buffer E (60 mM Tris/HCl, pH 7.5, 1 M NaCl, 60 mM MgCl₂, 10 mM DTT), 3 μ l 10 $\times$ BSA (100 μ g ml⁻¹) and 1 μ l *Hind*III restriction endonuclease (Promega; 10 U μ l⁻¹). The digestion mixture was incubated overnight at 37 °C. Endonuclease *Hph*I cleavage was carried out as above, except that the digestion mixture contained 3 μ l 10 $\times$ NE Buffer 4 (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9) and 1 μ l *Hph*I restriction endonuclease (NEB; 5 U μ l⁻¹). For endonuclease *Bcl*I, the digestion mixture contained 3 μ l 10 $\times$ NE Buffer 1 (10 mM Bistris propane-HCl, 10 mM MgCl₂, 1 mM DTT, pH 7.0) and 1 μ l *Bcl*I restriction endonuclease (NEB; 10 U μ l⁻¹). All digests were analysed on 2.5 % (w/v) agarose gels alongside a 100 bp ladder (NEB).

Sequencing of PSE1–PSE4 PCR product. PCR was performed for the PSE1–PSE4 amplimer of strain A900322. The PCR product was purified using the High Pure PCR Product Purification kit (Roche) according to the manufacturer's instructions. The PCR amplimer was sequenced by GATC Biotech. The derived sequence (accession no. DQ993159) was aligned with the complete *egc* locus sequence of strain A900322 (AF285760) and with the *sei-seg* intergenic region of strain FRI572 (AF156894) using the CLC Free Workbench software version 3.0.2. The BLAST program (<http://www.ncbi.nlm.nih.gov/blast/>) was used to interrogate the NCBI database for matches for the derived sequence.

RESULTS

In silico analyses of PSE1–PSE4, *sei-seln* intergenic regions of *egc* loci

Alignment of the PSE1–PSE4 region sequences of the four $\psi ent1^+$ $\psi ent2^+$ strains A900322 (1066 nt), and Mu50, N315 and FRI572 (each 1135 nt), revealed a 69 nt deletion in the $\psi ent1$ – $\psi ent2$ sequence of strain A900322 between nt G 3462 and nt G 3463 of the original *egc* cluster sequence AF285760 compared with those of the three other $\psi ent1^+$ $\psi ent2^+$ strains in which this 69 nt sequence is identical (5'-ACCGAGCATGATGGAAATCAAATAGATAAAAAT-AATTCAACTGATAACTCTCATAATATCTTAATTAAA-3') (Fig. 1). This 69 nt sequence is also 100 % conserved in the sequences of two of the *selu*⁺ strains (383F, 352E) and the two *selu_v*⁺ strains (382F and MRSA252) examined. In the other two *selu*⁺ strains RF122 and FRI137 there is an inversion of TC to CT at nt 37 and nt 38 of the 69 nt sequence (see TC bold type in 69 nt sequence above). The missing 69 nt in the strain A900322 sequence occur in the 23 nt $\psi ent1$ and $\psi ent2$ reading-frame overlap region that was originally described by Jarraud *et al.* (2001a) to harbour the start codon of pseudogene $\psi ent2$ and the stop codon of pseudogene $\psi ent1$, namely, 5' 3448-ATG[$\psi ent2$]TATG-GCGGTGTG[Δ 69 nt]GTTTATGA [$\psi ent1$]-3470 3' (bold type signifies features in the sequence mentioned above).

Fig. 1. Schematic diagram of PSE1–PSE4 PCR products bearing pseudogenes $\psi ent1$ and $\psi ent2$ and the *selu* or *selu_v* gene. The hatched area in the pseudogene-bearing PSE1–PSE4 PCR product (1135 bp) corresponds to the 69 bp sequence missing in the pseudogene overlap region of strain A900322. The cleavage sites of restriction endonucleases *Hind*III and *Bcl*I for a pseudogene-bearing PCR product, e.g. from strain Mu50, are shown with the sizes of the fragments indicated in bp. One of the *Bcl*I sites falls within the 69 bp sequence lacking in the strain A900322 sequence. The filled area in the *selu*- or *selu_v*-bearing PSE1–PSE4 PCR product (1149 or 1150 bp) corresponds to the signature 15 bp insert in the $\psi ent1$ pseudogene, which provides the unique cleavage site for restriction endonuclease *Bcl*I. The cleavage sites of restriction endonucleases *Hph*I and *Bcl*I for a *selu*- or *selu_v*-bearing PCR product, e.g. from strain RF122 and 382F, respectively, are shown with the sizes of the fragments indicated in bp.

In strain A900322, the $\psi ent1$ – $\psi ent2$ pseudogene sequence starts at nt 154 and ends at nt 856 of the PSE1–PSE4 amplimer sequence, whereas in strains Mu50, N315 and FRI572, the $\psi ent1$ – $\psi ent2$ sequence starts at nt 154 and ends at nt 925.

The PSE1–PSE4 sequences of three of the *selu*⁺ (RF122, 352E, FRI137) and the two *selu_v*⁺ (382F, MRSA252) strains are 1149 nt long. That of strain 383F (*selu*⁺) is 1 nt longer due to the insertion of a thymine in its PSE1–PSE4 sequence outside the *selu* ORF between three thymines (T 68–T 70). In PSE1–PSE4 sequences, the *selu* or *selu_v* gene begins at nt 154 (nt 155 in strain 383F) and ends at nt 939 (940 in strain 383F). Analysis of the 1149/1150 nt long *selu* and *selu_v* sequences compared with PSE1–PSE4 sequences of strains A900322, Mu50, N315 and FRI572 revealed the 15 nt insertion between G 218 and T 219 of the $\psi ent1$ sequences (5' 219-CTCTAAAATTGATGG-233 3' of the *selu* or *selu_v* genes) [this 15 nt sequence comprises nt 13–27 of the PSE2 primer of Letertre *et al.* (2003)]. All six of the *selu*- or *selu_v*-bearing sequences have a single adenine deletion compared with the $\psi ent1$ sequences of strains A900322, Mu50, N315 and FRI572 from a run of seven adenines (A 365–A 371 of the $\psi ent1$ pseudogenes corresponding to nt A 380–A 385 of the *selu* and *selu_v* genes). This single adenine deletion abolishes the opal/umber stop codon TGA present at the end of pseudogene $\psi ent1$.

Table 3. *In silico* analysis of restriction endonuclease cleavage of *sei*–*seln* intergenic sequences of the *egc* locus between primers PSE1 and PSE4 containing pseudogenes ψ *ent1* and ψ *ent2* or the *selu* or *selu_v* gene

Restriction endonuclease	Sizes (bp) of restriction fragments of PSE1–PSE4 regions for indicated strains*			
	A900322 (1066 bp, ψ <i>ent1</i> ψ <i>ent2</i>)	Mu50, N315, FRI572 (1135 bp, ψ <i>ent1</i> ψ <i>ent2</i>)	RF122, 352E, FRI137, 383F† (1149/1150 bp†, <i>selu</i>)	382F, MRSA 252 (1149 bp, <i>selu_v</i>)
<i>Hind</i> III	889 176	958 176	None	None
<i>Hph</i> I	None	None	850/851† 298	850 298
<i>Bcl</i> I	569 496	586 496	586 376/377† 135 51‡	586 376 135 51‡

*See Table 2 for accession numbers of sequences.

†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 outside the *selu* ORF between three thymines (T 68–T 70).

‡Deletion of a single adenine residue that abrogates the stop codon TGA of pseudogene ψ *ent1* leads to this fragment of the *selu* and *selu_v* genes being 1 nt shorter.

Each of the 10 PSE1–PSE4 sequences was analysed using the RestrictionMapper version 3 program (Table 3). Of the enzymes with one restriction site, endonuclease *Hind*III cut the PSE1–PSE4 regions between A 958 and A 959 in strains Mu50, N315 and FRI572 (Fig. 1), A 889 and A 890 in strain A900322, 33 nt downstream of the ψ *ent2* stop codon TAA and 5 nt upstream of the start codon of the *seln* gene in the strains with pseudogenes ψ *ent1* and ψ *ent2*, but did not cut PSE1–PSE4 regions in strains bearing the *selu* and *selu_v* genes (Table 3). In contrast, restriction endonuclease *Hph*I cut the PSE1–PSE4 region between T 850 and A 851 in strains with *selu* or *selu_v* genes (Fig. 1), 86 nt upstream of the stop codon, but did not cut PSE1–PSE4 regions in strains bearing the pseudogenes ψ *ent1* and ψ *ent2*.

The only enzyme found to cut the sequence of the 27-mer PSE2 forward primer (Letertre *et al.*, 2003) was restriction endonuclease *Bcl*I. The insertion of the 15 bp sequence creates a unique *Bcl*I cleavage site between A 376 and A 377 (nt 17 and nt 18 of the PSE2 primer) of PSE1–PSE4 amplicons of *selu*⁺ and *selu_v*⁺ strains (Fig. 1). PSE1–PSE4 regions bearing pseudogenes ψ *ent1* and ψ *ent2* are cleaved into three fragments, whereas those bearing *selu* and *selu_v* genes are cleaved into four fragments (Table 3). Pseudogene- and *selu*- or *selu_v*-bearing strains can be readily distinguished by the presence of a 496 nt *Bcl*I fragment and 376/377 nt and 135 nt *Bcl*I fragments, respectively (Table 3).

PCR-RFLP analysis of PSE1–PSE4 amplicons using restriction endonucleases *Hind*III and *Hph*I in tandem

Strains Mu50 and A900322 (ψ *ent1*⁺ ψ *ent2*⁺), RF122 (*selu*⁺) and MRSA 252 (*selu_v*⁺) were examined using the PSE1/PSE4, PSE2/PSE4 and PSE2/PSE6 primer sets of

Letertre *et al.* (2003) (Fig. 2a–c). All four strains yielded PCR products of approximately 1135–1150 bp with the PSE1/PSE4 primers. Strains Mu50 and A900322 did not give amplicons with the PSE2/PSE4 and PSE2/PSE6 primer pairs, confirming their ψ *ent1*⁺ ψ *ent2*⁺ status. In contrast, strains RF122 and MRSA 252 produced PCR products of approximately 790 and 142 bp with primers PSE2/PSE4 and PSE2/PSE6, respectively (Fig. 2b, c), confirming the presence of *selu* and *selu_v* genes, respectively. Strains NCTC 8325-4, MW2 and COL were subjected to PCR as negative controls for the *egc* locus. None yielded PSE1/PSE4, PSE2/PSE4 or PSE2/PSE6 amplicons.

To test whether isolates bearing either the pseudogenes ψ *ent1* and ψ *ent2* or the *selu* gene or the *selu_v* gene could be distinguished using restriction endonucleases *Hind*III and *Hph*I, the PSE1–PSE4 amplicons of strains A900322, Mu50, RF122 and MRSA 252 were incubated overnight with these restriction enzymes. As anticipated from the *in silico* analysis, amplicons of strains bearing the pseudogenes (A900322 and Mu50) were digested by endonuclease *Hind*III, but not by endonuclease *Hph*I, whereas amplicons of strains harbouring the *selu* or *selu_v* gene (RF122 and MRSA 252) were restricted by endonuclease *Hph*I, but not by endonuclease *Hind*III, yielding fragments of the expected approximate sizes (Fig. 2d, e).

Eight bovine isolates of RAPD type 7 (Fitzgerald *et al.*, 2000), which were known to possess or lack the *sec* and *tst* genes but had not been otherwise screened for SE or SEL genes, were tested for the presence of pseudogenes or the *selu*/*selu_v* gene by PCR and PCR-RFLP analysis. In all cases PSE1–PSE4, PSE2–PSE4 and PSE2–PSE6 amplicons characteristic of isolates possessing an *egc* locus with the *selu* or *selu_v* gene were obtained. These isolates were all confirmed to have the

Fig. 2. PCR amplification and PCR-RFLP analysis of *sei-seln* intergenic regions of *S. aureus* harbouring *egc* loci. PCR amplimers are shown of strains A900322 (lane 1), Mu50 (lane 2), RF122 (lane 3) and MRSA 252 (lane 4), using (a) primers PSE1 and PSE4 showing an amplimer of approximately 1150 bp, (b) primers PSE2 and PSE4 showing an amplimer of approximately 790 bp from strains RF122 and MRSA 252, and (c) primers PSE2 and PSE6 showing an amplimer of approximately 142 bp for strains RF122 and MRSA 252. PCR-RFLP analysis of PSE1–PSE4 amplimers digested with restriction endonucleases (d) *Hind*III, (e) *Hph*I and (f) *Bcl*I. PCR amplimers of strains A900322 and Mu50 bearing pseudogenes ψ ent1 and ψ ent2 were cut to give bands at approximately 958 and 176 bp by endonuclease *Hind*III (d) but not by endonuclease *Hph*I (e), whereas PCR amplimers of *selu*⁺ and *selu*_v⁺ strains RF122 and MRSA 252, respectively, were not cut by endonuclease *Hind*III (d) but were digested by endonuclease *Hph*I to give bands at approximately 850 and 298 bp. Undigested PSE1–PSE4 PCR products of 1135 bp from strains A900322 and Mu50 can be seen in lanes 1 and 2 of (d) and of 1150 bp from strains RF122 and MRSA 252 in lanes 3 and 4 of (e) (Fig. 1a). PCR products of pseudogene-positive strains A900322 and Mu50 were cut by endonuclease *Bcl*I to give bands at approximately 496 and 586 bp (f), while those of *selu*-positive strains RF122 and MRSA 252 were cut to show bands at approximately 586, 376 and 135 bp (white arrow). Lanes M contain 100 bp ladders; 500 bp markers are asterisked.

selu or *selu*_v gene on the basis of the *Hph*I susceptibility and *Hind*III resistance of their PSE1–PSE4 amplimers (data not shown).

PCR-RFLP analysis of PSE1–PSE4 amplimers using restriction endonuclease *Bcl*I

PCR-RFLP analysis of PSE1–PSE4 amplimers using endonuclease *Bcl*I was carried out for the *egc* archetypal strain A900322 and the genome-sequenced strains Mu50, RF122 and MRSA 252 (Fig. 2f). The pseudogene-positive strains A900322 and Mu50 yielded a restriction product of approximately 496 bp, whereas strains RF122 and MRSA 252, with *selu* and *selu*_v genes, respectively, yielded restriction products of approximately 376 and 135 bp (Fig. 2f, Table 3).

Ten animal isolates known to possess an *egc* locus (the *selo*, *selm*, *sei*, *seln* and *seg* genes) – five from cows, two from goats and three from chickens – were identified as having pseudogenes and the *selu* or *selu*_v gene using PCR with the PSE primer sets and PCR-RFLP with endonucleases *Hind*III and *Hph*I in tandem. The three chicken isolates and

two of the bovine isolates (DS37 and 1007) possessed pseudogenes, while the two goat isolates and three of the bovine isolates had the *selu* or *selu*_v gene (Table 1). Their PSE1–PSE4 amplimers were then examined by PCR-RFLP using restriction endonuclease *Bcl*I in an operator-blinded manner. The *egc* loci were all correctly identified, those bearing pseudogenes giving a restriction product of approximately 496 bp and those bearing a *selu* or *selu*_v gene giving restriction products of approximately 376 and 135 bp, respectively (data not shown).

Sequencing of the PSE1–PSE4 amplimer of strain A900322

There was no evidence from the PCR-RFLP studies to suggest that the *egc* locus of strain A900322 differed from that of the ψ ent1⁺ ψ ent2⁺ strain Mu50. Given that the missing 69 nt were present in all other published sequences of *selu* or *selu*_v genes and ψ ent1– ψ ent2 pseudogenes in the NCBI database, resequencing of the pseudogene region of the *egc* locus of strain A900322 was performed. The 694 nt-long sequence obtained was from nt A 64 to nt G 757 of the

PSE1–PSE4 amplicon (accession no. DQ993159). This encompasses all of pseudogene $\psi ent1$ and the first 288 nt of pseudogene $\psi ent2$, including the crucial pseudogene $\psi ent1$ – $\psi ent2$ overlap region in which the 69 nt sequence in question would occur, if present.

Using the CLC Free Workbench software version 3.0.2, this 694 nt sequence was aligned with the complete *egc* locus sequence of strain A900322 (AF285760; 6418 nt) and the *sei*–*seln* intergenic spacer region of strain FRI572 (AF156894; 2016 nt). With the exception of 1 nt difference (C 342 in AF156894 versus T 189 in DQ993159), the derived sequence showed complete nucleotide concordance with nt A 154–G 847 of the strain FRI572 intergenic spacer region of Monday & Bohach (2001). In the case of the complete *egc* locus sequence of Jarraud *et al.* (2001a), there was a 100 % match between the derived sequence DQ993159 and the complete *egc* locus sequence AF285760 of strain A900322 from nt A 2982 to nt G 3606, barring the absence of the 69 nt between nt G 3462 and nt G 3463 of the AF285760 sequence. Using BLAST, the 694 nt DQ993159 sequence gave a 100 % match with $\psi ent1$ – $\psi ent2$ regions of the genomic sequences of strains Mu50 and N315, both of which possess the 69 nt sequence.

Taken together, these findings revealed that the missing 69 nt were present in the resequenced pseudogene $\psi ent1$ – $\psi ent2$ overlap region of strain A900322. The 23 nt $\psi ent1$ and $\psi ent2$ reading-frame overlap region of strain A900322 originally described by Jarraud *et al.* (2001a) to harbour the start codon of pseudogene $\psi ent2$ and the stop codon of pseudogene $\psi ent1$ is thus 26 nt long, as it is in the other $\psi ent1^+$ $\psi ent2^+$ strains Mu50, N315 and FRI572.

DISCUSSION

To date, confirmation of PCR findings in relation to the nature of the *egc* locus has only been possible by sequencing of PCR products. As demonstrated herein, PCR-RFLP can be utilized for verification of the nature of *egc* loci determined by PCR when applying the primer sets of Letertre *et al.* (2003). Chini *et al.* (2006) have recently used restriction endonuclease *HindIII* to distinguish MRSA strains bearing the *egc1* locus (*selo selm sei $\psi ent1$ $\psi ent2$ seln seg*) and the *egc2* locus (*selo selm sei selu seln seg*). Using a clearly defined amplicon, the present findings not only confirm the value of using endonuclease *HindIII*, but further extend the use of restriction enzymes by inclusion of endonuclease *HphI* in tandem for reverse susceptibility of the *egc1* and *egc2* loci. Furthermore, the usefulness of endonuclease *BclI* is pinpointed by the fact that the 15 bp insert leading to the formation of the *selu* and *selu_v* genes from pseudogenes $\psi ent1$ and $\psi ent2$ creates a unique cleavage site. PCR-RFLP either with endonucleases *HindIII* and *HphI* in tandem or with endonuclease *BclI* alone with the PSE1–PSE4 amplicon can also be used as a primary screening test for these *egc* loci. The described PCR-RFLP procedure is rapid and amenable to the screening of batches

of clinical isolates. However, strains with atypical *egc* loci are known to occur (Blaiotta *et al.*, 2004; Thomas *et al.*, 2006). Both the PCR procedure of Letertre *et al.* (2003) and the PCR-RFLP procedure described herein may be affected by insertion sequences and by *egc* gene recombination or deletion events such as those reported by Thomas *et al.* (2006).

Direct repeats ATTT, AAGG and CATGAT were identified as the insertion sites of the newly described transposase 8-like/retrovirus integrase-like cassette in the *sei*, *seln* and *seg* genes of isolates apparently lacking these genes by PCR analysis (Thomas *et al.*, 2006). Analysis of the *sei*–*seln* intergenic region reveals that the nucleotide motif ATTT occurs 21 times in strain Mu50 and 22 times in strains RF122 and MRSA 252 (equivalent to 21 % of the total ATTT direct repeats in the *egc* locus of archetypal strain A900322). Thus, on this basis the *sei*–*seln* intergenic region would seem to be a potential hot spot for insertion sequences such as the transposase 8-like/retrovirus integrase-like cassette.

The *in silico* analysis of *egc* loci based on the available NCBI database sequences annotated for the first time that the *sei*–*seln* intergenic region of the archetypal *egc*⁺ strain A900322 (AF285760; Jarraud *et al.*, 2001a) differed from the inter-pseudogenic sequences of three other $\psi ent1^+$ $\psi ent2^+$ strains, one of which (strain FRI572) is the *egc* prototype strain of Monday & Bohach (2001), by the absence of a 69 nt-long stretch, as well as from the sequences of six *selu*⁺ or *selu_v*⁺ strains. This strongly suggested either that the *sei*–*seln* intergenic region of strain A900322 was atypical due to a unique deletion or that the sequence lodged in the database was incorrect or faulty. Resequencing (DQ993159) and sequence alignments confirmed the presence of the missing 69 nt in the $\psi ent1$ – $\psi ent2$ overlap region of strain A900322.

Thomas *et al.* (2006) have reported a new *sei*–*seln* intergenic gene in *S. aureus* strain A900624 that they designate *selu2*. The *selu2* gene results from a single adenine deletion which converts the 772 nt $\psi ent1$ – $\psi ent2$ region into a 771 nt ORF. However, as pointed out herein, this same single adenine deletion is present in all the *selu* and *selu_v* gene sequences in the NCBI database. Moreover, the *selu2* ORF differs in crucial respects from all published *selu* and *selu_v* sequences. The latter are 786 nt long and possess the 15 nt signature sequence 5' 219-CTCTAAAATTGATGG-233 3' between nt G 218 and nt T 219 of pseudogene $\psi ent1$. From the description of the *selu2* gene, namely, that nt 1 to nt 402 demonstrated 99.3 % (399/402) identity with pseudogene $\psi ent1$ of strain Mu50, and that nt 376 to nt 771 had 100 % identity to pseudogene $\psi ent2$ of strain Mu50, the *selu2* gene is not a *selu* or *selu_v* gene *sensu stricto* but merely a 1 nt frameshift of the $\psi ent1$ – $\psi ent2$ sequence that deletes the opal/umber stop codon of pseudogene $\psi ent1$.

Furthermore, while the natures of three nucleotide differences in the first 402 nt of the *selu2* gene are not available through the NCBI database, use of RestrictionMapper version 3 *in silico* with the PSE1–PSE4

sequence of strain Mu50 with the single adenine removed confirmed that the *selu2* gene is cleaved by endonuclease *Hind*III, but not by endonuclease *Hph*I, and by endonuclease *Bcl*I to yield a 496 bp fragment. It is thus not possible to distinguish *selu2*-bearing from ψ ent1- ψ ent2-bearing isolates using the PCR-RFLP procedures described herein. Since the *selu2* ORF is distinctly different from all *selu* genes described to date in the absence of the signature 15 nt descriptor of *selu* genes and its endonuclease *Hind*III, *Hph*I and *Bcl*I cleavage characteristics, we propose that this gene be redesignated the *selw* gene. This is coincidentally appropriate, since the single adenine deletion from the ψ ent1- ψ ent2 sequence removes the sixth-from-last tryptophan residue of the translated ψ ent1 sequences of strains A900322, Mu50, N315 and FRI572.

Neither the PCR-RFLP analyses herein nor the procedure of Chini *et al.* (2006) distinguishes *selu*- from *selu_v*-bearing isolates. However, this appears to be possible using the PCR-RFLP scheme of Blaiotta *et al.* (2004, 2006). These authors demonstrated seven different PCR-RFLP groups based on restriction of a 3375 bp *selm*-*seg* amplicon using endonucleases *Eco*RI, *Taq*I, *Alu*I and *Cfo*I. Strain A900322 of the *egc1* locus type belonged to REA group 2, strain FRI137 of the *egc2* locus type belonged to REA group 2, and strain AB-8802, probably of the *egc3* locus type because it possesses the *sei_v* and *seg_v* genes that co-exist with the *selu_v* gene in strain 382F, belonged to REA group 6. However, these PCR-RFLP differences are not based on differences in the *sei*-*seln* intergenic regions of *egc* loci, as endonucleases *Taq*I, *Alu*I and *Cfo*I do not cut the PSE1-PSE4 amplicon, and endonuclease *Eco*RI has the same single cleavage site on the basis of the restriction analyses carried out herein using RestrictionMapper version 3, but rather are dependent on the presence or absence of sequence variation in other genes, e.g. *sei_v*, *seg_v* and *seln_v*.

While there has been a plethora of reports on the occurrence of the five common genes of the *egc* locus (*selo selm sei seln seg*) in strains of *S. aureus* of human and animal origin over the past 5 years, very limited data exist on the relative occurrence of the ψ ent1 and ψ ent2 pseudogenes and of the *selu* and *selu_v* genes within the *egc* locus (Letertre *et al.*, 2003; Blaiotta *et al.*, 2004; Bania *et al.*, 2006a, b; Chini *et al.*, 2006; Thomas *et al.*, 2006). The combined PCR and PCR-RFLP methodology described should aid in the determination of the occurrence of *egc1* and *egc2/egc3* type loci in isolates of different human and animal clinical origin, and in the evaluation of their association, if any, with the disease-causing potential of *S. aureus* harbouring these different intergenic *sei*-*seln* *egc* loci types.

ACKNOWLEDGEMENTS

M. M. C. was in receipt of a studentship from the Sarah Purser Medical Research Fund and of a Trinity College postgraduate research studentship. Thanks to Amy Wong and Marie-Laure De Buyser, who provided information on the origins of Food Research Institute (FRI), University of Wisconsin, and Agence Française de Sécurité

Sanitaire des Aliments (AFSSA) isolates, to Tim Foster and Sophie Jarraud, who provided control strains of known *egc* type, and to Davida Smyth and Ross Fitzgerald, who originally characterized the animal isolates used.

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