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Novel insights into the enterotoxigenic potential and genomic background of *Staphylococcus aureus* isolated from raw milk

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ABSTRACT

In this study, 53 *Staphylococcus* (*S.*) *aureus* strains were typed by 16S–23S rDNA intergenic spacer region (ISR) typing and staphylococcal enterotoxin gene (SEg) typing for all the staphylococcal enterotoxin (*se*) and staphylococcal enterotoxin-like toxin (*sel*) genes known to date, revealing a higher discriminatory power than that of multi locus sequence typing. Six strains, one of each ISR- and SEg-type, were genome sequenced and the ability to produce some classical and new SEs when growing in milk was investigated.

The manual analysis of the six genomes allowed us to confirm, correct and expand the results of common available genomic data pipelines such as VirulenceFinder. Moreover, it enabled us to (i) investigate the actual location of *se* and *sel* genes, even for genes such as *sel*Y, whose location (in the core genome) was so far unknown, (ii) find novel allelic variants of *se* and *sel* genes and pseudogenes, (iii) correctly annotate *se* and *sel* genes and pseudogenes, and (iv) discover a novel type of enterotoxin gene cluster (*egc*), i.e. the *egc* type 5 in strains 356P and 364P, while *S. argenteus* MSHR1132 harbored the *egc* type 6.

Four of the six *S. aureus* strains produced sufficient amounts of SEA, SEC, SED and SEH in milk to cause staphylococcal food poisoning (SFP), with *S. aureus* 372 P being the highest producer of SED in milk found to date, producing as much as ca. 47,300 ng/mL and 49,200 ng/mL of SED, after 24 and 48 h of incubation in milk at 37 °C, respectively. *S. aureus* 372 P released a low amount of SER in milk, most likely because the *se*R gene was present as a pseudogene, putatively encoding only 51 amino acids. These findings confirm that not only the classical SEs, but also the new ones can represent a potential hazard for the consumers' health if produced in foods in sufficient amounts. Therefore, the detection of SEs in foods, especially if involved in SFP cases, should focus not only on classical, but also on all the new SEs and SEls known to date. Where reference methods are unavailable, the presence of the relevant genes, by using the conventional and real time PCR protocols we exhaustively provided herein, and their nucleotide sequences, should be investigated.

1. Introduction

Staphylococcus (*S.*) *aureus* is one of the major etiological agents of bacterial diseases worldwide. Among the various diseases caused by this pathogen, staphylococcal food poisoning (SFP), a food-borne intoxication due to the consumption of food contaminated with sufficient amounts of staphylococcal enterotoxins (SEs) mainly produced by certain *S. aureus* strains, continues to be a public health concern. Although European regulations (EC regulations no. 2073/2005, 1441/2007) require the enumeration of coagulase-positive staphylococci (CPS) at specific steps of the production process (process hygiene criteria), i.e. when the number of staphylococci is expected to be the highest, as well as the screening of samples for the presence of staphylococcal enterotoxins above well specified M values (food safety criteria), the rate of SFP has still increased over the last years (EFSA and ECDC, 2105a, b; Cady et al., 2016; Fusco et al., 2018). Considering that SEs are thermo-stable and may thus persist in a food matrix after the vegetative cells have been inactivated, samples in which CPS are absent or below the levels established by the EU regulation may still contain SEs in sufficient amounts to cause SFP (Fusco et al., 2018). Moreover,

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beyond the five classical SEs (SEA-SEE), novel SEs and staphylococcal enterotoxin-like toxins (SELs) have been discovered (Spoor et al., 2015; Fusco et al., 2018; Zhang et al., 2018). Certain newly described SEs have also been demonstrated to cause SFP, or their genes have been detected in *S. aureus* strains involved in this type of food poisoning (Chiang et al., 2008; Yan et al., 2012; Gallina et al., 2013; Hait et al., 2014; Johler et al., 2015a; Roussel et al., 2015; Sato'o et al., 2015; Suzuki et al., 2015; Hu et al., 2017; Umeda et al., 2017; Zhao et al., 2017; Ciupescu et al., 2018; Guidi et al., 2018).

These findings suggest that the new SE/SELs are a potential cause of foodborne outbreaks and call for studies aimed at investigating the actual enterotoxigenic potential of *S. aureus* harboring these toxins, as well as the prevalence of these toxins.

Whole genome sequencing is being widely used to characterize *S. aureus* strains, but incorrect annotations and the use of pipelines targeting only a limited number of virulence genes provide only a partial and often misleading description of the actual and overall virulence potential of these pathogenic microorganisms (Copin et al., 2018). Confusion has further been created by the incorrect nomenclature given to the latest SELs discovered (Tuffs et al., 2018). In this study, we investigated the actual and up-to-date enterotoxigenic potential of 53 *S. aureus* strains isolated from raw milk, and assessed the genomic background, as well as the ability to produce some classical and new SEs, when growing in milk, of 6 selected *S. aureus* strains with different enterotoxigenic potential.

2. Materials and methods

2.1. Strains, growth conditions and DNA extraction

The strains used in this study are listed in Tables 1 and 2. Table 1 lists the *S. aureus* reference strains used as controls for the PCR of the *se* and *sel* genes, while Table 2 shows the 53 *S. aureus* wilde-type strains isolated from raw milk in previous investigations in our laboratories. In particular, all the typical colonies present in the selective agar plates with the highest dilutions of each raw milk sample were isolated and screened by species-specific PCR of the *hsp*60 gene (Blaiotta et al., 2004). The resulting 53 confirmed *S. aureus* isolates were stored in our collection and used for the present study. Preparation of working cultures and DNA extraction were performed using the protocols described by Fusco et al. (2011a). DNA quality and quantity were evaluated as described by Fusco et al. (2016).

2.2. PCR detection of the mec and tsst-1 genes and real time PCR of the enterotoxin gene cluster

Methicillin-resistance genetic determinants were detected by applying the PCR protocol of Oliveira and de Lencastre (2002), using DNA aliquots of IMM1-T002 01–04 methicillin-resistant *S. aureus* (MRSA) as positive control, kindly provided by Prof. Karsten Becker (University Hospital Münster, Institute of Medical Microbiology, Münster, Germany). PCR targeting the gene encoding the TSST-1 was performed using the protocol described by Johnson et al. (1991) with *S. aureus* RIMD 31092 (Blaiotta et al., 2004) DNA as positive control. PCRs were performed on a 9700 thermocycler (Applied Biosystems, Milan, Italy) and the resulting amplicons were detected by agarose gel electrophoresis as described by Fusco et al. (2011b). The presence of the *enterotoxin gene cluster* (*egc*) was investigated by the real time PCR described by Fusco et al. (2011a), using a Viia 7 Real-Time PCR System (Life Technologies, Milan, Italy).

2.3. Staphylococcal enterotoxin gene (SEg) typing and PCR of the 16S–23S rDNA intergenic spacer region (ISR-PCR)

The SEg typing was carried out using DNA of the 53 *S. aureus* strains and the control strains listed in Tables 1 and 2 by conventional and real time PCRs, following protocols reported previously with some modifications (Table S1). For the PCR detection of *sel*Z, *sel*27 and *sel*28 genes, the primers, reaction mixture and thermocycling were custom designed and optimized in the present study (Table S1). DNA of the above-mentioned strains (Table 2) was also used for 16S–23S rDNA intergenic spacer region PCR (ISR-PCR) as described by Jensen et al. (1993), with modifications. Specifically, 50 μL of each reaction mixture contained MegaMix (Microzone Limited, Haywards Heath, UK), 0.25 μM of each primer and 25 ng of DNA. Thermocycling included 95 °C for 3 min; 35 cycles of 94 °C for 30 s, 55 °C for 7 min, 72 °C for 3 min; final extension of 72 °C for 7 min, using a 9700 thermocycler (Applied Biosystems, Milan, Italy). Four microliters of PCR mixture were loaded on agarose gel (1.7% w/v) and the resulting amplicons were separated by electrophoresis in TAE buffer at 60V for 6.5 h. The resulting patterns were visualized as described by Fusco et al. (2011b) and analysed using the Bionumerics software version 5.1 (Applied Maths, Sint Martens Latem, Belgium) (Di Lena et al., 2015), using the Pearson correlation as fingerprint similarity coefficient with default parameters for position tolerance settings.

2.4. Whole genome sequencing and analysis

Two mL of fresh cultures of the *S. aureus* strains were washed with TE buffer (pH 7.5) and resuspended in TE buffer (pH 7.5) containing 20 μL lysozyme (10 mg/mL). Genomic DNA was extracted by using the peqGOLD bacterial DNA kit (Peqlab, Erlangen, Germany), according to the manufacturer's instructions.

The integrity, purity and quantity of DNA were assessed by agarose gel electrophoresis, Nanodrop photometer (Peqlab), and Qubit 3.0 fluorometer (Life Technologies). To prepare sequencing libraries, the Illumina Nextera XT paired-end and mate pair library prep kits (Illumina, San Diego, USA) were used according to the manufacturer's

Table 1
Reference *S. aureus* strains used as positive controls for PCR detection of genes encoding staphylococcal enterotoxins (*se*) and staphylococcal enterotoxin-like toxins (*sel*).

Reference strains	harbored *se* and *sel* genes	Reference
DSM 20231^T	-	Blaiotta et al. (2004)
ATCC 14458	*seB, seK, selQ*	Blaiotta et al. (2004); Sergeev et al. (2004)
ATCC 27664 (FRI326)	*seE*	Blaiotta et al. (2004)
A900322	*seP, egc*1 (*seG, seI, seM, seN, seO, ψent*1, *ψent*2)	Jarraud et al. (2001); Blaiotta et al. (2004); Sergeev et al. (2004); Collery et al. (2009); Schubert et al. (2016)
NCTC 9393	*seD, selJ, egc*1 (*seG, seI, seM, seN, seO, ψent*1, *ψent*2)	Blaiotta et al. (2004)
RIMD 31092	*seB, seC, egc*1 (*seG, seI, seM, seN, seO, ψent*1, *ψent*2), *tsst*-1	Jarraud et al. (2001); Blaiotta et al. (2004); Sergeev et al. (2004)
ATCC 19095 (FRI137)	*seC, seH, seL, egc*2 (*seG, seI, seM, seN, seO, selU*)	Blaiotta et al. (2004); Collery et al. (2009); Schubert et al. (2016)
ATCC 25923	*egc*1 (*seG, seI, seM, seN, seO, ψent*1, *ψent*2)	Blaiotta et al. (2004)
AB-8802	*egc*3 (*seG$_Y$, seI$_Y$, seM$_Y$, seN$_Y$, seO$_Y$, selU$_Y$*)	Blaiotta et al. (2004); Collery et al. (2009)
A900624	*seP, egc*4 (*seO, selV, selU2, seN, seG*)	Thomas et al. (2006); Collery et al. (2009)

Table 2
Designation and origin of the 53 *S. aureus* strains used in this study.

Milk sample[a]	CPS count[b]	*S. aureus* strains designation
A	3.70 ± 0.11	178P, 179P, 180P, 181P, 182P, 183P, 184P, 185P, 186P, 187P, 188P, 189P, 190P, 191P, 192P, 193P, 194P, 195P, 196P, 197P, 198P, 199P, 356P, 357P, 358P, 359P, 360P, 361P, 362P, 363P, 372P
B	3.20 ± 0.25	364P, 365P, 366P
C	4.00 ± 0.15	236P, 237P, 238P, 239P, 240P, 241P, 242P, 243P, 244P, 367P, 373P, 374P
D	3.70 ± 0.04	200P, 201P
E	3.80 ± 0.10	211P, 212P, 213P, 214P
F	4.10 ± 0.20	234P

[a] Raw bulk tank milk samples (A-F) taken from six different farms in the province of Cosenza (Calabria, Italy).
[b] Results of coagulase positive staphylococci (CPS) counts in milk expressed as log cfu/mL ± standard deviation. CPS were enumerated on Baird Parker with Egg Yolk Tellurite Emulsion (BP-EY, Oxoid), according to the reference method ISO 6888–1: 1999.

instructions, and then sequenced on the Illumina MiSeq platform with the 2 × 250 pair procedure. Reads were then trimmed with the NxTrim (V2) (O'Connell et al., 2015) and the Trimmomatic (Bolger et al., 2014), and then *de novo* assembly was performed using SPAdes version 3.10.1 (Bankevich et al., 2012).

The completeness of the *de novo* assemblies was measured by the presence of 111 single-copy genes that are observed across almost all prokaryotic genomes by using MiGA (Rodriguez et al., 2018). Quality scores were then calculated as completeness percentage minus five times contamination percentage.

All the proteins sequences used in this study were retrieved from GenBank (NCBI). The homology-based relationship of *S. aureus* strains towards selected proteins was determined by BLASTP algorithm on the NCBI site (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Gene models were manually determined, and clustering and orientation were subsequently deduced for the closely linked genes.

In silico analysis of virulence determinants, multi locus sequencing typing (MLST), genomic island, prophage sequences and plasmid detection were manually performed and compared with results obtained by using VirulenceFinder database (version 2.0) (Joensen et al., 2014), PubMLST (Bartual et al., 2005), IslandViewer 4 (Bertelli et al., 2017), Phaster (Baba et al., 2008) and PlasmidFinder (Carattoli et al., 2014) pipelines.

Nucleotide and amino acid sequences of genes annotated as exotoxins, enterotoxins, toxins and hypothetical proteins were also analysed for their homology with reference *se* and *sel* genes and relevant proteins using the Bionumerics software, version 5.1 (Applied Maths), as well as by using nucleotide BLAST and protein BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

The phylogenetic tree was constructed using the entire *egc* loci determined within this study (in the *S. aureus* strains 356P, 363P and 364P) as well as the ancestral *egc* locus of *S. aureus* A900322 [AN: AF285760 as corrected in DQ993159 (Collery and Smyth, 2007)] and that retrieved from complete genome sequence of *S. argenteus* MSHR1132 (AN: FR821777). The phylogenetic analysis was performed by the maximum likelihood (ML) method. A consensus tree was inferred using the neighbor-joining method and MEGA v7.0.18 (http://www.megasoftware.net). Phylogenetic robustness was inferred from 1000 replications to obtain the confidence value for the aligned sequence dataset.

2.5. Staphylococcal enterotoxins production in milk

2.5.1. Growth conditions in milk and sampling

In order to assess the ability to produce SEA, SEC, SED, SEH, SEP and SER in milk, five out of the six *S. aureus* strains chosen for whole genome sequencing, namely *S. aureus* 200P, 211P, 356P, 363P and 372P, were grown at 37 °C under agitation (230 rpm) in UHT (Ultra High Temperature) skimmed (0.0% fat) milk (Mlekpol, Grajewo, Poland) as described by Schubert et al. (2016).

To assess SEs production by *S. aureus* strains, milk aliquots were sampled after 24 and 48 h of incubation and milk pH was also measured at the same times using FE20-FiveEasy™ pHmeter (Mettler-Toledo, Greifensee, Switzerland).

Bacterial concentration in UHT milk at time zero (immediately after the inoculum) and after 24 h and 48 h of incubation was determined by plating appropriate volumes of serial decimal dilutions in sterile saline solution (0.9% NaCl) on Brain Heart infusion (BHI) agar plates. Colonies were enumerated after 24 h of incubation at 37 °C in aerobiosis.

2.5.2. Sandwich ELISA

The production of SEA, SEC, SED, SEH, SEP and SER was assessed by sandwich ELISA as described by Schubert et al. (2016). Native SE (SEA) (Sigma Aldrich) or recombinant SEs previously produced (rSEC, rSER and rSEH), or produced just before performing the ELISA (rSED) as described by Schubert et al. (2017), were used as standards in ELISA essays. Data analysis was carried out using GraphPad Prism (GraphPad Software Inc., La Jolla, CA) by means of a 4-parameter logistic curve.

2.6. Statistical analysis

Results of microbial counts and SEs' production in milk were reported as mean ± standard deviation (the latter displayed as error bar in histograms) of three replicates.

Statistical analysis was performed using the Student *t*-test, assuming $P < 0.05$ as significance level.

3. Results

3.1. Enterotoxin gene cluster (egc), tsst gene and mec element detection

Fourteen out of the 53 *S. aureus* strains analysed in this study tested positive for the real time PCR assay of the *egc* developed by Fusco et al. (2011a) (Table 3), while all of these were negative for the presence of the *tsst* and *mec* element genes.

3.2. Staphylococcal enterotoxin gene (SEg)-typing and 16S–23S rDNA intergenic spacer region (ISR)-PCR

The SEg-typing, based on PCR detection of 28 genes coding for SEs and SEls, and the ISR-PCR allowed the identification of 6 different SEg-(Table 3) and 6 different ISR-types (Table 3 and Fig. 1). As shown in Table 3, these two molecular methods revealed the same discriminatory power in *S. aureus* typing. The SEg and ISR features of the six *S. aureus* strains selected for whole genome sequencing are shown in Table 4.

3.3. Genomic features of whole genome sequenced S. aureus

Genomic features of the six whole genome sequenced *S. aureus* are reported in Table S2. Genomes were assembled using the SPAdes software version 3.10.1 (Bankevich et al., 2012) resulting in a total of 7–18

Table 3
Distribution of the 53 *S. aureus* isolates from raw bulk tank milk and corresponding genotypes obtained by staphylococcal enterotoxin gene (SEg) typing and 16S–23S rDNA intergenic spacer region (ISR) analysis.

ISR type	SEg type (harbored *se/sel* genes)	Origin[a] and number of *S. aureus* isolates for each raw milk sample (% of total isolates)						TOTAL
		A[a]	B[a]	C[a]	D[a]	E[a]	F[a]	
I	1 (*egc*[b], *se*C, *se*L, *sel*W, *sel*X)	9 (29%)						9
II	2 (*egc*[b], *se*P, *sel*W, *sel*X)	1 (3.2%)						1
III	3 (*egc*[b], *sel*W, *sel*X, *sel*Y, *sel*27, *sel*28)		3 (100%)	1 (8.3%)				4
IV	4 (*se*A, *se*D, *sel*J, *se*R, *sel*W, *sel*X)	21 (67.8%)		1 (8.3%)			1 (100%)	23
V	5 (*se*A, *sel*W, *sel*X)				2 (100%)			2
VI	6 (*se*H, *sel*W, *sel*X)			10 (83.4%)		4 (100%)		14
TOTAL		31 (100%)	3 (100%)	12 (100%)	2 (100%)	4 (100%)	1 (100%)	53

[a] A-F: raw bulk tank milk samples taken from six different farms in the province of Cosenza (Calabria, Italy).
[b] *egc*: *enterotoxin gene cluster* harboring *se*O, *se*M, *se*I, *se*N, *se*G and *sel*U2 or *ψent*1 and *ψent*2 genes.

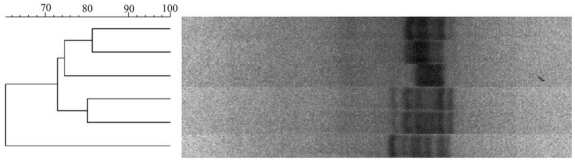

Fig. 1. UPGMA dendrogram generated by similarity analysis of fingerprints obtained by 16S–23S rDNA intergenic spacer region (ISR)-PCR performed on the 53 *S. aureus* isolates. ISR-type I: 356P, 357P, 358P, 359P, 360P, 361P, 362P, 188P, 189P (harboured genes: *egc*[a], *se*C, *se*L, *sel*W, *sel*X); ISR-type II: 363P (harboured genes: *egc*[a], *se*P, *sel*W, *sel*X); ISR-type III: 364P, 365P, 366P, 367P (harboured genes: *egc*[a], *sel*W, *sel*X, *sel*Y, *sel*27, *sel*28); ISR-type IV: 178P, 179P, 180P, 372P, 374P, 181P, 182P, 183P, 184P, 185P, 186P, 187P, 190P, 191P, 192P, 193P, 194P, 195P, 196P, 197P, 198P, 199P, 234P (harboured genes: *se*A, *se*D, *sel*J, *se*R, *sel*W, *sel*X); ISR-type V: 200P, 201P (harboured genes: *se*A, *sel*W, *sel*X); ISR-type VI: 373P, 211P, 212P, 213P, 214P, 236P, 237P, 238P, 239P, 240P, 241P, 242P, 243P, 244P (harboured genes: *se*H, *sel*W, *sel*X); [a]enterotoxin gene cluster (*egc*) harboring the following genes or pseudogenes: *se*O, *se*M, *se*I, *se*N, *se*G and *sel*U2 or *ψent*1 and *ψent*2.

Table 4
Origin, staphylococcal enterotoxin gene (SEg)-type and 16S–23S rDNA intergenic spacer region (ISR)-type of the six whole genome sequenced *S. aureus*.

Strain	Origin[a]	SEg-type	harboured *se/sel* genes	ISR-type
356P	A	1	(*egc*[b], *se*C, *se*L, *sel*W, *sel*X)	I
363P	A	2	(*egc*[b], *se*P, *sel*W, *sel*X)	II
364P	B	3	(*egc*[b], *sel*W, *sel*X, *sel*Y, *sel*27, *sel*28)	III
372P	A	4	(*se*A, *se*D, *sel*J, *se*R, *sel*W, *sel*X)	IV
200P	D	5	(*se*A, *sel*W, *sel*X)	V
211P	E	6	(*se*H, *sel*W, *sel*X)	VI

[a] A-E: raw bulk tank milk samples taken from different farms in the province of Cosenza (Calabria, Italy).
[b] *egc*: enterotoxin gene cluster harboring *se*O, *se*M, *se*I, *se*N, *se*G and *sel*U2 or *ψent*1 and *ψent*2 genes.

contigs (> 500 bp) and a mol GC% of ca. 32% for all strains. The total length of the assembly was around of 2.7Mbp for all genomes. The completeness of the assemblies was of 95.5% and the quality score was high at 77.5 for all the strains.

The six *S. aureus* genome sequences were submitted to GenBank as Bioproject PRJNA413759 under the following accession numbers: *S aureus* 200P: PDIW00000000; *S aureus* 211P: PDIV00000000; *S aureus* 356P: PDIR00000000; *S aureus* 363P: PDIT00000000; *S aureus* 364P: PDIS00000000; *S aureus* 372P: PDIU00000000. The version described in this paper are PDIW00000000.1, PDIV00000000.1, PDIR00000000.1, PDIT00000000.1, PDIS00000000.1 and PDIU00000000.1, respectively.

3.4. In silico multilocus sequence typing (MLST)

Multilocus sequence type (ST) of the six whole genome sequenced *S. aureus* was determined by using the PubMLST database (Bartual et al., 2005). Results are reported in Table 5. All strains belong to different ST's, with the exception of 372P and 200P, which both belong to ST-8. *S. aureus* 364P harbors a novel *pta* allele, with ST-9 being the most closely related ST type.

Table 5
Allelic profile of *S. aureus* isolates.

isolate	*arc*C	*aro*E	*glp*F	*gm*K	*pta*	*tpi*	*yqil*	ST
200P	3	3	1	1	4	4	3	ST-8
211P	1	1	1	1	1	1	1	ST-1
356P	10	14	8	6	10	3	2	ST-45
363P	1	4	1	4	12	1	10	ST-5
364P	3	3	1	1	1[a]	1	10	ST-9[a]
372P	3	3	1	1	4	4	3	ST-8

[a] Novel allele, ST may indicate the nearest ST.

3.5. Enterotoxin genes

Table 6 shows *ψent*1 and *ψent*2 pseudogenes and *se* and *sel* genes identified by VirulenceFinder 2.0 (Joensen et al., 2014) or detected by NCBI automatic annotation or by manual annotation.

As shown in Table 6, VirulenceFinder 2.0 was able to identify only 13 of the 19 *se* and *sel* genes (which were unspecifically annotated by the NCBI automatic annotation as exotoxins, enterotoxins, toxins or hypothetical proteins and in some cases, with an incorrect position), which could be identified by manual annotation.

Nucleotide and amino acid differences between *se* and *sel* genes, reference genes and predicted proteins are shown in Table S3.

Aung et al. (2017), discriminated 6 groups by analysing the open reading frame of *sel*W of 16 *S. aureus* strains. The six *S. aureus* strains studied here belonged to 4 of these 6 groups (Table 6). In particular, the nucleotide sequences of the *sel*W gene of *S. aureus* strains 200P and 372P exhibit 100% identity to that of the strain TD112, showing that they belong to the phylogenetic group 2 (Aung et al., 2017) (Table 6). *S. aureus* strain 211P, with its *sel*W gene showing 100% identical nucleotide sequence to that of the strain TD160, belongs to the group 3. The *sel*W gene of *S. aureus* strain 356P displays 99% identity to that of *S. aureus* TD158 (Tables 6 and S3), making it to belong to the group 1 of Aung et al. (2017). The *sel*W genes of *S. aureus* strains 363P and 364P share 100% (754/754 nucleotides) and 98% (739/754 nucleotides) identity with the *sel*W gene of the strain *S. aureus* TD97 (Table 6 and S3), respectively. They thus belong to the group 4 of Aung et al. (2017) and in these strains *sel*W is a pseudogene that most likely encodes an incomplete and non-functional protein with no superantigenic or enterotoxigenic properties. The manual annotation allowed us to confirm the location of *sel*W genes of our *S. aureus* strains in the core genome and in synteny with other *S. aureus* strains (Okumura et al., 2012).

The nucleotide sequence alignment of the *sel*X genes of the 6 *S.*

Table 6
Position of enterotoxin (*se*) genes and enterotoxin-like toxin (*sel*) genes in whole genome sequenced *S. aureus* strains 200P, 211P, 356P, 363P, 364P, 372P and percentage of identity with reference genes (whose accession numbers are shown in brackets) using three different annotation methods (NCBI Prokaryotic Genome Annotation Pipeline, VirulenceFinder 2.0 analysis, manual analysis).

	NCBI			VirulenceFinder 2.0			Manual analysis		
se/sel genes	position	protein ID	annotation	position	annotation	Ref. Gene identity % (Query/Template length) (accession number)	position	annotation	Ref. Gene identity % ([a]matching nucleotides subject/query) (accession number)
				200P					
***se*A**	PDIW01000001.1: c1073929..1074702[b]	PGG91782.1	enterotoxin	PDIW01000001.1:1073929..1074711[b]	*se*a	100 (783/783) (AP009324.1)	PDIW01000001.1: 1073929..1074702[b]	*se*A	100 (774/774) (KP402066)
***sel*W**	PDIW01000001.1: c694880..695584[b]	PGG91428.1	exotoxin	N.D.	N.D.	N.D.	PDIW01000001.1: 694880..695632[b]	*sel*W	100 (753/753) (KX655710)
***sel*X**	PDIW01000005.1: 79411..80022	PGG88680.1	toxin	N.D.	N.D.	N.D.	PDIW01000005.1: 79411..80022	allelic variant 19 *sel*X	99.84 (611/612) (ACKD01000059.1: 35256-35867)
				211P					
***se*H**	PDIV01000002.1: 598943..599668	PGG85894.1	enterotoxin	PDIV01000002.1: 598943..599668	*se*h	100 (726/726) (BX571857.1)	PDIV01000002.1: 598943..599668	*se*H	100 (726/726) (AJ937549)
***sel*W**	PDIV01000001.1: 282578..283282	PGG86982.1	exotoxin	N.D.	N.D.	N.D.	PDIV01000001.1: 282578..283282	*sel*W	100 (705/705) (KX655704)
***sel*X**	PDIV01000002.1: 929376..929987	PGG86180.1	toxin	N.D.	N.D.	N.D.	PDIV01000002.1: 929376..929987	allelic variant 5 *sel*X	100 (612/612) (NC_002953.3: 393545-394156)
				356P					
***se*C**	PDIR01000001.1: c1038243..1039043	PGG84009.1	enterotoxin	PDIR01000001.1: 1038243..1039043	*sec/sec*3	100 (801/801) (AB860417.1/ M28364.1)	PDIR01000001.1: c1039043..1038243	*se*C	100 (801/801) (AB860417.1: 14292..15092)
***se*L**	PDIR01000001.1: 1037354..1038076	PGG84008.1	exotoxin	PDIR01000001.1: 1037354..1038076	*se*l	99.45 (723/723) (AF217235.1/ BA000018.3)	PDIR01000001.1: 1037354..1038076	*se*L	100 (723/723) (AB860417.1: c15259..15981)
***sel*W**	PDIR01000001.1: 256212..256916[b]	PGG83275.1	exotoxin	N.D.	N.D.	N.D.	PDIR01000001.1: 256164..256916[b]	*sel*W	99.87 (752/753) (KX655717)
***sel*X**	PDIR01000002.1: 895742..896359[b]	PGG82685.1	toxin	N.D.	N.D.	N.D.	PDIR01000002.1: 895749..896359[b]	truncated *sel*X	100 (611/611) (KT943499.1)
***se*O**	PDIR01000001.1: 36044..36808[b]	PGG83079.1	exotoxin	PDIR01000001.1: 36026..36808[b]	*se*o	100 (783/783) (BA000018.3)	PDIR01000001.1: 36023..36808[b]	*se*O	100 (786/786) (AF285760.1)
***se*M**	PDIR01000001.1: 37088..37807	PGG83080.1	exotoxin	PDIR01000001.1: 37088..37807	*se*m	99.58 (720/720) (BA000018.3)	PDIR01000001.1: 37088..37807	*se*M	99.44 (716/720) (AF285760.1)
***se*I**	PDIR01000001.1: 37842..38570	PGG83081.1	exotoxin	PDIR01000001.1: 37842..38570	*se*i	99.59 (729/729) (BA000018.3/ CP011147.1)	PDIR01000001.1: 37842..38570	*se*I	99.45 (725/729) (AF285760.1)
***sel*U2**	PDIR01000001.1: 38724..39494	PGG83082.1	exotoxin	PDIR01000001.1: 38724..39494	*se*u	99.74 (771/771) (HE681097.1)	PDIR01000001.1: 38724..39494	*sel*U2	100 (771/771) (EF030428.1)
***se*N**	PDIR01000001.1: 39533..40288[b]	PGG83083.1	exotoxin	PDIR01000001.1: 39512..40288[b]	*se*n	100 (777/777) (BA000018.3)	PDIR01000001.1: 39512..40288[b]	*se*N	100 (777/777) (AF285760.1)
***se*G**	PDIR01000001.1: 40571..41347	PGG83084.1	enterotoxin	PDIR01000001.1: 40571..41347	*se*g	99.87 (777/777) (CP001844.2)	PDIR01000001.1: 40571..41347	*se*G	99.87 (776/777) (AF285760.1)
				363P					

(*continued on next page*)

Table 6 (*continued*)

	NCBI			VirulenceFinder 2.0			Manual analysis		
se/sel genes	position	protein ID	annotation	position	annotation	Ref. Gene identity % (Query/Template length) (accession number)	position	annotation	Ref. Gene identity % ([a]matching nucleotides subject/query) (accession number)
***se*P**	PDIT01000001.1: 2569309..2570091[b]	PGG74829.1	exotoxin	PDIT01000001.1: 2569309..2570086[b]	*se*p	100 (778/778) (BA000018.3)	PDIT01000001.1: 2569309..2570091[b]	*se*P	100 (783/783) (BA000018.3: c2011380..2012162)
***sel*W**	PDIT01000001.1: 198132..198605 /198703..198942[b]	PGG72573.1	enterotoxin/ carboxylate–amine ligase	N.D.	N.D.	N.D.	PDIT01000001.1: 198189..198942[b]	*sel*W	100 (754/754) (KX655714)
***sel*X**	PDIT01000001.1: c1440642..1441253	PGG73761.1	toxin	N.D.	N.D.	N.D.	PDIT01000001.1: 1440642..1441253	allelic variant 1 *sel*X	100 (612/612) (NC_013450.1: 377804-378415)
***se*O**	PDIT01000001.1: 2740341..2741105[b]	PGG75020.1	exotoxin	PDIT01000001.1: 2740323..2741105[b]	*se*o	100 (783/783) (BA000018.3)	PDIT01000001.1: 2740320..2741105[b]	*se*O	100 (786/786) (AF285760.1)
***se*M**	PDIT01000001.1: 2741386..2742105	PGG75021.1	exotoxin	PDIT01000001.1: 2741386..2742105	*se*m	100 (720/720) (BA000018.3)	PDIT01000001.1: 2741386..2742105	*se*M	99.86 (719/720) (AF285760.1)
***se*I**	PDIT01000001.1: 2742140..2742868	PGG75022.1	exotoxin	PDIT01000001.1: 2742140..2742868	*se*i	100 (729/729) (BA000018.3/ CP011147.1)	PDIT01000001.1: 2742140..2742868	*se*I	99.86 (728/729) (AF285760.1)
***ψent*1**	PDIT01000001.1: 2743022..2743423[b]	PGG75024.1	enterotoxin	PDIT01000001.1: 2743022..2743793[b]	*se*u	99.61 (772/771) (HE681097.1)	PDIT01000001.1: 2743022..2743423[b]	*ϕent*1	100 (402/402) (AF285760.1 as corrected in DQ993159)
***ψent*2**	PDIT01000001.1: 2743398..2743793[b]	PGG75024.1	enterotoxin	PDIT01000001.1: 2743022..2743793[b]	*se*u	99.61 (772/771) (HE681097.1)	PDIT01000001.1: 2743398..2743793[b]	*ϕent*2	100 (396/396) (AF285760.1 as corrected in DQ993159)
***se*N**	PDIT01000001.1: 2743832..2744587[b]	PGG75025.1	exotoxin	PDIT01000001.1: 2743811..2744587[b]	*se*n	100 (777/777) (BA000018.3)	PDIT01000001.1: 2743811..2744587[b]	*se*N	100 (777/777) (AF285760.1)
***se*G**	PDIT01000001.1: 2744870..2745646	PGG75026.1	enterotoxin	PDIT01000001.1: 2744870..2745646	*se*g	99.87 (777/777) (CP001844.2)	PDIT01000001.1: 2744870..2745646	*se*G	99.87 (776/777) (AF285760.1)
					364P				
***sel*W**	PDIS01000001.1: c1144987..1145691[b] note = internal stop;	pseudo	exotoxin	N.D.	N.D.	N.D.	PDIS01000001.1: 1144987..1145739[b]	*sel*W	98 (739/754) KX655714)
***sel*X**	PDIS01000002.1: 1072164..1072775	PGG76248.1	toxin	N.D.	N.D.	N.D.	PDIS01000002.1: 1072164..1072775	allelic variant 20 *sel*X	99.67 (610/612) (HQ850969.1)
***sel*Y**	PDIS01000002.1: c492185..492850	PGG75740.1	toxin	N.D.	N.D.	N.D.	PDIS01000002.1: c492850..492185	*sel*Y	98.80 (658/666) (AB924045)
***sel*27**	PDIS01000001.1: 1358180..1358950[b]	PGG77771.1	exotoxin	N.D.	N.D.	N.D.	PDIS01000001.1: 1358207..1358950[b]	*sel*27	99.87 (743/744) (MF370876.1: 2650..3393)
***sel*28**	PDIS01000001.1: 1358977..1359729	PGG77772.1	hypothetical protein	N.D.	N.D.	N.D.	PDIS01000001.1: 1358977..1359729	*sel*28	100 (753/753) (MF370876.1: 3420..4172)
***se*O**	PDIS01000001.1: c1380275..1381039[b]	PGG77790.1	exotoxin	PDIS01000001.1: 1380275..1381057[b]	*se*o	100 (783/783) (CP003979.1)	PDIS01000001.1: 1380275..1381060[b]	*se*O	99.87 (785/786) (AF285760.1)
***se*M**	PDIS01000001.1: c1379275..1379994	PGG77788.1	exotoxin	PDIS01000001.1: 1379275..1379994	*se*m	100 (720/720) (BA000018.3)	PDIS01000001.1: 1379275..1379994	*se*M	99.86 (719/720) (AF285760.1)
***se*I**	PDIS01000001.1: c1378512..1379240	PGG77788.1	exotoxin	PDIS01000001.1: 1378512..1379240	*se*i	100 (729/729) (BA000018.3/ CP011147.1)	PDIS01000001.1: 1378512..1379240	*se*I	99.86 (728/729) (AF285760.1)
***sel*U2**	PDIS01000001.1: c1377588..1378358	PGG77787.1	exotoxin	PDIS01000001.1: 1377588..1378358	*se*u	99.87 (771/771) (HE681097.1)	PDIS01000001.1: 1377588..1378358	*sel*U2	99.87 (770/771) (EF030428.1)

(*continued on next page*)

Table 6 (*continued*)

	NCBI			VirulenceFinder 2.0			Manual analysis		
se/sel genes	position	protein ID	annotation	position	annotation	Ref. Gene identity % (Query/Template length) (accession number)	position	annotation	Ref. Gene identity % ([a]matching nucleotides subject/query) (accession number)
***se*N**	PDIS01000001.1: c1376794..1377549[b]	PGG77786.1	exotoxin	PDIS01000001.1: 1376794..1377570[b]	*se*n	100 (777/777) (AP014653.1)	PDIS01000001.1: 1376794..1377570[b]	*se*N	99.87 (776/777) (AF285760.1)
***se*G**	PDIS01000001.1: c1375735..1376511	PGG77785.1	exotoxin	PDIS01000001.1: 1375735..1376511	*se*g	99.74 (777/777) (CP001844.2)	PDIS01000001.1: 1375735..1376511	*se*G	99.74 (775/777) (AF285760.1)
					372P				
***se*A**	PDIU01000003.1: 201067..201840[b]	PGG78518.1	enterotoxin	PDIU01000003.1: 201058..201840[b]	*se*a	100 (783/783) (AP009324.1)	PDIU01000003.1: 201067..201840[b]	*se*A	100 (774/774) (KP402066)
***se*D**	PDIU01000005.1: c14659..15435[b]	PGG78176.1	exotoxin	PDIU01000005.1: 14661..15435[b]	*se*d	99.87 (775/775) (KF007920.1)	PDIU01000005.1: 14659..15435[b]	*se*D	100 (777/777) (KX168620.1)
***sel*J**	PDIU01000005.1: 16330..17136	PGG78177.1	exotoxin	PDIU01000005.1: 16330..17136	*se*j	99.75 (807/807) (GQ900405.1)	PDIU01000005.1: 16330..17136	*sel*J	100 (807/807) (KF831356.1: 15..821)
***se*R**	PDIU01000005.1: c17226..17864/c17825..18006 note = frameshifted[b]	PGG78178.1	exotoxin/ hypothetical protein	PDIU01000005.1: 17226..18006[b]	*se*r	99.23 (781/780) (AB075606.1)	PDIU01000005.1: 17226..18006[b]	*se*R pseudogene	99.74 (781/779) (KF831356.1: c911..1689 misc_feature, note = "similar to EntR; enterotoxin type R)
***sel*W**	PDIU01000001.1: 227951..228655[b]	PGG80090.1	exotoxin	N.D.	N.D.	N.D.	PDIU01000001.1: 227903..228655[b]	*sel*W	100 (753/753) (KX655710)
***sel*X**	PDIU01000002.1: 968304..968915	PGG79622.1	toxin	N.D.	N.D.	N.D.	PDIU01000002.1: 968304..968915	allelic variant 2 *sel*X	100 (612/612) (ACKD01000059.1: 35256-35867)

N.D.: not detected.

misc_feature: region of biological interest which cannot be described by any other feature key; a new or rare feature (https://www.ebi.ac.uk/ena/WebFeat/misc_feature_s.html).

KX655710: *sel*W gene belonging to group 2; KX655704: *sel*W gene belonging to group 3; KX655717: *sel*W gene belonging to group 1; KX655714: non functional *sel*W gene belonging to group 4; ACKD01000059.1: *sel*X gene allele 2; NC_002953.3: *sel*X gene allele 5; KT943499.1: truncated *sel*X gene; NC_013450.1: *sel*X gene allele 1; HQ850969.1: *sel*X gene allele 11; KP402066: accession number (AN) for *S. aureus* strain Sa17 enterotoxin A and hypothetical protein genes, complete cds; AB860417.1: AN for complete sequence of pathogenicity island SaPITokyo12571 of *S. aureus* Tokyo12571 strain; KX168620.1: AN for complete cds of *se*D variant v2 gene of *S. aureus* strain BW10; AJ937549: AN for *se*H gene of *S. aureus* strain VI 50695; KF831356.1: AN for complete plasmid pBU108b sequence of *S. aureus* strain A960649; AF285760.1: AN for *S. aureus* enterotoxin gene cluster, complete sequence; EF030428.1: AN for *S. aureus sel*U2 gene, complete cds; DQ993159: AN for *S. aureus* strain A900322 *se*I-*se*N intergenic region, partial sequence; *ψent*1 pseudogene, complete sequence; and *ψent*2 pseudogene, partial sequence.

[a] matching nucleotides subject/query: number of matching nucleotides between the reference gene sequence length (query) and the corresponding sequence in *S. aureus* genomes presented in this study.

[b] No agreement in gene position between the different annotation methods.

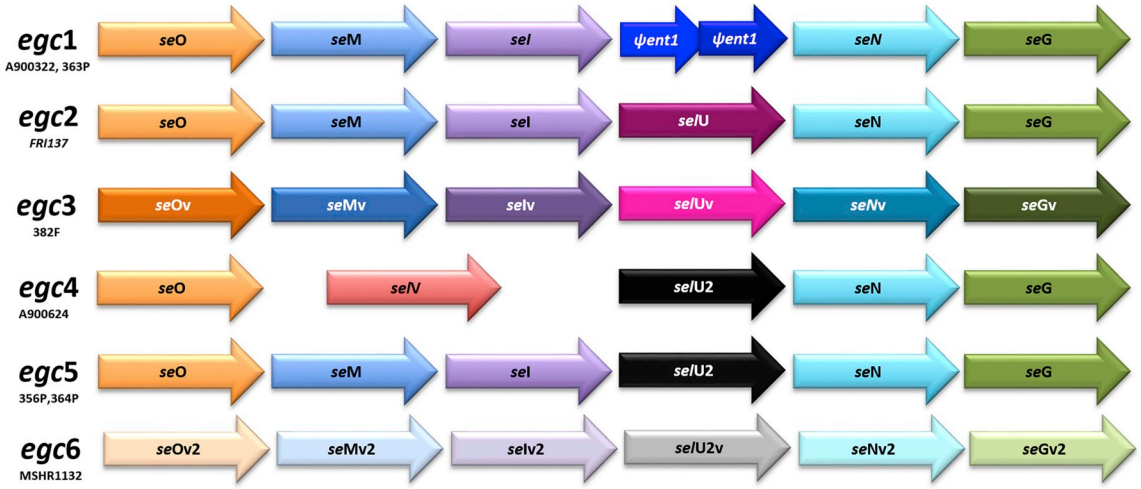

Fig. 2. Structure of the six *enterotoxin gene cluster (egc)* variants known to date [Up-dated from Collery et al. (2009)].

aureus strains with the reference ones (Wilson et al., 2011; Roetzer et al., 2016a) allowed us to detect the following allelic variants: *sel*X1 (363P), *sel*X2 (372P), *sel*X5 (211P) and *sel*X "truncated" (356P) (Table 6). This latter variant, discovered by Roetzer et al. (2016a), results from a single nucleotide deletion that leads to a premature stop codon, thus preventing SElX production (Roetzer et al., 2016a). One and 2 nucleotide substitutions in the *sel*X genes of *S. aureus* strains 200P and 364P, respectively (Table 6), led to one amino acid substitution when compared to the relevant amino acid sequences of the allelic variants *sel*X2 and *sel*X11 (Table S3). Thus, these strains harbor novel allelic variants, different from the 18 known to date (Wilson et al., 2011; Roetzer et al., 2016a), namely *sel*X19 and *sel*X20, respectively (Table 6).

The *sel*26 and *sel*27 genes in the genome of *S. aureus* 364P could be detected and thus correctly annotated as *sel*27 and *sel*28, respectively, only by manual annotation. The *sel*27 gene shared 99% identity with that reported by Zhang et al. (2018) (AN: MF370876.1), corresponding to a I195V substitution in the predicted primary amino acid sequence (Table S3), whereas *sel*28 of our strain showed 100% identity to that of Zhang et al. (2018) (AN: MF370876.1) (Table 6). Moreover, *sel*27 and *sel*28 are located in a pathogenicity island νSaβ, harboring also the *egc* and sharing the same genetic content and organization of that of the methicillin-resistant *S. aureus* DAR4145 [CP010526 (Zhang et al., 2018);].

The *se*L gene of *S. aureus* 356P is 100% identical to that of *S. aureus* Tokyo12571, whose production of SEL in broth has been described (Suzuki et al., 2015) (Tables 6). Moreover, this gene shares 99% (719/723) nucleotide identity and 99% (238/240) amino acid identity with the *se*L gene and coded protein, respectively, discovered by Fitzgerald et al. (2001) (AF217235.1; AAG29598.1).

In our *egc*-positive *S. aureus* strains we detected *se*O, *se*M, *se*I, *se*N and *se*G genes by SEg-typing. Since we obtained negative results by the PCR of the *sel*U gene (Letertre et al., 2003), but positive results by using the primers PSE1/PSE4, which amplified a region that is present in the genomes of the three *egc* + strains as suggested by Collery et al. (2009), we hypothesized the presence of either the two pseudogenes (*egc*1) or the *selU2* (*egc*4) gene, which indeed derives from an adenine deletion within the *ψent*1 and *ψent*2 pseudogenes. On the other hand, the absence of the *sel*V gene [all the *egc* + strains were negative for the *sel*V gene PCR of Thomas et al. (2006)], was also supported by the presence of *se*M and *se*I genes (whose recombination creates the *sel*V), leading to hypothesize that these *egc* + isolates harboured the *egc* type 1 (comprising the two pseudogenes and *se*O, *se*M, *se*I, *se*N and *se*G genes). The manual analysis of the genomic sequences of our strains clarified this questionable scenario, allowing us to discriminate the *sel*U2 gene, *sel*U and the two pseudogenes *ψent*1 and *ψent*2 (Table 6) (of which only the *sel*U is detectable by the VirulenceFinder) and to detect any polymorphism in the nucleotide and amino acid sequences of the target with respect to the reference genes and their relevant proteins (Table S3).

S. aureus strain 363P was shown to harbor the two pseudogenes (Table 6) and thus it belongs to the *egc* type 1 (AN: MN450303). At the nucleotide 3067 of the *se*O-*se*G region of *S. aureus* A900322, *S. aureus* strains 356P and 364P have an adenine deletion in the *ψent*1 and *ψent*2 locus, which generates the *sel*U2 gene (Table S4). The *sel*U2 gene of *S. aureus* strain 356P revealed 100% identity to that of *S. aureus* A900624 discovered by Thomas et al. (2006) (Table 6), whereas the nucleotide sequence of the *sel*U2 gene of *S. aureus* 364P is different with respect to that of *S. aureus* A900624 due to only one point mutation (Tables 6, S3 and S4). The presence of the *sel*U2 genes in these strains led us to hypothesize that they harbor the *egc*4 (*se*O, *sel*V, *sel*U2, *se*N, *se*G), which differs from the ancestral *egc*1 of *S. aureus* A900322 (Jarraud et al., 2001; Collery et al., 2009) due to the absence of *se*I and *se*M genes and *ψent*1 and *ψent*2 pseudogenes, as well as the presence of the *sel*V *and sel*U2 genes. But, as indicated by the SEg-typing results, and confirmed by our genomic analyses, the two strains have *se*I and *se*M genes, while they do not have the *sel*V gene (Table 6). Therefore our *S. aureus* strains 356P (AN: MN450302) and 364P (AN: MN450304) harbor a new variant of the *egc* comprising the *se*O, *se*M, *se*I, *sel*U2, *se*N and *se*G genes, which, based on the classification of Collery et al. (2009) can be categorized as *egc* type 5 (Fig. 2).

We found the same organization in the *egc* of *S. argenteus* MSHR1132, but, as shown in Table S4, in Fig. 2 and in Fig. 3, it harbored variants of the above mentioned genes.

As shown in the alignment reported in Table S4, more than 200 nucleotide differences among substitutions, deletions and insertions were found in the *egc* region of *S. argenteus* MSHR1132. Most of the substitutions are located in the −35 region, the −10 region of the hypothetical promoter of the cluster, in *se*O (56 nt, 22 aa changes), in *se*M (40 nt, 16 aa changes), in *se*I (19 nt, 9 aa changes), in *sel*U2 (31 nt, 15 aa changes), in *se*N (63 nt, 30 aa changes), and in *se*G (27 nt, 15 aa changes). Insertions were found upstream seO (14 nt), in the intergenic region *se*N-*se*G (2 nt), upstream the hypothetical terminator of the cluster. Finally, an 11 nt deletion in the intergenic region *se*I-*sel*U2 was found. Thus, we named this *egc* as type 6 (Fig. 2).

3.6. Enterotoxin genes location

As shown in Fig. 4, the *enterotoxin gene cluster* (*egc*) is located in three different genomic contexts in the 3 strains harboring this cluster.

In *S. aureus* strain 364P, the *egc* is located in a *S. aureus*

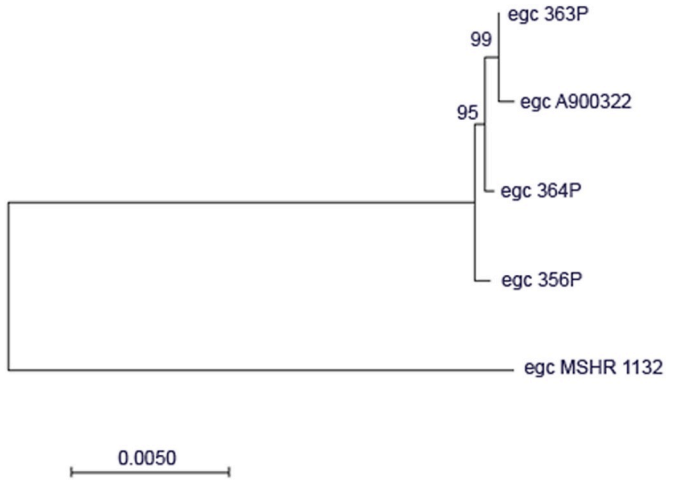

Fig. 3. Evolutionary relationships of taxa inferred by the Neighbor-Joining method. The analysis involved the nucleotide sequences of the *egc* of: *S. aureus* 356P: PDIR01000001.1, 35655..42147. *S. aureus* 363P PDIT01000001.1, 2739952..2746446; *S. aureus* 364P PDIS01000001.1, 1381428..1374935. *S. aureus* A900322 [AN: AF285760 as corrected in DQ993159 (Collery and Smyth, 2007) and *S. argenteus* MSRH 1132 (AN: FR821777.2). All positions containing gaps and missing data were eliminated. There was a total of 6150 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

pathogenicity island (SaPI) (AN: MN450304), sharing the same genetic content and organization of the SaPI reported for the *S. aureus* subsp. *aureus* ST772-MRSA-V strain DAR4145 [CP010526 (Zhang et al., 2018);]; downstream the *egc* there are eight tRNA (tRNA-Ser, tRNA-Glu, tRNA-Asn, tRNA-Gly, tRNA-His, tRNA-Phe, tRNA-Asp, tRNA-Met) and upstream are the restriction modification system genes (hsdS and hsdM), *sel*27 (CRU83_06940) and *sel*28 (CRU83_06940) located.

In *S. aureus* strain 363P, the *egc* is located in a *ν*Saβ of type I SaPI, (MN450303) homologue to that of *S. aureus* N315 island (Baba et al., 2008). This region (31 kbp) includes few virulence determinants such as the bi-component leukocidin LukED subunit D (CRU81_14345) and the serine proteases *spl*A, *spl*B, *spl*C, *spl*D and *spl*F (CRU81_14375–14395).

In *S. aureus* strain 356P (SaPI AN: MN450302), as in the other two strains, the *egc* is located close to the eight tRNA genes, but the genomic context is quite different than in other *S. aureus* strains. Indeed, only the calcium binding protein and few hypothetical proteins are homologous to other *S. aureus* strains, whereas the region upstream the *egc* is not a homologue to the other enterotoxin cluster loci. This organization was, however, retrieved by BLAST analysis in a few *S. aureus* strains, such as e.g. the clinical isolate 1549-WT (GenBank: LT992434.1).

The *se*A gene, which is present in *S. aureus* 200P and 372P, is located in a putative prophage region.

In *S. aureus* strain 200P this region was predicted as a 49.7 kbp intact region by Phaster (Arndt et al., 2016) and homologue to the *Staphylococcus* phage ϕNM3 (95.6% identity) (NC_008617); it comprises *se*A (CRU84_05550), one MBL fold metallo-hydrolase (CRU84_05725), the staphylokinase (CRU84_05525), several phage proteins as well as few XRE transcriptional regulators.

In *S. aureus* strain 372P the region of 56.5 kbp was predicted as intact by Phaster (NODE_3:160966–217507) and incorporates 63 proteins (identity 96.5% with the *Staphylococcus* phage ϕNM3); the region includes the *se*A gene (CRU82_13285), the MBL XRE family transcriptional regulators, the staphylokinase (CRU82_13320) and the MBL fold metallo-hydrolase (CRU82_13110).

In *S. aureus* strain 364P, in other *S. aureus* genomes, *sel*Y (CRU83_10795) is located in the core genome, close to the two holin-like proteins *Cid*B and *Cid*A, a reductase, the ferrous iron transport proteins A and B, one copper exporting ATPase gene and the gene coding for the copper resistance protein CopZ (Fig. S1A).

In the six *S. aureus* strains *sel*W genes are present in the same genomic context, located in the core genome, proximal to the *pfs* gene encoding 5′-methylthioadenosine nucleosidase/S-adenosylhomocysteine nucleosidase. Close to this gene, there are the competence operon *com*E, one lactam utilization protein, and the gene coding for the filamentation protein FiC. The only difference among these genomic regions is that in *S. aureus* 200P, 356P and 372P located upstream the elongation factor 4 gene, there is a gene encoding for a nitrogen fixation protein *Nif*R (CRU84_03370, CRU80_01510, and CRU82_01180 in 200P, 356P and 372P, respectively), which is instead located elsewhere in the other strains.

In *S. aureus* strain 211P, the *se*H gene (CRU85_10275) is present in a region comprising also two tRNA genes and two MBL metallo-fold hydrolase (Fig. S1B). Although the predicted IS3 family transposase is frameshifted and there is a contig boundary downstream of *se*H, we did not detect the non-*mec*A containing SCC element harbored by the SCC*mec* type IV of *S. aureus* MW2 which is generally located close to *se*H (Hu et al., 2018).

In all strains, *sel*X is located in the same genomic content, close to a CAAX protease encoding gene, one integrase and several hypothetical proteins (Fig. S1C) coding genes. In *S. aureus* 200P, 211P, 356P and 364P, some elements of this locus (hypothetical proteins and binding proteins) are included in a region predicted as incomplete by Phaster and homologue to *S. aureus* phage B20-like (NC_028821).

In *S. aureus* strain 372P, *se*D, *sel*J and *se*R are located on a putative plasmid, which is PIB485-like (Bayles and Iandolo, 1989). The island comprises several Rep proteins [apart from rep 7 and rep21 found by PlasmidFinder (Carattoli et al., 2014)] (Table S5), one cadmium

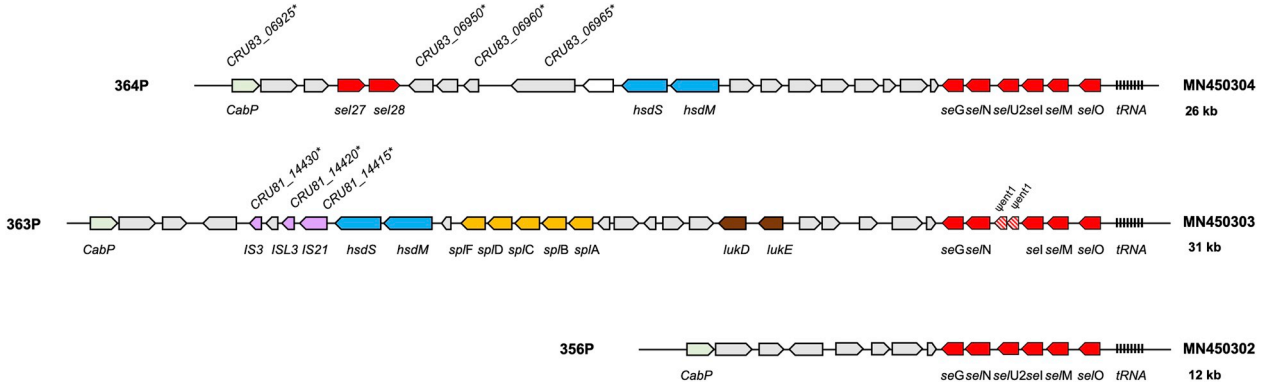

Fig. 4. *Enterotoxin gene cluster* (*egc*) localization in *S. aureus* strains. Accession numbers are indicated in the figure. Red = enterotoxins; yellow = serine proteases; brown = leukocidins; grey = hypotetical proteins; light blue = restriction endonucleases; pink = transposases; *Cap*B = Calcium binding protein; * indicates a pseudogene. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

transporter, one type I toxin-antitoxin system Fst family toxin, and, downstream a contig boundary, the locus contains the two invertases and genes coding for the penicillase repressor *Bla*I, the beta-lactam sensor/signal transducer *Bla*I, and the penicillin-hydrolyzing class A beta-lactamase *Bla*Z (Fig. S1D). Due to several interruptions in the assembly, we cannot reconstruct the entire locus, but within the other short contigs putatively mapped close to this location there are several tRNA genes, one transposase and few *rep* genes. The transposon Tn552 insertion site is indeed partially mapped in the NODE_6 (6687..6862) and partially in the NODE_2 (894827..896134).

In *S. aureus* strain 363P, the *se*P gene (CRU81_13075) is comprised in a region predicted as intact by Phaster and Island Viewer (Bertelli et al., 2017) (Table S6), which is homologue to the *S. aureus* phage ϕN315 and located in the NODE_1: 2531835..2576023 (CRU81_12790-CRU81_13140); it comprises several phage structural proteins, the staphylokinase and the MBL fold metallo-hydrolase (CRU81_12895). The organization and content of this region is almost identical to that of the phage ϕNM3 in strains 200P and 372P: in this case *se*P is present instead of *se*A and there are few differences in the prediction of hypothetical proteins.

In *S. aureus* strain 356P, *se*C and *se*L genes (CRU80_05315 and CRU80_05310) are located in a pathogenicity island (AN: MN450305) sharing 99% identity with the *S. aureus* pathogenicity island SaPITokyo12571 (AB860417.1). In Fig. 5, we highlighted few differences generated by the annotation pipeline between the two SaPIs. In *S. aureus* strain 356P two nearby pathogenicity island proteins were predicted (PGG84023.1 and PGG84022.1) while in SaPITokyo12571 only the hypothetical protein coded by the ORF5 was predicted, although another CDS was found to be present nearby (indicated as 5′ in Fig. 5). The same happens for the hypothetical protein PGG84334.1 (indicated in SaPITokyo12571 as 19' in Fig. 5) while the opposite occurs for ORF14, present but not predicted in *S. aureus* 356P. The gene coding for the pathogenicity island protein PGG84335.1 was annotated shorter than ORF16, although manual analysis confirmed the presence of a longer ORF also in *S. aureus* strain 356P. The predicted protein is 43 aa shorter than the hypothetical protein predicted for SaPITokyo1257. The terminase small subunit in *S. aureus* 356P is a pseudogene frameshifted.

3.7. Growth in milk and pH variations

The tested *S. aureus* strains significantly ($p < 0.05$) showed growth in milk, during 24 h of incubation at 37 °C, with an average increase of ca. 1.8 log CFU/mL starting from ca. 6.3 log CFU/mL. No significant growth was detected during the following 24 h of incubation (Fig. 6).

S. aureus strain 363P did not significantly change the milk pH during 48 h of incubation (Fig. 7), while *S. aureus* strain 356P only slightly decreased milk pH ($p < 0.05$) by ca. 0.18 units during the 24 h of incubation (Fig. 7). However, *S. aureus* strains 372P, 200P and 211P significantly decreased ($p < 0.05$) the milk pH, after 24 h and 48 h of incubation, respectively, when compared to the uninoculated milk that was used as a control. Specifically, after 24 h the average pH decrease was ca. 1.03 units and after 48 h it was ca. 1.45 units (Fig. 7). *S. aureus* strain 211P, harbouring *se*H, *sel*W and *sel*X genes, decreased the pH of the milk more than the other strains, i.e. 1.34 units after 24 h and 1.64 units after 48 h of incubation, when compared to the uninoculated milk (Fig. 7).

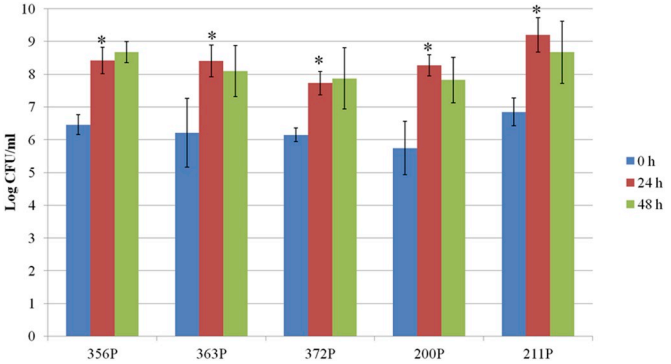

Fig. 6. Concentrations of *Staphylococcus aureus* strains in milk during 24 h and 48 h of incubation at 37 °C under agitation (230 rpm). Error bars represent standard deviation. Asterisk indicates significant difference ($P < 0.05$) compared to time zero (0 h).

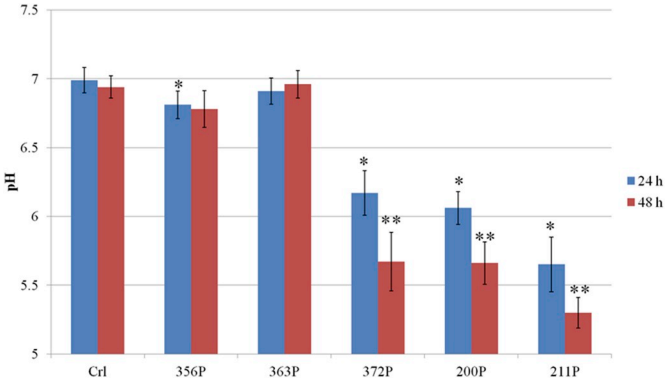

Fig. 7. pH variations of milk inoculated with *Staphylococcus aureus* strains (ca. 6.3 log CFU/mL) during 48 h of incubation at 37 °C under agitation (230 rpm). Asterisk (*) and double asterisk (**) indicate a significant difference ($P < 0.05$) compared to negative control (Crl, uninoculated milk sample) after 24 h and 48h of incubation, respectively. Error bars represent standard deviation.

3.8. Staphylococcal enterotoxins (SE) production in milk

The *S. aureus* strains 200P, 211P, 356P, 363P and 372P (Table 7) were tested for the production of certain SEs in milk, namely the

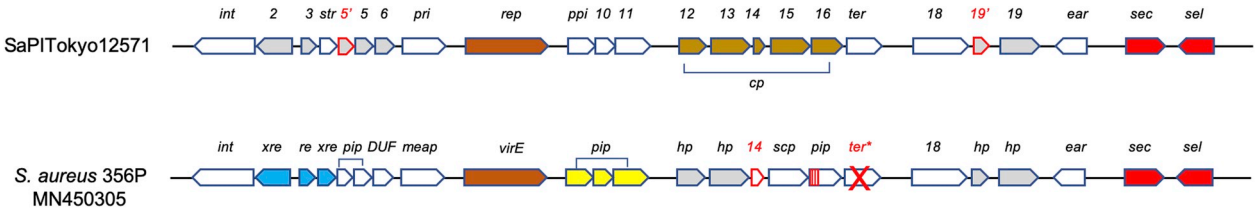

Fig. 5. Representation of a pathogenicity island (SaPI) of *S. aureus* strain 356P (AN: MN450305) and comparison with SaPITokyo12571 (Suzuki et al., 2015). Arrowheads represent the location and orientation of open reading frames (ORFs). Genes are presented according to their sequences and functions. Genes present but not predicted by the annotation pipeline are indicated in red outlines. *int*: integrase; *str*: regulatory protein; *pri*: primase; *rep*: replication initiator; *ppi*: phage interference; *cp*: packaging genes; *ter*: terminase small subunit; *ear*: penicillin-binding protein; *sec*: staphylococcal enterotoxin C; *sel*: staphylococcal enterotoxin L; *xre*: XRE family transcriptional regulator; *re*: transcriptional regulator; *pip*: pathogenicity island protein; *DUF*: DUF1474 domain-containing protein; *meap*: mobile element-associated protein; *vir*E: virulence factor E; *hp*: hypotetical protein; *scp*: spore coat protein; *indicates a pseudogene (frameshifted). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 7
Production in milk of staphylococcal enterotoxins SEA, SEC, SED, SEH, SEP and SER by *S. aureus* 200P, 211P, 356P, 363P, 372P.

Strain	SEA [ng/mL]		SEC [ng/mL]		SED [ng/mL]		SEH [ng/mL]		SEP [ng/mL]		SER [ng/mL]	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
200P	43 ± 8	44 ± 5	–	–	–	–	–	–	–	–	–	–
211P	–	–	–	–	–	–	580 ± 84	751 ± 39	–	–	–	–
356P	–	–	20 ± 2	78 ± 29	–	–	–	–	–	–	–	–
363P	–	–	–	–	–	–	–	–	0	0	–	–
372P	56 ± 9	56 ± 3	–	–	47,277 ± 5,524	49,160 ± 15,592	–	–	–	–	< 46.9	< 46.9

classical SEA, SEC, and SED and the recently discovered SEH, SEP and SER. Results are showed in Table 7.

S. aureus strain 356P produced ca. 20 ng/mL of SEC after 24 h and 78 ng/mL after 48 h of incubation at 37 °C. *S. aureus* 372P was tested for the concomitant production of SEA, SED and SER. SEA reached concentrations of ca. 56 ng/mL both at 24 h and 48 h of incubation, SED amounts were ca. 47,300 ng/mL and 49,200 ng/mL after 24 h and 48 h of incubation, respectively, whereas SER reached amounts below 46.9 ng/mL at both experimental times. SEA production was tested also for *S. aureus* strain 200P, which produced ca. 43 ng/mL of SEA after 24 h and 44 ng/mL after 48 h of incubation. *S. aureus* strain 211P produced ca. 580 ng/mL of SEH and ca. 751 ng/mL of this toxin after 24 h and 48 h of incubation, respectively. *S. aureus* strain 363P was tested for the production of SEP. For this purpose the presumed cross-reactivity of anti-SEA antibodies to perform the ELISA assay for the detection of SEP was exploited. This SE was not detected in milk after 24 h and 48 h of incubation.

4. Discussion

Raw milk is a very nutritious food but can be vehicle for transmission of several pathogenic bacteria (Fusco and Quero, 2014), including enterotoxigenic *S. aureus* able to cause the staphylococcal food poisoning (SFP) (Fusco et al., 2011a).

Although some new SEs have caused SFP outbreaks (Ikeda et al., 2005; Jorgensen et al., 2005) or genes coding for new SEs and SEls have been identified in *S. aureus* strains involved in SFP cases (Chiang et al., 2008; Yan et al., 2012; Gallina et al., 2013; Hait et al., 2014; Johler et al., 2015a; Roussel et al., 2015; Sato'o et al., 2015; Suzuki et al., 2015; Hu et al., 2017; Umeda et al., 2017; Ciupescu et al., 2018; Guidi et al., 2018), no reference methods are to date available to detect all of these. In this study, we provide the conventional and real time PCR protocols to detect all the *se* and *sel* genes known to date. The staphylococcal enterotoxin gene (SEg) typing is a valuable tool to simoultaneously type *S. aureus* isolates and to obtain information on their enterotoxigenic potential. However, given their potential to cause SFP, all the known *se* and *sel* genes should be targeted. Not considering the recently identified *sel*27 and *sel*28 (Zhang et al., 2018), after the discovery of SElZ in 2015 (Spoor et al., 2015) numerous studies focused on the characterization of enterotoxigenic *S. aureus* isolated from milk and dairy products, but none of these investigated the presence of all the *se* and *sel* genes (Carfora et al., 2015; Basanisi et al., 2016, 2017; Ferreira et al., 2016; Parisi et al., 2016; Kümmel et al., 2016; Mehli et al., 2017).

The SEg typing adopted in this study, which targeted 28 *se* and *sel* genes, allowed grouping the 53 *S. aureus* isolates into 6 SEg-types (Table 3). Moreover, detecting the *egc*-encoding genes in the 14 *egc*-positive isolates, it allowed to confirm the reliability of the real time PCR assay of Fusco et al. (2011a). The 16S–23S rDNA intergenic spacer region PCR (ISR-PCR), widely utilized to characterize *S. aureus* isolated from milk (Fournier et al., 2008; Boss et al., 2011; Syring et al., 2012; Cosandey et al., 2016), displayed the same discriminatory power as the SEg-typing (Fig. 1 and Table 3). Indeed, the *S. aureus* isolates belonging to the same SEg-type belonged to the same ISR-type. This is in agreement with Fournier et al. (2008) and Graber et al. (2009), who highlighted that the ISR-type of *S. aureus* isolated from milk of cows suffering of mastitis was highly associated to their virulence gene profiles. The *in silico* multilocus sequence typing (MLST) of the strains subjected to whole genome sequencing displayed a lower discriminatory power than the other two typing techniques used in this study (SEg typing and ISR-PCR). Indeed, *S. aureus* strain 200P [ISR-type V and SEg-type 5 (*se*A, *sel*W, *sel*X)] and *S. aureus* strain 372P [ISR-type IV and SEg-type 4 (*se*A, *se*D, *sel*J, *se*R, *sel*W, *sel*X)] share the same sequence type (ST), i.e. ST8 (Table 5).

Detecting different *S. aureus* strains in the same milk sample (Table 3) highlights the importance of isolating and characterizing as many typical colonies as possible to provide an actual and representative picture of the population structure and enterotoxigenic potential of *S. aureus* in each analysed sample. In all the previous studies which aimed at isolating and characterizing enterotoxigenic *S. aureus* from milk-dairy matrices (Carfora et al., 2015; Basanisi et al., 2016, 2017; Ferreira et al., 2016; Kümmel et al., 2016; McMillan et al., 2016; Mehli et al., 2017), only 1 to 5 typical *S. aureus* colonies were isolated per each sample, despite the possibilities of finding a higher intraspecific diversity and detecting strains with a different enterotoxigenic potential, if higher numbers of typical colonies were to be analysed.

As mentioned above, numerous SE and SEls have been discovered following the classical ones (SEA-SEE), but, although the nomenclature of SEs has been defined (Lina et al., 2004), a considerable confusion in naming of the last *sel* genes discovered resulted. Thomas et al. (2006) discovered $SElU_2$ in the *egc* type 4 and, since Collery and Smyth (2007) found its sequence to differ from that of SElU, they proposed to call it SElW. However, thereafter, another enterotoxin like-toxin with a different nucleotide and amino acid sequence and a different chromosomal location was discovered and was also termed SElW (Okumura et al., 2012). SElX, SElY and SElZ were discovered in 2011 and 2015 (Wilson et al., 2011; Ono et al., 2015; Spoor et al., 2015). Two further SEls have been found by Zhang et al. (2018), who named these SEl26 and SEl27, but annotated them as SEl27 and SEl28 (ANs: AVX35650.1; AVX35651.1), adding a further dimension to this already confusing scenario.

Herein, we provide the correct annotation of all the *se* and *sel* genes we found in the genome sequenced *S. aureus* strains of this study (Table 6), keeping to the nomenclature of SElU2 and SElW as such, in agreement with Tuffs et al. (2018). The availability of correctly annotated SEs and SEls will contribute to improve and standardize comparative genomic analyses of *S. aureus* strains.

Whole genome sequencing allowed us to confirm the presence of *se* and *sel* genes in the six *S. aureus* strains but for this purpose the VirulenceFinder 2.0 failed as the latest update of this database (on February 18, 2016 https://cge.cbs.dtu.dk/services/VirulenceFinder), contained only *se*A-*se*E, *se*G-*se*O, *se*R, *sel*U and *se*Q genes. The manual annotation we performed was therefore crucial to confirm the presence of all the genes we detected by conventional and real time PCRs (Table 6) and to provide correctly annotated SE and SEls. This will simplify the search of these toxins on other whole genome sequenced *S. aureus* strains.

100% of *S. aureus* isolates harboured the *sel*W and *sel*X genes. The

high prevalence of these genes, which are localized in the core genome, is in agreement with the literature. Indeed, the *sel*X gene has been found in 95% of the 66 *S. aureus* strains isolated from human and clinical infections (Wilson et al., 2011), whereas the *sel*W gene was found in 98% of 144 *S. aureus* isolates from food handlers in Myanmar analysed by Aung et al. (2017). Novel allelic variants of the *sel*X gene different from the 18 known to date (Wilson et al., 2011; Roetzer et al., 2016a), namely *sel*X19 and *sel*X20, were found in both *S. aureus* strains 200P and 364P, respectively (Table 6). Moreover, differences in the location of *sel*X genes, in respect to those found by Wilson et al. (2011), were found in our strains. As shown in Fig. S1C, the *sel*X locus comprises hypothetical proteins, a DNA-binding protein involved in DNA replication, one transcriptional regulator, two ribosomal proteins, one peptidase, one CAAX protease and one integrase. This latter was predicted as a pseudogene due to mutations (leading to a shift in the frame or to an internal stop) in strains 211P, 356P and 363P, as in other *S. aureus* strains (Wilson et al., 2011). In the other study it was postulated that the genetic linkage between *sel*X and the pseudogene integrase, which is partially homologue to an integrase of the *S. aureus* phage PT1028 (Accession YP_239446.1), indicates an ancient horizontal acquisition during *S. aureus* speciation (Wilson et al.., 2011). However, in *S. aureus* strain 200P, two adjacent integrases were predicted, one of which is a pseudogene, while in *S. aureus* 364P two adjacent genes were predicted to code for proteins with integrase-domains, with no frameshift mutation or internal stop. Thus, it cannot be excluded that both these domains might be individually functional. In *S. aureus* strain 364P the translated locus comprising these genes leads to a longer amino acid sequence with two stops in the reading frame.

The genomic analyses allowed us to confirm the presence (revealed by SEg-typing) of a *sel*Y gene in *S. aureus* 364P, which is a novel allelic variant, since it shares 98.80% (658/666 nucleotides) identity with the *sel*Y identified by Ono et al. (2015) (AN: AB924045). We were also able to find its location, so far unknown (Benkerroum, 2018), in the core genome (Fig. S1A).

The *se*G, *se*I, *se*M, *se*N, *se*O genes and *sel*V, *sel*U/*sel*U2 genes or *ψent*1 and *ψent*2 pseudogenes are present in tandem in an operon known as *enterotoxin gene cluster* (*egc*), which is localized in the highly variable island *ν*Saβ (Jarraud et al., 2001) (Fig. 4). Holtfreter et al. (2004) demonstrated that superantigens encoded by the *egc* are neutralized by human sera less than classical SEs or toxic shock syndrome toxin-1, suggesting a potential for increased severity of clinical diseases (Fusco et al., 2011a). Two *egc*-encoded toxins, namely SEI and SEN, have been found crucial for the induction of human blood cell proliferation and pathogenicity in rabbits (Roetzer et al., 2016b). Moreover, several *S. aureus* strains harboring *egc*-encoded toxins have been frequently detected in SFP cases but the co-occurrence of classical SEs did not allowed to ascertain their role in SFP. Only recently, the involvement of *S. aureus egc*+ in two SFP outbreaks occurred in Switzerland (Johler et al., 2015a), and one in Japan (Umeda et al., 2017) was proven. Moreover, the emetic activity of the *egc*-encoded SEG, SEI, SEM, SEN and SEO has been determined (Munson et al., 1998; Hu et al., 2003; Omoe et al., 2013; Ono et al., 2017). To date, four variants of the *egc* are known (Fig. 2). Genetic rearrangements of *se* genes in the *egc* type 1, discovered by Jarraud et al. (2001), harboring the genes *se*G, *se*I, *se*M, *se*N, *se*O and the two pseudogenes *ψent*1 and *ψent*2, lead to the emergence of the *egc* types 2 and 4. Particularly, in certain *S. aureus* strains the insertion of 15 bp into the *ψent*1 pseudogene and some other point mutations in the two pseudogenes led to an open reading frame of 261 amino acids called *sel*U (Letertre et al., 2003) (*egc* type *2*). Furthermore, a recombination event between *se*M and *se*I led to the *sel*V gene, whereas the deletion of an adenine in the *ψent*1 and *ψent*2 pseudogenes led to the *sel*U2 gene (Thomas et al., 2006) (*egc* type 4). The *egc* type 3 harbours the allelic variants of *se*G, *se*I, *se*M, *se*N, *se*O and *sel*U (Letertre et al., 2003; Abe et al., 2000; Blaiotta et al., 2004, 2006).

In the present study, two novel types of *egc* were detected, namely the *egc* type 5, present in *S. aureus* strains 356P (AN: MN450302) and 364P (AN: MN450304) and harboring *se*O, *se*M, *se*I, *sel*U2, *se*N and *se*G genes, and the *egc* type 6, harboring the allelic variants of the aforementioned genes (Fig. 2 and Table S4) present in *S. argenteus* MSHR1132 (Wakabayashi et al., 2018; Suzuki et al., 2017). As highlighted in Fig. 3 and suggested by Holt et al. (2011), the *egc* in *S. argenteus* as well as the whole genomic island *ν*Saβ containing this locus has been acquired before its separation from *S. aureus*.

S. aureus strain 364P harbors the *egc* cluster in the *ν*Saβ (AN: MN450304), which has the same genetic content and organization of that of the methicillin-resistant *S. aureus* strain SAR4145 of Zhang et al. (2018), whereas in *S. aureus* strain 363P, it is located in a *ν*Saβ (AN: MN450303) homologue to that of *S. aureus* N315 (Baba et al., 2008) while in *S. aureus* strain 356P (AN: MN450302) it is located in a genomic context quite different from that of the *ν*Saβ of the other two strains (Fig. 4).

Recently, several variants of *se* and *sel* genes (Kauffman and Roberts, 2006; Suzuki et al., 2015; Johler et al., 2016; Aung et al., 2017), that could affect the superantigenic and enterotoxigenic activity of SE and SEls, have been described.

S. aureus strain 211P produced ca. 580 ng/mL and 751 ng/mL of SEH in milk after 24 and 48 h at 37 °C, respectively (Table 7), amounts which are much higher than those found in reconstituted powder milk and mashed potatoes that caused SFP outbreaks in Japan and Norway, respectively (Ikeda et al., 2005; Jorgensen et al., 2005). Moreover, *S. aureus* strain 211P lowered the pH of the milk incubated at 37 °C, reaching values of 5.65 and 5.30 after 24 and 48 h, respectively (Fig. 7). This finding is in agreement with Schubert et al. (2016) who reported that *se*H positive strains able to proliferate and acidify milk are good producer of SEH in this matrix, with values ranging from 107 up to 4246 ng/mL of SEH in 48 h of incubation, whereas less acidifying strains produce only small amounts of SEH (1–36 ng/mL in 48 h). The nucleotide sequence of the *S. aureus* strain 211P *se*H gene shares 100% identity with that of *S. aureus* VI 50695 (Tables 6) and *S. aureus* VI 50671, isolated by Jorgensen et al. (2005) from the SFP outbreak due to the ingestion of SEH in Norway from mashed potatoes and milk, respectively. However, the amount of SEH produced in milk by *S. aureus* strain 211P is similar to that produced by *S. aureus* FRI 137, i.e. ca. 328 and 894 ng/mL, after 24 and 48 h of incubation at 37 °C, respectively (Schubert et al., 2016). This despite the fact that the *se*H gene sequence of our strain differed from that of the *S. aureus* FRI 137 (AN: AY345144) in three substitutions, which result in a S54N substitution in the relative amino acid sequence.

The *se*A genes of *S. aureus* strains 372P and 200P (Tables 6) are 100% identical to the *se*A genes of the *S. aureus* strains FRI281A (AN: L22566.1) (Borst et al., 1993), Sa17 (Tables 6), Sa21 (KP402067.1), Sa45 (KP402068.1), Sa48 (KP402069.1) and Sa54 (KP402072.1), which harbor the *se*A1 gene and are classified as high producers of SEA (1470.25–1,0378.59 ng/mL in BHI broth). The only exception is strain *S. aureus* Sa54, which was classified as low producer (0.41–8.35 ng/mL in BHI broth) by Cao et al. (2012). Differences in gene expression and amount of SEA produced by the different *se*A$^+$ *S. aureus* strains can be regulated by the life-cycle of the temperate *Siphoviridae* bacteriophage encoding the *se*A gene (Cao et al., 2012; Zeaki et al., 2015) (in strains 200P and 372P this gene is located on a region sharing 95.6% and 96.5% identity to the *Staphylococcus* phage ϕNM3, respectively). *S. aureus* strains 200P and 372P produced an amount of SEA in milk of ca. 44 ng/mL and 56 ng/mL, respectively (Table 7) after 24 h and 48 h at 37 °C. The different media utilized [BHI broth by Cao et al. (2012) and milk in the present study], as well as the different amount of inoculum used, do not allow any comparison of the amount of SEA produced. Nevertheless, since Schubert et al. (2016), who utilized the same experimental conditions we used in this study, demonstrated that the production of SEA is from 1.3 to 13 times higher in BHI than in milk, the strains *S. aureus* 200P and 372P from this study could be classified as intermediate producers of SEA. Two huge SFP outbreaks were caused by SEA producing strains (Evenson et al., 1988; Asao et al., 2003). The

minimum poisoning dose reported by Evenson et al. (1988) is 0.5 ng/mL, whereas Asao et al. (2003) estimated that the ingestion of SEA *per capita* causing symptoms was approximately of 20–100 ng. Given the amount of SEA produced by our strains 200P and 372P (Table 7), only 2–3 mL of contaminated milk could transmit almost 100 ng of SEA.

S. aureus strain 363P harbors a *se*P gene with 100% identity to that (AN: BA000018.3) originally discovered by Omoe et al. (2005) (Table 6) and is located in a region homologue to the *S. aureus* phage ϕN315. Moreover, it shares 99% identity (782/783 nucleotides [G with A in position 560]) with the *se*A gene of the strain SA51 (accession number KP402070.1), which was identified by Zeaki et al. (2015) as a low producer of SEA and is divergent from all the other *se*A genes as it actually is a *se*P gene and not a *se*A gene (the deduced SEP of SA51 share 99% identity with the SEP for 260/261 amino acids [R with K in position 547]). Wrong annotations like this one complicates genomic-based comparative analyses, whose consistency can be standardized and improved only by correct annotations.

We did not detect SEP in the milk inoculated with *S. aureus* 363P. This could be due to the absence of cross-reactivity between anti-SEA antibodies against the SEP. Indeed, in order to detect the SEP in milk, given the high homology of the SEP and SEA (78%) (Benkerroum, 2018) and thus the likely cross-reaction of anti-SEA antibodies with SEP, we used anti-SEA antibodies and the native SEA (to construct the standard curve) for the sandwich ELISA.

S. aureus strain 372P harbours *se*D, *sel*J and *se*R genes, which have been always detected together in the *S. aureus* isolates characterized in the present study. This finding suggests the hypothesis that these isolates harbor a pIB485-*like* plasmid where these three genes are localized (Zhang et al., 1998; Omoe et al., 2003; Argudin et al., 2010). Plasmid replicon types that could be predicted using PlasmidFinder in 372P were rep 20 and rep 7 (Table S6) replicons, which suggest the presence of a plasmid belonging to the plasmid group 31 (McCarthy and Lindsay, 2012). However, we identified a region sharing a high identity with Plasmid SAP048A, which is one of pIB485-like plasmids, carrying the *se*D, *sel*J, and *se*R genes (Matyi et al., 2013) and more *rep* genes than those detected by PlasmidFinder. *S. aureus* strain 372P produced in milk SED and SER in combination with SEA. In particular, it produced ca. 47,300 ng/mL and 49,200 ng/mL of SED, after 24 and 48 h of incubation in milk at 37 °C, respectively (Table 7). These amounts are much higher than the 200 ng of SED per g of raw milk soft cheese that caused SFP in a Swiss college on 2014 (Johler et al., 2015b) and are 34 and 20 times higher, respectively, than the average amounts produced under the same experimental conditions, by five strains of *S. aureus* that Schubert et al. (2017) reported as high producers of SED, whereas are similar to those produced in meat juice by the *S. aureus* strains tested by Schubert et al. (2017). In contrast with the findings of Schubert et al. (2017), who did not find any significant variation of the pH during the 48 h of incubation of the *se*D + strains in milk, our SED producer significantly decreased the pH of milk after 24 and 48 h (Fig. 7) and produced high amounts of SED, leading us to hypothesize a correlation between the high levels of SED production and the pH reduction of milk, similar to that found by Schubert et al. (2016) for high SEH producing *S. aureus* strains in milk. Further studies are required to unveil the mechanism that regulate the production of this enterotoxin in milk.

The *se*D gene of *S. aureus* strain 372P shares 100% identity to the variant v2 detected by Johler et al. (2016) (Table 6), which differs by a single nucleotide and a single amino acid from the reference SED of *S. aureus* RN4220 (Johler et al., 2016). To date, other genetic variants of the *se*D gene have also been discovered, which encode for proteins with shorter amino acid sequences, of 154 (Suzuki et al., 2015), 180 (Johler et al., 2016) and 150 amino acids (Kauffman and Roberts, 2006), respectively, and strains harboring these variants have been demonstrated to produce low amounts of SED, not detectable by ELISA, SET-RPLA or TECRA (Kauffman and Roberts, 2006; Suzuki et al., 2015; Johler et al., 2016). Our *S. aureus* 372P, which harbors an intact variant of the *se*D gene, was able to produce a higher amount of SED after incubation at 37 °C for 24 or 48 h with respect to other strains of *S. aureus* (ranging from 500 to 2000 ng/mL in BHI or TSB), harboring intact *se*D genes (Kauffman and Roberts, 2006; Suzuki et al., 2015).

S. aureus strain 372P harbors a genetic variant of the *se*R gene described by Omoe et al. (2003) (AN: AB075606.1). The latter encodes a predicted protein of 259 amino acids (AN: BAC97795.1). By contrast, in our *S. aureus* 372P *se*R gene, the insertion of one adenosine in the stretch of eight adenosines [position 124–131 of the *se*R gene of Omoe et al. (2003), AN: AB075606.1] causes a shift in the frame that leads to a predicted protein of 51 amino acids, shorter than the 59 amino acid coded by the pseudogene described by Hait et al. (2018). This finding could explain the low amount of SER (less than the limit of quantification of 46.9 ng/mL) detected in milk by sandwich ELISA.

In conclusion, the *S. aureus* strain 372P was able to produce SEA and SED in milk in sufficient amounts to cause SFP. The concurrent production of different enterotoxins in food is known and has been detected in several outbreaks of SFP. As an example, a SFP outbreak involving SEA and SED occurred in Austria on 2007 (Schmid et al., 2009), and one involving SEA and SEH in Japan in 2000 (Jorgensen et al., 2005). A further outbreak in which SEA, SEB and SEC were produced by a *S. aureus* strain in a local cheese (minas) occurred in Brazil (Simeão do Carmo et al., 2002). These findings highlight the importance of using methods able to detect the production of all SEs and SEls known to date and, in absence of such methods, of using tools which are able to detect *in vitro* and *in silico* all *se* and *sel* genes.

As *se*D, *sel*J and *se*R genes, also *se*C and *se*L genes always found in association in the *S. aureus* isolates characterized in this study led us to assume the presence of a pathogenicity island harboring these genes (e.g., in SaPIm1, SaPIn1, SaPImw2, SaPIbov1, SaPITokyo12571, SaPITokyo12381) (Suzuki et al., 2015; Benkerroum, 2018). Indeed, by analysing the flanking region of the *se*C_3 and *se*L genes in our *S. aureus* strain 356P it was possible to locate these genes in a putative pathogenicity island, namely SaPI356P (AN: MN450305), which shares 99% identity (16300/16305 nucleotides) with the SaPITokyo12571 (AN: AB860417.1), recently discovered by Suzuki et al. (2015) in *S. aureus* Tokyo12571 isolated from a SFP case (Fig. 5).

The *S. aureus* strain 356P harbors the *se*C_3 variant, whose nucleotide sequence has been provided by Couch and Betley, (1989). This gene encodes for a 266 amino acid protein that represents the precursor of the SEC3, which after cleavage, is released as a mature protein of 238 amino acids (Couch and Betley, 1989). Moreover, the *se*C gene of strain 356P is 100% identical to the *se*C1 variant described by Johler et al. (2016), which is harbored by all foodborne outbreaks and the nasal strains they analysed, but which these authors erroneously reported identical to the SEC-2.

The *S. aureus* Tokyo12571 harbors the same SEC$_3$ variant of *S. aureus* strain 356P and was able to produce discrete amount of SEC in broth, similar to those produced by our strain in milk (ca 20 ng and 78 ng of SEC per mL of milk, after 24 and 48 h of incubation at 37 °C, respectively). The role of SEC in SFP outbreaks was investigated (Carmo et al., 2002; Jones et al., 2002) and, considering that generally the amount of enterotoxins able to cause disease is in the range of 94–200 ng *per capita* (Benkerroum, 2018), we assume that the ingestion of ca 5–10 mL and 1.2–2.6 mL of milk once contaminated with *S. aureus* strain 356P and incubated at 37 °C for 24 and 48 h respectively may lead to production of sufficient SEC to cause SFP.

5. Conclusion

The SEg-typing and ISR-PCR characterization showed a higher discriminatory power than the MLST and allowed us to group a collection of 53 raw milk *S. aureus* isolates into 6 groups. Furthermore, four out of the six sequenced *S. aureus* strains were able to produce in milk sufficient amounts of SEA, SEC, SED and SEH to cause SFP.

These findings confirm that not only the classical SEs but also the

newly described ones can represent a hazard for the consumers' health and that the detection of SEs in raw milk and other matrices, especially if involved in SFP cases, should focus not only on classical, but also on all the new SEs and SEls known to date. Where reference methods are unavailable for the detection of these toxins, the presence of the relevant genes should be investigated using the conventional and real time PCR protocols provided in the present study.

Manual genomic analyses must be used to confirm, correct, or widen the results of the common available pipelines such as VirulenceFinder. Indeed, the manual genomic analyses allowed the detection of all the *se* and *sel* genes and their novel allelic variants, as well as their locations. Moreover, the analyses of the *sel* and *se* genes enabled us to correctly annotate these, despite the numerous incorrect or unspecific annotations we found in previous published work by other investigators, making available the correctly annotated *se* and *sel* genes as well as SaPIs, which will allow more rigorous reference-based comparative analyses.

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Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2020.103482.

Availability of data and materials

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher. The six *S. aureus* genome sequences were submitted to GenBank as Bioproject PRJNA413759 under the following accession numbers: *S. aureus* 200P: PDIW00000000; *S. aureus* 211P: PDIV00000000; *S. aureus* 356P: PDIR00000000; *S. aureus* 363P: PDIT00000000; *S. aureus* 364P: PDIS00000000; *S. aureus* 372P: PDIU00000000. The version described in this paper are PDIW00000000.1, PDIV00000000.1, PDIR00000000.1, PDIT00000000.1, PDIS00000000.1 and PDIU00000000.1, respectively. The accession numbers of the nucleotide sequences of the *Staphylococcus aureus* pathogenicity islands (SaPIs) harboring the *enterotoxin gene cluster* of *S. aureus* 356P, 363P and 364P are MN450302, MN450303 and MN450304, respectively, while that of the SaPI356P is MN450305.

Authors' contributions

VF conceived the work and interpreted the data. VF, DC and FF performed the bioinformatic work. DC performed the ISR-typing and the SEg-typing. DC and JS tested the production of SEs in milk. G-SC performed the genomic sequencing. GB performed the alignment of the *egc*s and their cluster analysis. VF, DC, FF wrote the manuscript. All the authors contributed to the revision of the manuscript, read and approved the submitted manuscript.

Contribution to the field

Staphylococcus (*S.*) *aureus* is a pathogenic bacterium able to cause a wide range of diseases in humans. Certain strains of *S. aureus* can produce staphylococcal enterotoxins (SEs) that, if ingested in appropriate amounts, may cause staphylococcal food poisoning, a gastrointestinal illness characterized by a sudden start of nausea, vomiting, stomach cramps and diarrhea. The availability of genomic technologies has greatly improved the study of this microorganism, although confusion in naming genes related to the production of these harmful compounds and the use of tools that are not specifically implemented for detecting the newly discovered ones, lead to a very confused scenario and may underestimate the risk associated with *S. aureus* contamination.

In this study we characterized 53 *S. aureus* isolated from raw milk based on the presence of enterotoxin genes and provided an exhaustive protocol to detect them. We also sequenced six strains to deeply investigate their toxigenic potential, discovering new types and combination of enterotoxin genes. Moreover we investigated the production of SEs in milk by five of the six genome sequenced strains and found that four of them were sufficiently virulent to cause staphylococcal food poisoning if present as contaminants in milk.

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