Molecular typing of nasal carriage isolates of *Staphylococcus aureus* from an Irish university student population based on toxin gene PCR, *agr* locus types and multiple locus, variable number tandem repeat analysis

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Forty-eight *Staphylococcus aureus* isolates collected from a young, healthy, Irish university student population from 1995 to 2004 were screened for 16 enterotoxin (SE) and enterotoxin-like (SEI) genes (*sea*–*see*, *seg*–*sei*, *selj*–*selo*, *selq*, *selu*), and for the toxic shock toxin syndrome toxin-1 gene, *tst*. All of the isolates harboured at least one SE or SEI gene and 66.7 % possessed a classical SE gene (*sea*, *seb*, *sec*), the commonest being the *seb* gene. Most of the isolates (85.4 %) had a complete *egc* locus (*selo*, *selm*, *sei*, *seln*, *seg*). The intergenic *sei*–*seln* region of the *egc* locus was typed by PCR-RFLP in 34 isolates, 15 possessing pseudogenes ψ *ent1* and ψ *ent2* and 19 having the *selu* gene. The *seh* and *sell* genes, the *selk*–*selq* gene combination, and the *tst* gene were each found in <15 % of isolates. The *agr* genotype distribution was *agr* type III, 37.5 %; *agr* type I, 35.4 %; *agr* type II, 25 %; and *agr* type IV, 2.1 %. There was no association between SE–SEI genotype and *agr* type. All *tst* gene-positive isolates were of *agr* type III and harboured a classical SE gene. Multiple locus, variable number tandem repeat analysis (MLVA) produced 47 different patterns. While the *sdr* locus was present in all isolates, half of them lacked one or two of the *sdr* gene amplimers. Twenty isolates harboured the *bbp* gene, its presence being associated with *agr* type III, but not with the SE–SEI gene profile. The *agr* types of isolates were associated with MLVA subclusters. Selective MLST analysis revealed seven novel sequence types and a new *aroE* allele. Five clonal clusters (CCs), including CCs comprising major pandemic clones CC30, CC5 and CC22 and minor lineages CC6 and CC9, and three singletons were identified.

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INTRODUCTION

Staphylococcus aureus is an opportunistic pathogen that can persist in the anterior nares of healthy human carriers (Kluytmans *et al.*, 1997; Peacock *et al.*, 2001; Nouwen *et al.*, 2004; Wertheim *et al.*, 2005). The diseases caused by *S. aureus* range in severity from superficial skin infections and food poisoning to life-threatening conditions such as toxic

shock syndrome and septicaemia (Ferry *et al.*, 2005; Murray, 2005; Todd, 2005).

Many molecular methods have been employed to type, differentiate, and group isolates of *S. aureus* (Shopsin & Kreiswirth, 2001; Trindade *et al.*, 2003; Robinson & Enright, 2004; Vivoni & Moreira, 2005). While currently pulsed-field gel electrophoresis (PFGE), usually with *Sma*I-generated genomic DNA fragments, is considered the ‘gold’ standard method, it is time-consuming, requires specific and expensive equipment, and is relatively labour intensive (Arshad *et al.*, 1993; Wagner & Lai, 1994). In contrast, PCR-based techniques are simple to perform. Multiple locus, variable number tandem repeat analysis (MLVA) has been used to type *S. aureus* utilizing multiplex PCRs that target intragenic regions with variable numbers of tandem

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Abbreviations: CC, clonal cluster; MLVA, multiple locus, variable number tandem repeat analysis; PFGE, pulsed-field gel electrophoresis; SE, staphylococcal enterotoxin; SEI, staphylococcal enterotoxin-like; slv, single locus variant; ST, sequence type; TSST-1, toxic shock syndrome toxin-1.

repeats, namely *clfA*, *clfB*, *sdrC*, *sdrD*, *sdrE*, *sspA*, *spa*, *mecA*, *fnbA*, *fnbB* and *cna* (Sabat *et al.*, 2003, 2006; Francois *et al.*, 2005; Lindstedt, 2005; Malachowa *et al.*, 2005). This method has been shown to have a high level of inter-laboratory reproducibility (Lindstedt, 2005) and a discriminatory power and clustering capacity equivalent to PFGE (Malachowa *et al.*, 2005).

The most widely characterized of *S. aureus* regulatory loci is the accessory gene regulator (*agr*) (Novick, 2003; Robinson *et al.*, 2005; Wright *et al.*, 2005). Within the *agr* locus there is a variable region comprising the 3'-end of the *agrB* gene, the *agrD* gene and the 5'-end of the *agrC* gene. *S. aureus* has been divided into four main *agr* genotypes based on this sequence variation (Gilot *et al.*, 2002; Gilot & van Leeuwen, 2004; Wright *et al.*, 2005; Robinson *et al.*, 2005). Associations between the *agr* genotype of isolates and specific staphylococcal diseases have been shown for toxic shock syndrome and staphylococcal scalded skin syndrome (Jarraud *et al.*, 2002), and for abscesses and soft tissue infections in intravenous drug users (Monk *et al.*, 2004).

A survey by the National Food Centre in Dublin into microbial contamination of Irish household refrigerators revealed that 298/726 (41%) yielded *S. aureus* (Kennedy *et al.*, 2005). Analysis of 157 isolates from this survey demonstrated that 101 (64%) harboured at least two out of the *sea*, *seb*, *seg* and *sei* genes encoding enterotoxins with proven emetic activity (Smyth *et al.*, 2006). However, the animal or human origins of these enterotoxigenic *S. aureus* remain uncertain. While a study of *S. aureus* from cows, sheep, goats, rabbits and chickens had revealed isolates with enterotoxin gene profiles identical to those from Irish domestic refrigerators (Smyth *et al.*, 2005), no data were available on the frequencies of such genes in human carriage isolates in Ireland or on the population structure of Irish nasal isolates based on molecular typing methods.

In order to address this deficiency and thus enable epidemiological analysis, the present study was undertaken using PCR-based techniques (i) to characterize the enterotoxin (SE) and enterotoxin-like (SEI) gene profiles of *S. aureus* from a young, healthy Irish university student population, (ii) to determine the prevalence of the *tst* gene, encoding toxic shock syndrome toxin-1 (TSST-1), (iii) to group these isolates by *agr* typing, (iv) to determine the MLVA types and population structure, and (v) to ascertain the frequency of the *bbp* gene, an allelic variant of the *sdrE* gene amplified by the universal primers for *sdr* genes analysed by MLVA.

METHODS

Strain collection. As part of student laboratory exercises from 1995 to 2004, nasal swabs were taken from science students, dental students and dental nursing/dental hygiene students. These swabs were plated on Mannitol Salt agar (Oxoid) and incubated for 36–48 h. Single,

well-isolated colonies with the typical appearance of *S. aureus* were subcultured onto Tryptic Soya agar (Oxoid). Identification of these putative isolates as *S. aureus* was confirmed by sugar fermentation tests (arabinose, glucose, maltose, mannitol, sucrose, trehalose, xylose), the Voges–Proskauer test, DNase production, by a tube coagulase test using standard methods, and by the Staphaurex rapid latex test kit, which detects protein A and clumping factor (fibrinogen-binding protein) (Murex Diagnostics), according to the manufacturer's instructions. Primers specific for the *femA* gene encoding the *S. aureus* FemA peptidyltransferase (Schneider *et al.*, 2004) and generating a 132 bp amplicon were used for additional confirmation, as described by Mehrotra *et al.* (2000). Only isolates from students from the Republic of Ireland and Northern Ireland were retained.

Approximately 26–47% of the total number of nasal *S. aureus* isolates obtained in the years 1995, 1997, 2000 and 2002 were selected at random from those available. Only isolates from seven dental students were available for 2003, five of which were used. All of the isolates recovered during 2004 were included. The distribution of isolates by year of isolation was 1995, 7 isolates; 1997, 7 isolates; 2000, 8 isolates; 2002, 5 isolates; 2003, 5 isolates; and 2004, 16 isolates. The overall nasal carriage rate of the 492 students surveyed was 20.7%, with a range of 17–31% of the Irish students sampled in any year. These rates are very similar to those reported for medical students in three different years at Trinity College Dublin (Kingdom *et al.*, 1983). The numbers of isolates screened in this survey reflected approximately 10% of the students in each of the three student groups surveyed for *S. aureus* nasal carriage (15 isolates from 146 science students, 22 isolates from 245 dental science students, and 11 isolates from 101 dental nursing/dental hygiene students).

DNA extraction. Preparation of genomic DNA from *S. aureus* was performed as previously described (Fitzgerald *et al.*, 1997). The procedure involved 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 custom synthesized by MWG Biotech.

(i) MLVA. Primers used for MLVA were those described by Sabat *et al.* (2003). Primer pairs were tested individually and concentrations in the multiplex PCR primer mix optimized. Each multiplex PCR reaction contained 3–12 pmol of each primer. PCR was performed in a final volume of 25 µl. The PCR mix contained 16.2 µl H₂O, 1 µl dNTP mix (0.2 µM each dNTP), 0.3 µl *Taq* DNA polymerase (5 U µl⁻¹; Promega), 2.5 µl 10× buffer (10 mM Tris/HCl, pH 9.0), 3 µl 25 mM MgCl₂, 1 µl genomic DNA sample, and 1 µl primer mix. The thermal cycling conditions were those described by Sabat *et al.* (2003).

(ii) *agr* typing. An *agr* group-specific multiplex PCR was performed using the primers and thermal cycling conditions described by Gilot *et al.* (2002). The reaction mix contained 7–14 pmol of each primer. The following control strains for *agr* typing were kindly provided by university colleagues T. J. Foster and A. Rossney: strains Newman and COL (*agr* type I); strains MRSA252, MW2 and NCTC 8325-4 (*agr* type III) and strain RN4850 (*agr* type IV). Strain RF122 (*agr* type II) was from Fitzgerald *et al.* (2001).

(iii) *bbp* gene. The primers employed to detect the *bbp* gene encoding the bone sialoprotein-binding protein were those described by Tristan *et al.* (2003) using the thermal cycling conditions that Sabat *et al.* (2003) described for MLVA fingerprinting. The reaction mix contained 3–4 pmol of each primer. Strain MRSA252 was used as a positive control (Holden *et al.*, 2004).

(iv) **SE and SEI genes.** The standard nomenclature for SE (enterotoxin) and SEI (enterotoxin-like) genes recommended by Lina *et al.* (2004) is used throughout. All isolates were screened for the *sea*–*see*, *seg*–*sei*, *selj*–*selo*, *selq* and *tst* genes using the protocols and primers described by Smyth *et al.* (2005). These assays were done in a series of multiplex PCRs: mix 1, *sea*, *sec*, *sed*, *see* and 16S rRNA genes; mix 2, *seg*, *seh*, *sei*, *selj*, *tst* and 16S rRNA genes; mix 3, *sell*, *selm*, *seln*, *selo*, *selq* and 16S rRNA genes. The *seb* and *selk* genes were screened for using single PCRs. When *egc* locus-associated genes (*seg*, *sei*, *selm*, *seln*, *selo*) appeared to be missing from any isolate using multiplex PCR mix 2 or mix 3, individual PCRs were employed to recheck for these genes. The *sei*–*seln* intergenic region has been shown to harbour either two pseudogenes or the *selu* gene (Letertre *et al.*, 2003). Accordingly, isolates that were positive for the *egc* locus were screened by PCR-RFLP for the presence of pseudogenes ψ ent1 ψ ent2 or the *selu* gene to provide data on their relative occurrence in *egc*⁺ isolates (Letertre *et al.*, 2003; Coltery & Smyth, 2007).

The 16S rRNA gene was amplified as a positive control for *S. aureus* DNA in all multiplex PCRs (Monday & Bohach, 1999; Smyth *et al.*, 2005). DNA from SE and SEI gene-positive control strains was included with these multiplex PCR tests: RF122 (*sec*-bovine, *sell*, *tst*) (Fitzgerald *et al.*, 2001); NY19 (*seb*), FRI913 (*sea*, *sec*3, *see*, *tst*), FRI569 (*seh*), FRI472 (*sed*, *seg*, *sei*, *selj*), kindly provided by G. A. Bohach, University of Idaho, Moscow, USA, and A900322 (*selo*, *selm*, *sei*, ψ ent1, ψ ent2, *seln*, *seg*), kindly provided by S. Jarraud, Université Lyon 1, Centre National de Référence des Staphylocoques, Lyon, France (Smyth *et al.*, 2005). Strains A900322 and RF122 were used as controls for pseudogenes ψ ent1 ψ ent2 and the *selu* gene, respectively, in PCR-RFLP (Coltery & Smyth, 2007).

(v) **MLST.** MLST was performed selectively on isolates from MLVA clusters and subclusters by PCR amplification of internal fragments of seven housekeeping genes by using a previously described procedure and primers (Enright *et al.*, 2000). Sequencing of both DNA strands was performed commercially by Lark Technologies, using an automated Applied Biosystems 373A DNA sequencer, dye-labelled terminators and the primers used for PCR amplification. Analysis of sequences was performed using the BioNumerics software version 4.6 (Applied Maths). The alleles at each of the seven housekeeping loci were identified by comparing the sequences obtained from the test isolates with sequences held in the MLST database (<http://saureus.mlst.net>). This database was also used to identify the allelic profile and hence the sequence type (ST) of each isolate. STs were assigned to clonal complexes (CCs) using the clustering algorithm eBURST (based upon related sequence types) (Feil *et al.*, 2004; <http://eburst.mlst.net>). Using eBURST, isolates with specific STs are assigned to a particular CC if they are related to at least one other ST in that CC at six out of the seven MLST loci used. Isolates that do not share alleles at six of the seven MLST loci with any other ST in the MLST database are deemed singletons.

Computer-assisted analyses of MLVA band profiles. All PCR products were analysed on 2% (w/v) agarose gels, stained with ethidium bromide and visualized under UV light. For the purposes of generating dendrograms, PCR products from MLVA of *S. aureus* reference strain NCTC 8325-4, a derivative of a human isolate NCTC 8325 that is cured of prophages (Novick, 1967), were run in the first, middle and last lanes of gels as a universal gel standard. Computer-assisted analyses of MLVA band profiles were performed using the DENDRON software package version 2.4 (Solltech) as described previously (Soll, 2000; Gee *et al.*, 2002). Similarity coefficients (S_{AB} s) based on band position alone were calculated for each pairwise combination of isolate patterns according to the formula $S_{AB} = 2E / (2E + a + b)$, where E is the number of bands shared by isolates A and B, a is the number of bands unique to isolate A, and b is the number of bands unique to isolate B. An S_{AB} of 1.00 represents identical

patterns, and S_{AB} s ranging from 0.01 to 0.99 represent patterns with increasing proportions of bands at the same positions. Dendrograms were generated based on S_{AB} values using an unweighted pair-group method (Soll, 2000).

Statistics. Statistical significance was calculated using online calculators for the Fisher's exact test (<http://www.matforsk.no/ola/fisher.htm>) or χ^2 test (http://www.physics.csbsju.edu/stats/contingency_NROW_NCOLUMN_form.html).

RESULTS

SE, SEI and TSST-1 genes

All of the 48 nasal isolates of *S. aureus* screened for 16 SE and SEI genes possessed at least one of these; 66.7% had a classical SE gene (*sea*, *seb*, *sec*), of which 28 had a single gene and the remaining four had two of these genes in combination (3 *sea*⁺ *seb*⁺, 1 *seb*⁺ *sec*⁺) (Table 1). The commonest SE gene was *seb*, which was present in 75% of those possessing a classical SE gene. Of the isolates, 85.4% possessed the complete *egc* locus (*selo*, *selm*, *sei*, *seln*, *seg*); two other isolates lacked the *sei* gene and the *seln* and *selo* genes, respectively, as determined by both multiplex PCR and the use of primers for single genes (Table 1). The *seh*,

Table 1. Prevalence of enterotoxin genes, enterotoxin-like genes and the *tst* gene among 48 human nasal isolates of *S. aureus*

Gene	No. of isolates	Percentage of isolates
<i>sea</i> *	8	16.7
<i>seb</i> *	24	50.0
<i>sec</i> †	4	8.3
<i>sed</i>	0	0
<i>see</i>	0	0
<i>seg</i>	43	89.6
<i>seh</i>	4	8.3
<i>sei</i>	42	87.5
<i>selj</i>	0	0
<i>selk</i>	3	6.3
<i>sell</i>	7	14.6
<i>selm</i>	43	89.6
<i>seln</i>	42	87.5
<i>selo</i>	42	87.5
<i>selq</i>	3	6.3
<i>tst</i>	6	12.5
<i>egc</i> ‡	41	85.4
Incomplete <i>egc</i> §	2	4.2

*Three strains possessed the *sea* and *seb* genes in combination. The *sea* gene is carried by serotype F lysogenic converting bacteriophage (Carroll *et al.*, 1995).

†One isolate possessed the *seb* and *sec* genes in combination.

‡Complete *egc* locus comprising the *selo*, *selm*, *sei*, *seln* and *seg* genes. §*egc* locus lacking the *sei* gene or the *seln* and *selo* genes by multiplex PCR and the use of primers for individual genes.

selk, *sell*, *selq* and *tst* genes were each found in <15% of isolates (Table 1).

The *sei-seln* intergenic region of the *egc* locus generally harbours two pseudogenes or the *selu* gene (Letertre *et al.*, 2003). Of the 43 isolates with a complete or incomplete *egc* locus, 15 (34.9%) possessed pseudogenes ψ *ent1* and ψ *ent2* and 19 (44.2%) the *selu* gene as shown by PCR-RFLP (Collery & Smyth, 2007). None of the remaining 9 *egc*⁺ isolates, including the two with incomplete *egc* loci, gave PCR products with the primers of Letertre *et al.* (2003) despite several repeated attempts. Four of these 'non-typable' isolates lacked classical SE genes (1 *egc* only, 1 *egc seh*, 2 with incomplete *egc* loci). The other five 'non-typable' isolates all possessed the *seb* gene in combination with the *egc* locus (2 *seb egc*; 1 *seb egc selk selq*; 1 *seb sea egc*; 1 *seb sec egc sell tst*). Thomas *et al.* (2006) described isolates with *egc* loci harbouring insertion sequences and transposase genes, as well as an atypical *egc* locus with two new SEI genes, designated *selu2* and *selv*, generated by a limited deletion in pseudogenes ψ *ent1* and ψ *ent2* and by recombination between *selm* and *sei*, respectively. It is not known whether the primers described by Letertre *et al.* (2003) would type the *sei-seln* intergenic regions of such variant isolates.

The rates of occurrence of classical SE genes varied among the isolates examined from each of the six sampling years: 1995, 1 *seb*⁺, 14.3%; 1997, 5 *seb*⁺, 2 *sea*⁺ *seb*⁺, 100%; 2000, 7 *seb*⁺, 87.5%; 2002, 2 *sea*⁺, 1 *seb*⁺, 60%; 2003, 2 *seb*⁺, 3 *sec*⁺, 100%; and 2004, 3 *sea*⁺, 1 *sea*⁺ *seb*⁺, 4 *seb*⁺, 1 *seb*⁺ *sec*⁺, 56.3%. The differences in the numbers of isolates possessing classical SE genes between sampling years are statistically significant for 1995 versus 1997 and 1995 versus 2000 (Fisher's exact test, 2-tailed, *P*=0.005 and *P*=0.01, respectively). Humphreys *et al.* (1989) found that only 11.1% of 27 nasal isolates obtained from healthy medical students prior to arrival in hospitals for their clinical training and from biochemistry laboratory technicians in Dublin in the mid 1980s produced a classical SE (all SED-positive). Moreover, they found a statistically significant lower rate of SEB production among 17 community-acquired versus 35 hospital-acquired septicemia isolates (*P*<0.01). While the prior study tested for SEs and the present study for SE genes, the enterotoxin genotype data imply a real change in the rate of occurrence of *seb*⁺ isolates in the community from the late 1990s compared to the mid 1980s through mid 1990s, coupled with a more frequent occurrence of *sea*⁺ and *sec*⁺ isolates. Carriage of the *sed* gene-bearing plasmid may have imposed a fitness cost that was ameliorated by its loss.

Sixteen of the 22 dental students (72.7%), 5 of the 11 dental nursing/hygiene students (45.5%) and 9 of the 15 science students (60.0%) yielded isolates bearing one or two classical SE genes. The differences in the carriage rates of isolates bearing classical SE genes between these student groups are not statistically significant. The somewhat higher occurrence of isolates bearing SE genes in the

science and dental student groups may reflect the gender balance of these groups (see Methods) since male gender has been shown to be a candidate factor for persistent carriage in a student community (Bischoff *et al.*, 2004). The numbers of isolates bearing *sea:seb:sec* enterotoxin genes among nasal isolates from the dental, dental nurse/hygienist and science student groups were 3:13:3, 1:4:0 and 4:7:1, respectively. The differences in the frequencies of these genes between the three student groups are not statistically significant.

SE, SEI and TSST-1 gene combinations

The two commonest gene combinations were the *seb* gene with the *egc* locus and the *egc* locus alone (complete and incomplete), found in 13 isolates (27.1%) and in 12 isolates (25%), respectively (Table 2). Twenty-seven of the 32 isolates (84.4%) possessing a classical SE gene also had a complete *egc* locus. Of the five isolates possessing a classical SE gene but lacking the *egc* locus, two possessed the *sea* and *seb* genes together (one also with the *selk* and *selq* genes), and three possessed the *seb* gene only. The *egc* locus was present in 6 of the 8 isolates with the *sea* gene (75%) – 5 of these carried the *selu* gene, in 19 of the 24 isolates with the *seb* gene (79.2%) – 9 of these carried the *selu* gene, and in all 4 isolates with the *sec* gene – none of these carried the *selu* gene. Only 4 of the 14 isolates with a complete *egc* locus and lacking a classical enterotoxin gene had the *selu* gene. Neither of the isolates with an incomplete *egc* locus possessed a classical SE gene, additional SE or SEI genes, the *tst* gene or the *selu* gene.

Each of the six isolates containing the *tst* gene harboured at least one classical SE gene; all had the *egc* locus, three the *selu* gene, and three the *sell* gene. Of the three isolates possessing the *selk-selq* gene combination, two also had the *seb* gene and the *egc* locus (one with the *selu* gene), and the third isolate had the *sea* and *seb* genes. Of the four isolates with the *seh* gene, all had the *egc* locus – one with the *selu* gene, and two with the *sea* gene. All of the seven isolates with the *sell* gene had the *egc* locus – only one with the *selu* gene, three of which had classical SE genes – one with *seb*, one with *seb* and *sec* and one with *sec* – and the *tst* gene.

agr genotyping

The nasal isolates were *agr* genotyped (Table 2). The two commonest *agr* genotypes were types I and III. Only one strain was found to be of *agr* type IV. Eight of the *agr* type I isolates, seven of the *agr* type II isolates, 14 of the *agr* type III isolates and the single *agr* IV isolate harboured classical SE genes. These differences in the rates of occurrence of classical SE gene-positive isolates between *agr* genotypes I, II and III are not significant, nor are the rates of occurrence of individual classical SE genes between these *agr* types.

With respect to the commonest SE-SEI gene combination, namely *seb egc*, three isolates were of *agr* type I, four of *agr* type II, five of *agr* type III, and one of *agr* type IV (Table 2).

Table 2. Prevalence of *agr* genotypes and SE, SEI and TSST-1 genotypes among 48 human nasal isolates of *S. aureus*

<i>agr</i> genotype	SE, SEI, TSST-1 genes	No. of isolates	Percentage of isolates*
Type I		17	35.4
	<i>sea</i> †, <i>seb</i>	1	5.9
	<i>sea</i> †, <i>seb</i> , <i>selk</i> ‡, <i>selq</i> ‡	1	5.9
	<i>sea</i> †, <i>seb</i> , <i>egc</i> §	1	5.9
	<i>seb</i> , <i>egc</i>	3	17.6
	<i>seb</i> , <i>selk</i> , <i>selq</i> , <i>egc</i>	2	11.8
	<i>sec</i> , <i>sell</i> , <i>egc</i>	2	11.8
	<i>seh</i> , <i>egc</i>	2	11.8
	<i>sell</i> , <i>egc</i>	1	5.9
	<i>egc</i>	4	23.5
Type II		12	25.0
	<i>seb</i>	3	25.0
	<i>seb</i> , <i>egc</i>	4	33.3
	<i>egc</i>	3	25.0
	<i>egcII</i>	2	16.7
Type III		18	37.5
	<i>sea</i> †, <i>egc</i>	1	5.6
	<i>sea</i> †, <i>seh</i> , <i>egc</i>	2	11.1
	<i>sea</i> †, <i>egc</i> , <i>tst</i>	2	11.1
	<i>seb</i> , <i>egc</i>	5	27.8
	<i>seb</i> , <i>egc</i> , <i>tst</i>	1	5.6
	<i>seb</i> , <i>sell</i> , <i>egc</i> , <i>tst</i>	1	5.6
	<i>seb</i> , <i>sec</i> , <i>sell</i> , <i>egc</i> , <i>tst</i>	1	5.6
	<i>sec</i> , <i>sell</i> , <i>egc</i> , <i>tst</i>	1	5.6
	<i>sell</i> , <i>egc</i>	1	5.6
	<i>egc</i>	3	16.7
Type IV		1	2.1
	<i>seb</i> , <i>egc</i>	1	100.0

*Gene distribution within *agr* genotype calculated as percentage of isolates of that *agr* type.

†The *sea* gene is carried by serotype F lysogenic converting bacteriophage (Carroll *et al.*, 1995).

‡The *sek* and *seq* genes occur along with the *seb* gene on a pathogenicity island (Orwin *et al.*, 2002).

§Complete *egc* locus comprising the *selo*, *selm*, *sei*, *seln* and *seg* genes.

||Incomplete *egc* locus lacking either the *sei* gene or the *seln* and *selo* genes.

Of those with the complete *egc* locus alone, four isolates were of *agr* type I, and three each of *agr* types II and III. Of the seven harbouring the *egc* locus and the *sell* gene with or without a SE gene, three were of *agr* type I and four of *agr* type III. All six isolates with the *tst* gene were of *agr* type III. The difference in the rates of occurrence of the *tst* gene between *agr* types I, II and III is significant (χ^2 test, $P=0.017$). The three isolates that contained the *selk*–*selq* gene combination were of *agr* type I. Both the *seh* and *sell* genes were only associated with *agr* types I and III (Table 2).

The isolates from dental students were distributed as follows: *agr* type I, 10, 45.5 %; *agr* type II, 5, 22.7 %; and *agr* type III, 7, 31.8 %. For the dental nurse/hygienist group the distribution was *agr* type I, 4, 36.4 %; *agr* type II, 2, 18.2 %;

agr type III, 4, 36.4 %; and *agr* type IV, 1, 9.1 %. In the case of the science students the distribution was *agr* type I, 3, 20.0 %; *agr* type II, 5, 33.3 %; and *agr* type III, 7, 46.7 %. The differences in the rates of occurrence of *agr* genotypes I, II and III between these student groups are not significant. Nor are the differences in the rates of occurrence of the *agr* genotypes by year of isolation significant.

***bbp* gene**

When MLVA fingerprinting was performed according to Sabat *et al.* (2003) on the 48 nasal isolates, the PCR band profiles of 28 isolates (58.3 %) revealed only five or six bands against the seven anticipated on the basis of the primer sets used to amplify seven genes, while a few strains produced eight bands (Fig. 1). These MLVA patterns were highly reproducible. The differences in the numbers of bands were subsequently associated with differences in the presence or absence of *sdr* gene products. Sabat *et al.* (2006) reported that the PCR product of the *sdrC* gene was always present, but that one or both of the *sdrD* and *sdrE* gene PCR products was absent in 58.9 % of meticillin-sensitive isolates from asymptomatic nasal carriers in the UK, the Netherlands and Poland. In the latter respect the present observations mirror that report.

Universal forward and reverse primers for the *sdrC*, *sdrD* and *sdrE* genes are used in the MLVA multiplex PCR of Sabat *et al.* (2003). The *bbp* gene is an allelic variant of the *sdrE* gene (Tung *et al.*, 2000). The A-regions of the Bbp and SdrE proteins show 76 % amino acid sequence identity and the B-regions, 95–96 % identity (Tung *et al.*, 2000). Using the BLAST program (<http://www.ncbi.nlm.nih.gov/blast/>), the universal forward and reverse *sdr* primers of Sabat *et al.* (2003) have 100 % matches for *bbp* gene sequences of *S. aureus* strains 024 (Tung *et al.*, 2000), MRSA252 (Holden *et al.*, 2004) and NN1 (Otsuka *et al.*, 2006).

As the primers used by Sabat *et al.* (2003, 2006) and Malachowa *et al.* (2005) do not distinguish the *sdrE* gene from the *bbp* gene, the frequency of the *bbp* gene among the nasal isolates was determined using specific primers that do not match *sdrCDE* gene sequences (Tristan *et al.*, 2003). Twenty isolates were positive for the *bbp* gene. None of the *bbp*⁺ isolates yielded five bands on MLVA fingerprinting, while 11 gave six bands, six gave seven bands, and three gave eight bands, indicating that the *bbp* and *sdrE* genes were both present in the latter three MLVA groups of isolates. The *bbp* gene was present in one *agr* type I isolate (5.9 %), in 4 *agr* type II isolates (33.3 %), in 14 out of 18 *agr* type III isolates (77.8 %), and in the one *agr* type IV isolate. The differences in the frequencies of the *bbp* gene between *agr* type III and *agr* types I and II are statistically significant (Fisher's exact test, 2-tailed, $P=0.00002$ and $P=0.025$, respectively). There were no associations between the occurrence of the *bbp* gene and the *egc* locus or the *bbp* gene and classical SE genes either taken together or separately.

Fig. 1. MLVA of genetic polymorphisms within the *clfA*, *clfB*, *sdrC*, *sdrD*, *sdrE*, *sspA*, *spa* and *bbp* genes of nasal isolates of *S. aureus*. MLVA fingerprints of 12 nasal isolates are shown (lane numbers above gel: 2–7, 9–11 and 13–15). Lanes 1, 8 and 16 show the MLVA fingerprint of strain NCTC 8325-4 as an internal universal standard. Lane 12 shows a PCR negative control with no bacterial DNA present. The numbers of PCR products varied between five and eight (indicated below each lane). The differences in the number of bands are associated with differences at the *sdr* and *bbp* loci.

MLVA typing

The 48 *S. aureus* nasal isolates produced 47 MLVA patterns. S_{AB} values were computed for all possible pairwise combinations of isolates and the data used to construct a dendrogram showing the relationships between the nasal isolates (Fig. 2). The mean S_{AB} for 1128 pairwise comparisons was 0.25. Examination of the dendrogram reveals that the isolates were divided into two clusters, designated I and II, comprising 11 and 37 of the isolates with mean S_{AB} s of 0.15 ± 0.14 and 0.29 ± 0.21 , respectively. Seven of the cluster I isolates were of *agr* type I, three of *agr* type II, and one of *agr* type III. These isolates formed a heterogeneous population with limited relatedness.

MLVA cluster II could be subdivided into four subclusters, designated IIa to IId, at S_{AB} nodes of 0.37, 0.24 and 0.12. There was a marked distribution of isolates according to *agr* type within these subgroups. Subgroup IIa contained 13 isolates of *agr* type III and the single isolate of *agr* type IV. Subgroup IIb comprised the remaining four isolates of *agr* type III, two isolates of *agr* type I and one isolate of *agr* type II. Subgroups IIc and IId each contained eight isolates, with four each of *agr* types I and II. Within MLVA cluster II, 21 of the 37 isolates formed nine pairs and one triplet of the same *agr* types at different S_{AB} nodes. In MLVA cluster I, four of the 11 isolates paired with respect to *agr* type at different S_{AB} nodes.

Subgroups IIa and IIb (mean S_{AB} 0.71) are more related to each other than to subgroups IIc and IId (mean S_{AB} 0.58). The distribution of isolates of *agr* types I and II combined compared to *agr* types III and IV combined within MLVA subgroups IIa and IIb combined versus subgroups IIc and IId combined is highly statistically significant (Fisher's exact test, 2-tailed, $P=0.00000009$). Because of the strong association of the *bbp* gene with *agr* type III, the distribution of *bbp*⁺ isolates within MLVA subgroups IIa

and IIb combined versus subgroups IIc and IId combined is statistically significant (Fisher's exact test, 2-tailed, $P=0.00033$).

Of the isolates of *agr* type III that possessed the *tst* gene, four were in subgroup IIa and two in subgroup IIb. However, there was no association between the MLVA clusters or the four subclusters and the SE–SEI genotypes of isolates or between the MLVA clusters or the four subclusters and their year of isolation or the student group from which they were isolated.

MLST typing

On the basis of the MLVA dendrogram, 13 of the Irish nasal isolates were selected for MLST typing – four from subcluster IIa (all *agr* type III) and two from subcluster IIb (both *agr* type III), two from subcluster IIc and two from subcluster IId (three *agr* type II, one *agr* type I), and three from cluster I (all *agr* type I), reflecting 1:3 to 1:4 of the isolates in any cluster or subcluster. Selection within clusters or subclusters was based on diversity of enterotoxin gene profile and *agr* type.

The 13 isolates investigated were found to consist of 12 different STs, including seven novel STs (ST932, ST933, ST934, ST935, ST936, ST937 and ST938) (Fig. 2, Table 3). One novel *aroE* allele was found in ST937 and was designated 157. Using eBURST these 12 STs belonged to five CCs (CC30, $n=5$; CC6, $n=2$; CC5, $n=1$; CC22, $n=1$; CC9, $n=1$), and three were singletons.

Of the four isolates in subcluster IIa, two were ST30 (isolates MC15 and MC26) and one was ST484 (isolate MC19), a single locus variant (slv) of both ST30 and ST36 at the *pta* locus. The remaining isolate MC24 was of ST938, a slv of ST30 at the *gmk* locus. Three of the isolates formed pairs with other isolates with S_{AB} s of >0.82 by MLVA and

Fig. 2. Dendrogram generated from the S_{AB} values computed for every possible pairwise combination of 48 *S. aureus* nasal carriage isolates from an Irish university student population fingerprinted by MLVA. The S_{AB} scale is shown below the dendrogram. On the basis of their MLVA fingerprints the isolates are divided into two main populations, designated I and II, comprising 11 and 37 of the isolates, respectively. MLVA population II can be further divided into four subclusters, designated IIa (14 isolates), IIb (7 isolates), IIc (8 isolates) and IId (8 isolates), at S_{AB} nodes of 0.37, 0.24 and 0.12. Seventeen of the 18 *agr* type III isolates are in subgroups IIa and IIb, while all the isolates in subgroups IIc and IId are all of *agr* types I and II. Thirteen isolates were chosen for MLST analysis based on the major isolate groupings identified in the MLVA dendrogram. The sequence type (ST) and clonal complex (CC) of these isolates are shown in parentheses on the right of the figure. Table 3 shows the allelic profiles of the seven novel STs found in the present study, which are identified on the dendrogram in bold italics.

the fourth isolate, MC15, had an S_{AB} of 0.55 to one of these pairs. All belonged to CC30. Of those examined in subcluster IIb, isolate MC14 was ST936, a singleton with

Table 3. Allelic profiles of the seven novel STs found in the present study

	<i>arcC</i>	<i>aroE</i>	<i>glpF</i>	<i>gmk</i>	<i>pta</i>	<i>tpi</i>	<i>yqiL</i>
ST932	12	4	1	4	1	1	3
ST933	2	2	2	1	2	2	2
ST934	18	18	6	1	13	15	7
ST935	1	4	2	4	12	1	10
ST936	2	45	2	6	2	2	2
ST937	1	157	1	1	11	5	11
ST938	2	2	2	6	6	3	2

no close relatives in the database, and isolate MC16 was ST933, a slv of ST39 at the *gmk* locus and a member of CC30. Thus, of the six examined *agr* type III isolates within subclusters IIa and IIb, five belonged to CC30.

Of the two isolates in subcluster IIc, strain MC29 (*agr* type I) was of ST6 and the predicted ancestor of a small clonal complex, CC6. Strain MC38 (*agr* type II) was ST935, a slv of ST5 at the *glpF* locus, and belonged to CC5. Isolate MC31 (*agr* type II) in subcluster IId was ST109, a slv of ST9 at *aroE*, and thus belonged to CC9. The other isolate MC38 (*agr* type II) was ST937 and was predicted by eBURST to be a singleton.

Of the three isolates in subcluster I (all *agr* type I), strain MC49 was ST217, a slv of ST22 at the *tpi* locus, and belonged to CC22. Isolate MC43 was ST932, a slv of ST6 at the *pta* locus, and belongs to CC6. Isolate 40 was ST934 and predicted by eBURST to be a singleton. These isolates were distantly related by MLVA (S_{AB} of 0.04–0.14).

Thus, the more closely related isolates by MLVA (subclusters IIa and IIb) belonged to the same CC, while those in more distantly related MLVA groups (cluster I and subclusters IIc and IId) belonged to diverse CCs. Indeed, Malachowa *et al.* (2005) found the overall correlation between MLVA clusters and MLST CCs to be lower than between MLVA and PFGE clusters and MLVA and *spa* typing clusters.

DISCUSSION

Few data are available on SE and TSST-1 production by or on the presence of SE, SEI and TSST-1 genes in Irish human isolates of *S. aureus* (Humphreys *et al.*, 1989; Aucken *et al.*, 2006) or in animal isolates (Fitzgerald *et al.*, 2000; Smyth *et al.*, 2005, 2006). Herein 100% of nasal isolates from a young, healthy, Irish university student population collected over a 10-year period harboured a SE and/or SEI gene, 66.7% possessing at least one classical SE gene and 89.6%, a complete or incomplete *egc* locus. Of the isolates harbouring a classical SE gene, 84.4% had a complete *egc* locus. In contrast to the findings of Humphreys *et al.* (1989) on SED production, no isolate possessed the *sed* gene.

Five groups in Germany, Japan, Poland, New Zealand and Bulgaria have screened nasal carriage isolates for 12–18 of the SE and SEI genes in *S. aureus* discovered at the time of the present study (Becker *et al.*, 2003, 2004; Omoe *et al.*, 2005; Bania *et al.*, 2006; Boerema *et al.*, 2006; Nashev *et al.*, 2007). Comparison of these studies with the findings herein brings out several points. The percentage of isolates possessing an SE or SEI gene varied from 75 to 100 %. The proportion bearing a classical SE gene ranged from 30 to 67 %. The predominant classical SE gene varied from country to country – Ireland, *seb*; Germany, *sea*, *sec*; Japan, *seb*; New Zealand, *seb*; Poland, *sec*; and Bulgaria, *sea*. Moreover, of isolates with a classical SE gene, those possessing a complete (and incomplete) *egc* locus ranged from 38 to 84 % between these studies. Differences in the nature of the study population screened for nasal carriage, i.e. healthy individuals versus patients, and differences in PCR screening methodologies, e.g. primers, amplification cycles, single versus multiplex PCR (Monday & Bohach, 1999; Jarraud *et al.*, 2001; Becker *et al.*, 2003, 2004; Chen *et al.*, 2004; Smyth *et al.*, 2005; Bania *et al.*, 2006), may contribute to such variations, and as indeed may the makes of the thermal cyclers used (Saunders *et al.*, 2001; Schoder *et al.*, 2005).

Differences in the frequencies of incomplete *egc* loci among nasal isolates are particularly notable – 38.1 % (Becker *et al.*, 2003, 2004), 12.4 % (Omoe *et al.*, 2005), 5 % (Boerema *et al.*, 2006), 0 % (Bania *et al.*, 2006), 11.1 % (Nashev *et al.*, 2007) and 4.2 % herein. In an extensive study of human isolates collected during 2001–2003 from suppurative infections, acute toxemia and asymptomatic nasal carriage, Thomas *et al.* (2006) found incomplete *egc* loci to be uncommon (3.8 % of *egc* gene-positive isolates). While the Polish, New Zealand and Bulgarian investigations (Bania *et al.*, 2006; Boerema *et al.*, 2006; Nashev *et al.*, 2007) tested for *egc* locus genes individually, neither the Japanese study nor the German studies (Becker *et al.*, 2003, 2004; Omoe *et al.*, 2005) stated that they rechecked their *egc* loci with ‘missing’ genes using individual sets of primers. Detection of *egc* genes by PCR may be limited by primer specificity for individual alleles (Monday & Bohach, 1999; Jarraud *et al.*, 2001; Becker *et al.*, 2003, 2004; Smyth *et al.*, 2005; Bania *et al.*, 2006) as sequence variants of the *seg*, *sei* and *seln* genes have been described (Abe *et al.*, 2000; Letertre *et al.*, 2003; Blaiotta *et al.*, 2004).

The frequency of the *seh* gene in nasal isolates is generally low: 4.3 % (Jarraud *et al.*, 2002), 16.7 % and 4.1 % in Japan (Omoe *et al.*, 2002, 2005), 3.8 % in Poland (Bania *et al.*, 2006), 6.2 % in Germany (Becker *et al.*, 2003), 20 % in New Zealand (Boerema *et al.*, 2006), 6.7 % in Bulgaria (Nashev *et al.*, 2007) and 8.3 % herein. The *selq* gene is present on the SaPI3 pathogenicity island directly 5' of the *selk* gene and together with the *seb* gene (Orwin *et al.*, 2002). In nasal isolates the frequency of the *selk selq* gene combination is low: 5 % in Poland (Bania *et al.*, 2006), 6.2 % in Japan (Omoe *et al.*, 2005) and 6.3 % herein. The rates of occurrence of the *tst* gene in nasal isolates are fairly

consistent – 12.4 % in Japan (Omoe *et al.*, 2005), 12.5 % in Poland (Bania *et al.*, 2006), 22.4 % in Germany (Becker *et al.*, 2003), 24.3 % in the USA (Mehrotra *et al.*, 2000) and 13.7 % herein.

In two studies of nasal carriage isolates, the *agr* type distributions were: *agr* type I, 55.2 % and 52.3 %, versus 35.4 % herein; *agr* type II, 25.9 % and 29.2 %, versus 25.0 % herein; *agr* type III, 17.2 % and 10.8 %, versus 37.5 % herein; and *agr* type IV, 0 % and 7.7 %, versus 2.1 % herein (Cespedes *et al.*, 2005; Lina *et al.*, 2003). In a study of nasal isolates from children and their guardians, Shopsin *et al.* (2003) reported that the distribution of the four *agr* groups was *agr* type I, 41.6 %; *agr* type II, 24.7 %; *agr* type III, 33.8 %; and *agr* type IV, 0 %; this is somewhat similar to that found in the present study. All of the Irish isolates possessing the *tst* gene were of *agr* type III and belonged to MLVA subgroups IIa and IIb, four of these isolates possessing the *bbp* gene.

The diversity in the numbers of bands produced on MLVA fingerprinting had been unexpected, as such observations had not been specifically annotated in the original report of Sabat *et al.* (2003). Subsequently, Sabat *et al.* (2006) reported that, while all 142 MLVA-typed nasal isolates possessed the *sdrC* gene, only 55 (38.7 %) of these were both *sdrD*- and *sdrE*-positive, 76 (53.5 %) had the *sdrE* gene but not the *sdrD* gene, 3 (2.1 %) had the *sdrD* but not the *sdrE* gene, and 8 (5.6 %) lacked both the *sdrD* and *sdrE* genes, i.e. 94.4 % would yield six or seven bands versus 40 out of 48 nasal isolates (83.3 %) yielding six to eight bands herein. The percentage of isolates herein possessing the *bbp* gene (41.7 %) is somewhat higher than that reported by Tristan *et al.* (2003) for nasal isolates (20.7 %).

Three isolates yielding eight bands on MLVA typing were *bbp*-positive. While the *sdrE* and *bbp* alleles have been found separately in isolates (Otsuka *et al.*, 2006; Stephens *et al.*, 2006; Taneike *et al.*, 2006), their dual occurrence has only been previously recorded in a single community-acquired MRSA isolate NN13 (Otsuka *et al.*, 2006). However, seven isolates belonging to CC30 of MLST types ST36, ST30 and ST39 have been found to possess the *sdrE* and *bbp* genes with *bbp* arranged tandemly immediately downstream of *sdrE* (Dr Edward Feil, University of Bath, personal communication). The three isolates in the present study possessing the *sdrE* and *bbp* alleles all had the *seb* gene and the *egc* locus with the *selu_v* gene (Letertre *et al.*, 2003; M. M. Collery, unpublished data), were of *agr* type III and grouped in MLVA subclusters IIa and IIb, two of them being confirmed to belong to CC30 by MLST.

The association seen between MLVA subgroups and *agr* genotype has not been previously recorded, although MLVA clusters have been shown to match well with those obtained by PFGE and to be consistent with *spa* typing (Malachowa *et al.*, 2005). PFGE patterns and *agr* types have been shown to be linked within certain CCs (Goerke *et al.*, 2005). PFGE has also revealed a pronounced diversity among nasal carriage isolates and that isolates (non-MRSA) from

unrelated individuals yield unique patterns (Hu *et al.*, 1995; Cespedes *et al.*, 2005).

The Irish nasal carriage isolates investigated in the present study belonged to diverse genotypes as determined by MLST analysis. They yielded 12 distinct STs, seven of which had not been described previously. Furthermore, 3 of the 13 isolates were singletons and the remainder belonged to five CCs, the most common being CC30. Interestingly, three of these CCs comprised major pandemic clones, i.e. CC30, CC5 and CC22, whereas the other two, CC6 and CC9, belonged to minor lineages. Grundmann *et al.* (2002) previously identified *S. aureus* isolates of CC30, CC22, CC5 and CC9 among nasal carriage isolates in the UK, similar to the present study. They also identified CC30 as the commonest, accounting for 25.6% of the nasal isolates examined. In addition, Peacock *et al.* (2002) reported that 26–33% of nasal isolates from a cohort of renal dialysis outpatients on any of three sampling occasions belonged to CC30. Based on the selective MLST typing of isolates within subclusters IIa and IIb herein, a similar overall percentage of nasal isolates would be predicted to belong to CC30.

As the outbreak incidence of food-borne disease declines, monitoring sporadic cases of staphylococcal food poisoning will be an important challenge for the 21st century (Mead *et al.*, 1999). The molecular genetic definition of a population of nasal carriage isolates from a healthy Irish population together with data on molecular genotyping of animal isolates (Smyth *et al.*, 2005) and of *S. aureus* from contaminated Irish domestic refrigerators (Smyth *et al.*, 2006) should add to the knowledge base for epidemiological analysis of staphylococcal food poisoning incidents in Ireland and of the roles, which are possibly underestimated, of the more newly described SEs and possibly SEIs in this toxin-mediated syndrome.

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REFERENCES

- Abe, J., Ito, Y., Onimaru, M., Kohsaka, T. & Takeda, T. (2000). Characterization and distribution of a new enterotoxin-related superantigen produced by *Staphylococcus aureus*. *Microbiol Immunol* **44**, 79–88.
- Arshad, M. F., Dunn, F. J., Vega, R., Valvano, J. W. & Serwer, P. (1993). Progress in developing improved programs for pulsed field agarose gel electrophoresis of DNA. *Electrophoresis* **14**, 344–348.
- Aucken, H. M., O'Neill, G., Ganner, M., Dinerstein, N., Ali, M. & Murchan, S. (2006). Irish-1 and Irish-2: UK-epidemic methicillin-resistant *Staphylococcus aureus* strains associated with Northern Ireland. *J Hosp Infect* **63**, 170–178.
- Bania, J., Dabrowska, A., Korzekwa, K., Zarczynska, A., Bystron, J., Chrzanowska, J. & Molenda, J. (2006). The profiles of enterotoxin genes in *Staphylococcus aureus* from nasal carriers. *Lett Appl Microbiol* **42**, 315–320.
- Becker, K., Friedrich, A. W., Lubritz, G., Weilert, M., Peters, G. & von Eiff, C. (2003). Prevalence of genes encoding pyrogenic toxin superantigens and exfoliative toxins among strains of *Staphylococcus aureus* isolated from blood and nasal specimens. *J Clin Microbiol* **41**, 1434–1439.
- Becker, K., Friedrich, A. W., Peters, G. & von Eiff, C. (2004). Systematic survey on the prevalence of genes coding for staphylococcal enterotoxins SEIM, SEIO, and SEIN. *Mol Nutr Food Res* **48**, 488–495.
- Bischoff, W. E., Wallis, M. L., Tucker, K. B., Reboussin, B. A. & Sherertz, R. J. (2004). *Staphylococcus aureus* nasal carriage in a student community: prevalence, clonal relationships, and risk factors. *Infect Control Hosp Epidemiol* **25**, 485–491.
- Blaiotta, G., Ercolini, D., Pennacchia, C., Fusco, V., Casaburi, A., Pepe, O. & Villani, F. (2004). PCR detection of staphylococcal enterotoxin genes in *Staphylococcus* spp. strains isolated from meat and dairy products. Evidence for new variants of *seG* and *seI* in *S. aureus* AB-8802. *J Appl Microbiol* **97**, 719–730.
- Boerema, J. A., Clemens, R. & Brightwell, G. (2006). Evaluation of molecular methods to determine enterotoxigenic status and molecular genotype of bovine, ovine, human and food isolates of *Staphylococcus aureus*. *Int J Food Microbiol* **107**, 192–201.
- Carroll, D., Kehoe, M. A., Cavanagh, D. & Coleman, D. C. (1995). Novel organization of the site-specific integration and excision recombination functions of the *Staphylococcus aureus* serotype F virulence-converting phages Φ 13 and Φ 42. *Mol Microbiol* **16**, 877–893.
- Cespedes, C., Saïd-Salim, B., Miller, M., Lo, S.-H., Kreiswirth, B. N., Gordon, R. J., Vavagiakis, P., Klein, R. S. & Lowy, F. D. (2005). The clonality of *Staphylococcus aureus* nasal carriage. *J Infect Dis* **191**, 444–452.
- Chen, T.-R., Chiou, C.-S. & Tsen, H.-Y. (2004). Use of novel PCR primers specific to the genes of staphylococcal enterotoxin G, H, I for the survey of *Staphylococcus aureus* strains isolated from food-poisoning cases and food samples in Taiwan. *Int J Food Microbiol* **92**, 189–197.
- Collery, M. M. & Smyth, C. J. (2007). 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. *J Med Microbiol* **56**, 208–216.
- Enright, M. C., Day, N. P. J., Davies, C. E., Peacock, S. J. & Spratt, B. J. (2000). Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. *J Clin Microbiol* **38**, 1008–1015.
- Feil, E. J., Li, B. C., Aanensen, D. M., Hanage, W. P. & Spratt, B. J. (2004). eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes with multilocus sequence typing data. *J Bacteriol* **186**, 1518–1530.
- Ferry, T., Perpoint, T., Vandenesch, F. & Etienne, J. (2005). Virulence determinants in *Staphylococcus aureus* and their involvement in clinical syndromes. *Curr Infect Dis Rep* **7**, 420–428.
- Fitzgerald, J. R., Meaney, W. J., Hartigan, P. J., Smyth, C. J. & Kapur, V. (1997). Fine-structure molecular epidemiological analysis of *Staphylococcus aureus* recovered from cows. *Epidemiol Infect* **119**, 261–269.
- Fitzgerald, J. R., Hartigan, P. J., Meaney, W. J. & Smyth, C. J. (2000). Molecular population and virulence factor analysis of *Staphylococcus*

- aureus* from bovine intramammary infection. *J Appl Microbiol* 88, 1028–1037.
- Fitzgerald, J. R., Monday, S. R., Foster, T. J., Bohach, G. A., Hartigan, P. J., Meaney, W. J. & Smyth, C. J. (2001). Characterization of a putative pathogenicity island from bovine *Staphylococcus aureus* encoding multiple superantigens. *J Bacteriol* 183, 63–70.
- Francois, P., Huyghe, A., Charbonnier, Y., Bento, M., Herzig, S., Topolski, I., Fleury, B., Lew, D., Vaudaux, P. & other authors (2005). Use of an automated multiple-locus, variable-number tandem repeat-based method for rapid and high-throughput genotyping of *Staphylococcus aureus*. *J Clin Microbiol* 43, 3346–3355.
- Gee, S. F., Joly, S., Soll, D. R., Meis, J. F. G. M., Verweij, P. E., Polacheck, I., Sullivan, D. J. & Coleman, D. C. (2002). Identification of four distinct genotypes of *Candida dubliniensis* and detection of microevolution in vitro and in vivo. *J Clin Microbiol* 40, 556–574.
- Gilot, P. & van Leeuwen, W. (2004). Comparative analysis of *agr* locus diversification and overall genetic variability among bovine and human *Staphylococcus aureus* isolates. *J Clin Microbiol* 42, 1265–1269.
- Gilot, P., Lina, G., Cochard, T. & Poutrel, B. (2002). Analysis of the genetic variability of genes encoding the RNA III-activating components AgrC and TRAP in a population of *Staphylococcus aureus* strains isolated from cows with mastitis. *J Clin Microbiol* 40, 4060–4067.
- Goerke, C., Esser, S., Kummel, M. & Wolz, C. (2005). *Staphylococcus aureus* strain designation by *agr* and *cap* polymorphism typing and delineation of *agr* diversification by sequence analysis. *Int J Med Microbiol* 295, 67–75.
- Grundmann, H., Hori, S., Enright, M. C., Webster, C., Tami, A., Feil, E. J. & Pitt, T. (2002). Determining the genetic structure of the natural population of *Staphylococcus aureus*: a comparison of multilocus sequence typing with pulsed-field gel electrophoresis, randomly amplified polymorphic DNA analysis, and phage typing. *J Clin Microbiol* 40, 4544–4546.
- Holden, M. T. G., Feil, E. J., Lindsay, J. A., Peacock, S. J., Day, N. P. J., Enright, M. C., Foster, T. J., Moore, C. E., Hurst, L. & other authors (2004). Complete genomes of two clinical *Staphylococcus aureus* strains: evidence for the rapid evolution of virulence and drug resistance. *Proc Natl Acad Sci U S A* 101, 9786–9791.
- Hu, L., Umeda, A., Kondo, S. & Amako, K. (1995). Typing of *Staphylococcus aureus* colonising human nasal carriers by pulsed-field gel electrophoresis. *J Med Microbiol* 42, 127–132.
- Humphreys, H., Keane, C. T., Hone, R., Pomeroy, H., Russell, R. J., Arbuthnott, J. P. & Coleman, D. C. (1989). Enterotoxin production by *Staphylococcus aureus* isolates from cases of septicaemia and from healthy carriers. *J Med Microbiol* 28, 163–172.
- Jarraud, S., Peyrat, M. A., Lim, A., Tristan, A., Bes, M., Mougél, C., Etienne, J., Vandenesch, F., Bonneville, M. & Lina, G. (2001). *egc*, a highly prevalent operon of enterotoxin gene, forms a putative nursery of superantigens in *Staphylococcus aureus*. *J Immunol* 166, 669–677 (Enterotoxin Nomenclature Correction: *J Immunol* 166, 4260).
- Jarraud, S., Mougél, C., Thioulouse, J., Lina, G., Meugnier, H., Forey, F., Nesme, X., Etienne, J. & Vandenesch, F. (2002). Relationships between *Staphylococcus aureus* genetic background, virulence factors, *agr* groups (alleles), and human disease. *Infect Immun* 70, 631–641.
- Kennedy, J., Jackson, V., Blair, I. S., McDowell, D. A., Cowan, C. & Bolton, D. J. (2005). Food safety knowledge of consumers and the microbiological and temperature status of their refrigerators. *J Food Prot* 68, 1421–1430.
- Kingdom, J. C. P., Joyce, S. M., Bradley, F. L., Jauch, W., Falkiner, F. R. & Keane, C. T. (1983). Staphylococcal nasal carriage in medical students with varying clinical exposure. *J Hosp Infect* 4, 75–79.
- Kluytmans, J., van Belkum, A. & Verbrugh, H. (1997). Nasal carriage of *Staphylococcus aureus*: epidemiology, underlying mechanisms, and associated risks. *Clin Microbiol Rev* 10, 505–520.
- Letertre, C., Perelle, S., Dilasser, F. & Fach, P. (2003). Identification of a new putative enterotoxin SEU encoded by the *egc* cluster of *Staphylococcus aureus*. *J Appl Microbiol* 95, 38–43.
- Lina, G., Boutite, F., Tristan, A., Bes, M. & Vandenesch, F. (2003). Bacterial competition for human nasal cavity colonization: role of staphylococcal *agr* alleles. *Appl Environ Microbiol* 69, 18–23.
- Lina, G., Bohach, G. A., Nair, S. P., Hiramatsu, K., Jouvin-Marche, E. & Mariuzza, R. (2004). Standard nomenclature for the superantigens expressed by *Staphylococcus*. *J Infect Dis* 189, 2334–2336.
- Lindstedt, B.-A. (2005). Multiple-locus variable number tandem repeats analysis for genetic fingerprinting of pathogenic bacteria. *Electrophoresis* 26, 2567–2582.
- Malachowa, N., Sabat, A., Gniadkowski, M., Krzyszton-Russjan, J., Empel, J., Miedzobrodzki, J., Kosowska-Shick, K., Appelbaum, P. C. & Hryniewicz, W. (2005). Comparison of multiple-locus variable-number tandem-repeat analysis with pulsed-field gel electrophoresis, *spa* typing, and multilocus sequence typing for clonal characterization of *Staphylococcus aureus* isolates. *J Clin Microbiol* 43, 3095–3100.
- Mead, P. S., Slutsker, L., Dietz, V., McCaig, L. F., Bresee, J. S., Shapiro, C., Griffin, P. M. & Tauxe, R. V. (1999). Food-related illness and death in the United States. *Emerg Infect Dis* 5, 607–625.
- Mehrotra, M., Wang, G. & Johnson, W. M. (2000). Multiplex PCR for detection of genes for *Staphylococcus aureus* enterotoxins, exfoliative toxins, toxic shock syndrome toxin 1, and methicillin resistance. *J Clin Microbiol* 38, 1032–1035.
- Monday, S. R. & Bohach, G. A. (1999). Use of multiplex PCR to detect classical and newly described pyrogenic exotoxin genes in staphylococcal isolates. *J Clin Microbiol* 37, 3411–3414.
- Monk, A. B., Curtis, S., Paul, J. & Enright, M. C. (2004). Genetic analysis of *Staphylococcus aureus* from intravenous drug user lesions. *J Med Microbiol* 53, 223–227.
- Murray, R. J. (2005). Recognition and management of *Staphylococcus aureus* toxin-mediated disease. *Intern Med J* 35 (Suppl. 2), S106–S109.
- Nashev, D., Toshkova, K., Bizeva, L., Akineden, Ö., Lämmle, C. & Zschöck, M. (2007). Distribution of enterotoxin genes among carriage- and infection-associated isolates of *Staphylococcus aureus*. *Lett Appl Microbiol* 45, 681–685.
- Nouwen, J., Boelens, H., van Belkum, A. & Verbrugh, H. (2004). Human factor in *Staphylococcus aureus* nasal carriage. *Infect Immun* 72, 6685–6688.
- Novick, R. P. (1967). Properties of a cryptic high-frequency transducing phage in *Staphylococcus aureus*. *Virology* 33, 155–166.
- Novick, R. P. (2003). Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Mol Microbiol* 48, 1429–1449.
- Omoe, K., Ishikawa, M., Shimoda, Y., Hu, D.-L., Ueda, S. & Shinagawa, K. (2002). Detection of *seg*, *seh*, and *sei* genes in *Staphylococcus aureus* isolates and determination of the enterotoxin productivities of *S. aureus* isolates harboring *seg*, *seh*, or *sei* genes. *J Clin Microbiol* 40, 857–862.
- Omoe, K., Hu, D.-L., Takahashi-Omoe, H., Nakane, A. & Shinagawa, K. (2005). Comprehensive analysis of classical and newly described staphylococcal superantigenic toxin genes in *Staphylococcus aureus* isolates. *FEMS Microbiol Lett* 246, 191–198.
- Orwin, P. M., Leung, D. Y. M., Tripp, T. J., Bohach, G. A., Earhart, C. A., Ohlendorf, D. H. & Schlievert, P. M. (2002). Characterization of a novel staphylococcal enterotoxin-like superantigen, a member of the group V subfamily of pyrogenic toxins. *Biochemistry* 41, 14033–14040.

- Otsuka, T., Saito, K., Dohmae, S., Takano, T., Higushi, W., Takizawa, Y., Okubo, T., Iwakura, N. & Yamamoto, T. (2006). Key adhesion gene in community-acquired methicillin-resistant *Staphylococcus aureus*. *Biochem Biophys Res Commun* **346**, 1234–1244.
- Peacock, S. J., de Silva, I. & Lowy, F. D. (2001). What determines nasal carriage of *Staphylococcus aureus*? *Trends Microbiol* **9**, 605–610.
- Peacock, S. J., de Silva, G. D. I., Justice, A., Cowland, A., Moore, C. E., Winearls, C. G. & Day, N. P. J. (2002). Comparison of multilocus sequence typing and pulsed-field gel electrophoresis as tools for typing *Staphylococcus aureus* in a microepidemiological setting. *J Clin Microbiol* **40**, 3764–3770.
- Robinson, D. A. & Enright, M. C. (2004). Multilocus sequence typing and the evolution of methicillin-resistant *Staphylococcus aureus*. *Clin Microbiol Infect* **10**, 92–97.
- Robinson, D. A., Monk, A. B., Cooper, J. E., Feil, E. J. & Enright, M. C. (2005). Evolutionary genetics of the accessory gene regulator (*agr*) locus in *Staphylococcus aureus*. *J Bacteriol* **187**, 8312–8321.
- Sabat, A., Krzyszton-Russjan, J., Strzalka, W., Filipek, R., Kosowska, K., Hryniewicz, W., Travis, J. & Potempa, J. (2003). New method for typing *Staphylococcus aureus* strains: multiple-locus variable-number tandem repeat analysis of polymorphism and genetic relationships of clinical isolates. *J Clin Microbiol* **41**, 1801–1804.
- Sabat, A., Melles, D. C., Martirosian, G., Grundmann, H., van Belkum, A. & Hryniewicz, W. (2006). Distribution of the serine-aspartate repeat protein-encoding *sdr* genes among nasal-carriage and invasive *Staphylococcus aureus* strains. *J Clin Microbiol* **44**, 1135–1138.
- Saunders, G. C., Dukes, J., Parkes, H. C. & Cornett, J. H. (2001). Interlaboratory study on thermocycler performance in controlled PCR and random amplified polymorphic DNA analyses. *Clin Chem* **47**, 47–55.
- Schneider, T., Senn, M. M., Berger-Bächi, B., Tossi, A., Sahl, H.-G. & Wiedemann, I. (2004). *In vitro* assembly of a complete, pentaglycine interpeptide bridge containing cell wall precursor (lipid II-Gly5) of *Staphylococcus aureus*. *Mol Microbiol* **53**, 675–685.
- Schoder, D., Schmalwiesser, A., Schaubberger, G., Hoorfar, J., Kuhn, M. & Wagner, M. (2005). Novel approach for assessing performance of PCR cyclers used for diagnostic testing. *J Clin Microbiol* **43**, 2724–2728.
- Shopsin, B. & Kreiswirth, B. N. (2001). Molecular epidemiology of methicillin-resistant *Staphylococcus aureus*. *Emerg Infect Dis* **7**, 323–326.
- Shopsin, B., Mathema, B., Alcabes, P., Said-Salim, B., Lina, G., Matsuka, A., Martinez, J. & Kreiswirth, B. (2003). Prevalence of *agr* specificity groups among *Staphylococcus aureus* strains colonizing children and their guardians. *J Clin Microbiol* **41**, 456–459.
- Smyth, D. S., Hartigan, P. J., Meaney, W. J., Fitzgerald, J. R., Deobald, C. F., Bohach, G. A. & Smyth, C. J. (2005). Superantigen genes encoded by the *egc* cluster and *SaPIbov* are predominant among *Staphylococcus aureus* isolates from cows, goats, sheep, rabbits and poultry. *J Med Microbiol* **54**, 401–411.
- Smyth, D. S., Kennedy, J., Twohig, J., Miajlović, H., Bolton, D. & Smyth, C. J. (2006). *Staphylococcus aureus* isolates from Irish domestic refrigerators possess novel enterotoxin and enterotoxin-like genes and are clonal in nature. *J Food Prot* **69**, 508–515.
- Soll, D. R. (2000). The ins and outs of DNA fingerprinting the infectious fungi. *Clin Microbiol Rev* **13**, 332–370.
- Stephens, A. J., Huygens, F., Inman-Bamber, J., Price, E. P., Nimmo, G. R., Schooneveldt, J., Munckhof, W. & Giffard, P. M. (2006). Methicillin-resistant *Staphylococcus aureus* genotyping using a small set of polymorphisms. *J Med Microbiol* **55**, 43–51.
- Taneike, I., Otsuka, T., Dohmae, S., Saito, K., Ozaki, K., Takano, M., Higushi, W., Takano, T. & Yamamoto, T. (2006). Molecular nature of methicillin-resistant *Staphylococcus aureus* derived from explosive nosocomial outbreaks of the 1980s in Japan. *FEBS Lett* **580**, 2323–2334.
- Thomas, D. Y., Jarraud, S., Lemerrier, B., Cozon, G., Echasserieu, K., Etienne, J., Gougeon, M.-L., Lina, G. & Vandenesch, F. (2006). Staphylococcal enterotoxin-like toxins U2 and V, two new staphylococcal superantigens arising from recombination within the enterotoxin gene cluster. *Infect Immun* **74**, 4724–4734.
- Todd, J. K. (2005). Staphylococcal infections. *Pediatr Rev* **26**, 444–450.
- Trindade, P. A., McCulloch, J. A., Oliveira, G. A. & Mamizuka, E. M. (2003). Molecular techniques for MRSA typing: current issues and perspectives. *Braz J Infect Dis* **7**, 32–43.
- Tristan, A., Ying, L., Bes, M., Etienne, J., Vandenesch, F. & Lina, G. (2003). Use of multiplex PCR to identify *Staphylococcus aureus* adhesins involved in human hematogenous infections. *J Clin Microbiol* **41**, 4465–4467.
- Tung, H., Guss, B., Hellman, U., Persson, L., Rubin, K. & Rydén, C. (2000). A bone sialoprotein-binding protein from *Staphylococcus aureus*: a member of the staphylococcal Sdr family. *Biochem J* **345**, 611–619.
- Vivoni, A. M. & Moreira, B. M. (2005). Application of molecular techniques in the study of *Staphylococcus aureus* clonal evolution – a review. *Mem Inst Oswaldo Cruz* **100**, 693–698.
- Wagner, L. & Lai, E. (1994). Separation of large DNA molecules with high voltage pulsed field gel electrophoresis. *Electrophoresis* **15**, 1078–1083.
- Wertheim, H. F. L., Melles, D. C., Vos, M. C., van Belkum, A., Verbrugh, H. A. & Nouwen, J. L. (2005). The role of nasal carriage in *Staphylococcus aureus* infections. *Lancet Infect Dis* **5**, 751–762.
- Wright, J. S., III, Traber, K. E., Corrigan, R., Benson, S. A., Musser, J. M. & Novick, R. P. (2005). The *agr* radiation: an early event in the evolution of staphylococci. *J Bacteriol* **187**, 5585–5594.