Novel Insights into Enterotoxigenic Potential of *Staphylococcus aureus* Isolated from Raw Milk

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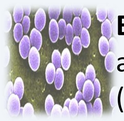
INTRODUCTION



Enterotoxigenic *Staphylococcus* (*S.*) *aureus* continue to be a great concern for raw milk and raw milk cheeses safety. Apart from the classical staphylococcal enterotoxins (SEs) (SEA-SEE), new SEs and staphylococcal enterotoxin-like toxins (Sels) have been detected and for most of them the emetic activity has been demonstrated or their genes have been found in *S. aureus* strains involved in staphylococcal food poisoning (SFP) cases, suggesting that the new SE/SEls are a potential cause of SFP outbreaks. 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 *se*/*sel* genes provide only a partial and often misleading description of the actual and overall enterotoxigenic potential of this pathogen (Copin et al., 2018). Confusion has further been created by the incorrect nomenclature given to the latest SEls discovered (Tuffs et al., 2018).

AIM: we investigated the actual and up-to-date enterotoxigenic potential of 53 *S. aureus* strains isolated from raw milk, employing also whole genome sequencing, and assessed the ability to produce some classical and new SEs when growing in milk, of *S. aureus* strains with different enterotoxigenic potential.

METHODS

53 *S. aureus* isolated from raw milk

Genetic characterization
- Enterotoxin gene (**Seg**-) **typing**
- 16S-23S rDNA intergenic spacer region (**ISR**-) **typing**

6 *S. aureus* strains one for each Seg- and ISR-type

Genome sequencing and analysis

SEA, SEC, SED, SEH, SEP, SER production in milk

Enterotoxin detection by ELISA

RESULTS AND DISCUSSION

Seg- and **ISR-typing** used to characterize **53 *S. aureus*** isolated from **raw milk**, showed the **same discriminatory power** which was **higher** than that of *in silico* multi locus sequence typing (Fig. 1)

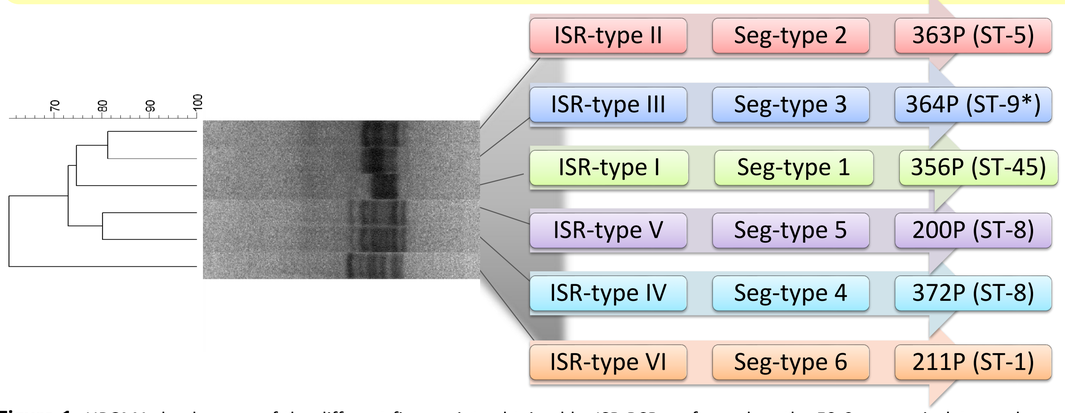


Figure 1. UPGMA dendrogram of the different fingerprints obtained by ISR-PCR performed on the 53 *S. aureus* isolates and correspondance with Seg-types. In the last column are listed the six *S. aureus* strains chosen for genome sequencing and their sequence type (ST). Seg-type: 1(*egc*[b]*, seC, seL, selW, selX*); 2 (*egc*[b]*, seP, selW, selX*); 3 (*egc*[b]*, selW, selX, selY, sel27, sel28*); 4 (*seA, seD, selJ, seR, selW, selX*); 5 (*seA, selW, selX*); 6 (*seH, selW, selX*). [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. *indicate the nearest ST

Great efforts were spent in **manual genomic analyses** of the **six sequenced *S. aureus*** strains to **confirm**, **correct** and **widen** the results of the common available pipelines (Tab. 1). In particular **novel allelic variants** of *se* and *sel* genes (Tab. 1) and **pseudogenes** were detected. The actual location of *se* and *sel* genes was found, such as that of *selY*, whose location in the core genome was so far unknown, and that of enterotoxin gene clusters (*egc*) (Fig. 2)

Table 1. Presence and position of *se* and *sel* genes in one of the six genome sequenced *S. aureus* using three different annotation methods

	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 % ([1]matching nucleotides subject/query) (accession number)**
364P									
***sel*W**	PDIS01000001.1: c1144987..1145691* note = internal stop;	pseudo	exotoxin	N.D.	N.D.	N.D.	PDIS01000001.1: 1144987..1145739*	*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*	PGG77771.1	exotoxin	N.D.	N.D.	N.D.	PDIS01000001.1: 1358207..1358950*	*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*	PGG77790.1	exotoxin	PDIS01000001.1: 1380275..1381057*	*seo*	100 (783/783) (CP003979.1)	PDIS01000001.1: 1380275..1381060*	*se*O	99.87 (785/786) (AF285760.1)
***se*M**	PDIS01000001.1: c1379275..1379994	PGG77788.1	exotoxin	PDIS01000001.1: 1379275..1379994	*sem*	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	*sei*	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	*seu*	99.87 (771/771) (HE681097.1)	PDIS01000001.1: 1377588..1378358	*sel*U2	99.87 (770/771) (EF030428.1)
***se*N**	PDIS01000001.1: c1376794..1377549*	PGG77786.1	exotoxin	PDIS01000001.1: 1376794..1377570*	*sen*	100 (777/777) (AP014653.1)	PDIS01000001.1: 1376794..1377570*	*se*N	99.87 (776/777) (AF285760.1)
***se*G**	PDIS01000001.1: c1375735..1376511	PGG77785.1	exotoxin	PDIS01000001.1: 1375735..1376511	*seg*	99.74 (777/777) (CP001844.2)	PDIS01000001.1: 1375735..1376511	*se*G	99.74 (775/777) (AF285760.1)

* no agreement in gene position between the different annotation methods; N.D.: not detected;
[1]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; 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)

Table 2. 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

A **novel** type of enterotoxin gene cluster (*egc*) in addition to the four described to date (Collery et al., 2009), namely ***egc*-type 5**, was detected in two genome sequenced ***S. aureus***, while *S. argenteus* MSHR1132 harbors variants of the same *se* and *sel* genes in the **novel *egc*-type 6** (Fig. 3)

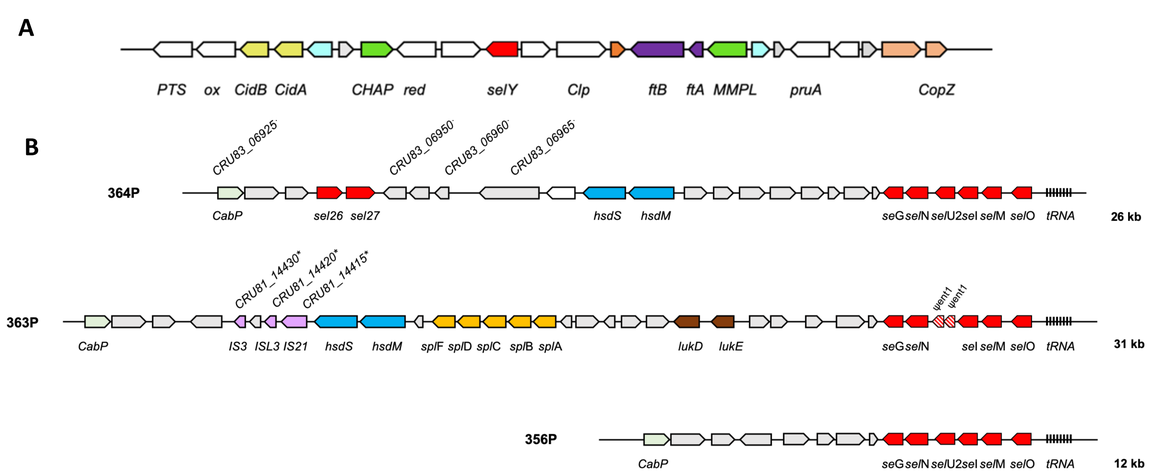


Figure 2. A: *SelY* location in *S. aureus* 364P. CidAB = holin-like proteins; ClP = Clp protease ATP-binding subunit; CopZ = copper resistance protein CopZ; PTS = PTS glucoside EIICBA component; pruA = dehydrogenase; MMPL = MMPL family transporter; ftAB = ferrous iron transporter AB. **B:** *Enterotoxin gene cluster* (*egc*) localization in *S. aureus* 356P, 363P, 364P. Red = enterotoxins; yellow = serine proteases; brown = leukocidins; grey = hypotetical proteins; light blue = restriction endonucleases; pink = transposases; *Cap*B = Calcium binding protein; * indicates a pseudogene

Four out of the six genome sequenced *S. aureus* strain produced in milk sufficient amounts of **SEA**, **SEC**, **SED** and **SEH** to cause **staphylococcal food poisoning** (SFP), with *S. aureus* **372P** as the **highest producer** of **SED** in milk reported to date (Tab. 2)

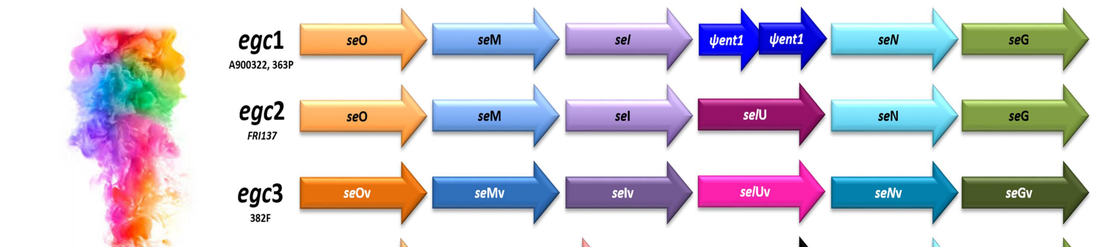


Figure 3. Structure of the six enterotoxin gene cluster (*egc*) types including the two new *egc*-types

CONCLUSION

The **correct annotation** of *se* e *sel* genes and pseudogenes herein performed on the six genome sequenced *S. aureus* will contribute to **improve and standardize** the **comparative genomics** of this pathogen and **clarify** the confused scenario of the *se* and *sel* **nomenclature**. As confirmed by our production study in milk not only the classical SEs but also the new ones, can represent an **hazard** for the consumers' health. For this reason the detection of SEs in raw milk and other matrices, should focus not only on classical but also on **all** the new SES and SEIs known to date and where reference methods are unavailable the presence of the relevant genes should be investigated.

REFERENCES
Collery et al., 2009. *J Med Microbiol*. 58, 13-25.
Copin et al., 2018. *Curr Opin Microbiol*. 41, 43-50.
Tuffs et al., 2018. *Pathogens*. 7(2)