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Genotype and enterotoxigenicity of *Staphylococcus epidermidis* isolate from ready to eat meat products

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ABSTRACT

We have previously shown that potentially pathogenic isolates of *Staphylococcus epidermidis* occur at high incidence in ready-to-eat food. Now, within 164 samples of ready-to-eat meat products we identified 32 *S. epidermidis* isolates. In 8 isolates we detected the genes encoding for staphylococcal enterotoxins, but in 7 *S. epidermidis* isolates these genes were not stable over passages. One isolate designated 4S was shown to stably harbour *sec* and *sel* genes. In the genome sequence of *S. epidermidis* 4S we identified 21,426-bp region flanked by direct-repeats, encompassing *sec* and *sel* genes, corresponding to the previously described composite staphylococcal pathogenicity island (SePI) in *S. epidermidis* FRI909. Alignment of *S. epidermidis* 4S and *S. epidermidis* FRI909 SePIs revealed 6 nucleotide mismatches located in 5 of the total of 29 ORFs. Genomic location of *S. epidermidis* 4S SePI was the same as in FRI909. *S. epidermidis* 4S is a single locus variant of ST561, being genetically different from FRI909. SEC_{epi} was secreted by *S. epidermidis* 4S to BHI broth ranging from 14 to almost 36 μg/mL, to milk ranging from 6 to 9 ng/mL, to beef meat juice from 2 to 3 μg/mL and to pork meat juice from 1 to 2 μg/mL after 24 and 48 h of cultivation, respectively. We provide the first evidence that *S. epidermidis* occurring in food bears an element encoding an orthologue to *Staphylococcus aureus* SEC, and that SEC_{epi} can be produced in microbial broth, milk and meat juices. Regarding that only enterotoxins produced by *S. aureus* are officially tracked in food in EU, the ability to produce enterotoxin by *S. epidermidis* pose real risk for food safety.

1. Introduction

Genus *Staphylococcus* comprises 52 species and 28 subspecies (http://www.bacterio.net) of gram-positive, non-motile bacteria that frequently occur as commensal colonizers of the mucocutaneous membranes of the warm-blooded animals and human (Becker et al., 2014; Fitzgerald and Penadés, 2008; Wertheim et al., 2005). With regard to the ability to coagulate rabbit plasma staphylococci fall into coagulase-positive (CPS) and coagulase-negative (CNS) groups, however this division does not reflect heterogeneity in the pathogenicity and habitat preferences within taxon. For decades, most of research was focused on coagulase-positive *Staphylococcus aureus*; hence, its virulence factors, population structure, pathogen-host interactions, and ability to cause life-threatening infections remain characterized to the greatest extent (Bergdoll, 1989; Lowy, 1998). *S. aureus* is also well known for its ability to evoke food poisoning due to the secretion of heat stable enterotoxins that may express superantigenic activity (Hennekinne et al., 2012; Le Loir et al., 2003). Several CNS species (e.g. *Staphylococcus carnosus*, *Staphylococcus xylosus*, and *Staphylococcus equorum*) are widely applied in food industry, exerting positive impact on fermentation processes and sensory characteristics of meat products (Nilsen and Rødbotten, 2007). Recent research highlighted the need of studies on involvement of CNS in human and animal disease. Enterotoxigenic CNS strains were already isolated from cases of human clinical infections (Ataee et al., 2011; da Cuhna et al., 2007; Vasconcelos et al., 2011), and foodstuffs (Even et al., 2010; Marín et al., 1992; Rall et al., 2010; Rodríguez et al., 1996; Zell et al., 2008). CNS isolates endowed with enterotoxigenic properties were also isolated from either healthy or diseased animals (Adesiyun and Usman, 1983; Park et al., 2011; Unal and Cinar, 2012; Valle et al., 1990). To date, the only well characterized enterotoxigenic CNS is *S. epidermidis* FRI909 strain isolated in the 1960s from a human clinical case (Madhusoodanan et al., 2011). FRI909 was shown to harbour *sec* and *sel* genes on an element similar to *S. aureus* pathogenicity island (hence designated SePI), and to express SEC and SEL. As it was first described by Park et al. (2011), some enterotoxin genes seem to occur in CNS in unstable form (Piette and Verschraegen, 2009). The significance of these genes for food safety, and public health remains unknown (Podkowik et al., 2013).

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Among CNS *Staphylococcus epidermidis* has gained the greatest attention, so far. Involvement of *S. epidermidis* in serious hospital infections especially in device-associated cases has been proven (Otto, 2004, 2009; Ziebuhr, 2001).Our previous studies have shown high incidence of *S. epidermidis* in ready-to-eat (RTE) meat products, and have confirmed significant prevalence of potentially pathogenic isolates among them (Podkowik et al., 2012a, 2012b). Therefore we aimed to describe incidence and characteristics of enterotoxigenic *S. epidermidis* isolates derived from RTE meat products.

2. Materials and methods

2.1. Isolation and identification of S. epidermidis

One hundred and sixty four samples of ready-to-eat porcine, bovine and chicken meat products were screened for presence of staphylococci. The samples were taken during a thirteen-month period from seven randomly selected supermarkets in Wrocław, Poland. Staphylococci from food samples were cultured on Giolitti-Cantoni enrichment broth (Thermo Fisher Scientific Inc., Waltham, MA, USA) and then subcultured onto Baird-Parker agar (Thermo Fisher Scientific Inc.). One isolate per product was taken for further analyses. The *S. epidermidis* isolates were identified by API Staph ID 32 (bioMerieux, Marcy l'Etoile, France), and additional tests for catalase, clumping factor and coagulase were done. Simultaneously, *tuf* (Martineau et al., 2001) and 16S rDNA genes (primers from htpp://rdna4.ridom.de) were partially sequenced.

2.2. Preparation of bacterial DNA

Two milliliters of bacterial cell suspension from an overnight culture grown in brain-heart infusion (BHI) broth (Thermo Fisher Scientific Inc.) were centrifuged for 5 min at 12,000 ×*g* and suspended in 100 μL of 100 mM Tris-HCl buffer, pH 7.4, containing 10 μg of lysostaphin (A&A Biotechnology, Gdynia, Poland). After 30-minute incubation at 37 °C, 10 μL of 10% SDS was added and the sample was incubated for another 30 min at 37 °C. Two hundred μL of 5 M guanidine hydrochloride were added and the sample was mixed and incubated at room temperature for 10 min. The DNA was extracted by phenol and chloroform, precipitated with ethanol, and dissolved in water.

2.3. Detection of enterotoxin genes

Detection of SEs genes (*sea-see*, *seg*, *seh*, *sei*, *selj*, *sek*, *sem*, *seo*, *tst1*, *sel*, *sen*, *sep*, *seq*, *ser*, *selu*) was performed with the use of primers and cycling conditions described by Park et al. (2011). The only modification was setting separate reactions for detection of every SE gene. All the amplicons in the size close to expected were sequenced. Sequencing was performed with the BigDye Terminator ready-reaction cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). *S. aureus* reference strains FRI913, A900322, FRI1151m, and CCM5757 served as PCR controls (Table 1).

Table 1
Reference *S. aureus* strains used for enterotoxin genes detection.

Strain	Enterotoxin gene content
FRI913	*sea*, *sec*, *see*, *sek*, *sel*, *tst*
FRI137	*sec*, *seh*, *sel*, *sem*, *sen*, *seo*, *seg*, *sei*, *selu*
CCM5757	*seb*, *sek*
A900322	*sep*, *sem*, *sen*, *seo*, *seg*, *sei*
FRI1151m	*sed*, *selj*, *ser*

2.4. Assessment of enterotoxin genes stability in S. epidermidis isolates

Enterotoxigenic *S. epidermidis* isolates were cultured on Columbia Agar with 5% sheep blood (BTL, Łódź, Poland) at 37 °C. The bacteria were subcultured every 24 h. The entire procedure comprised 10 consecutive passages. A wire loop of bacterial cells (several randomly selected colonies) was taken from each passage for further DNA isolation and analysis of enterotoxin gene content. During the cultivation colony morphology was assessed focusing on potential colony heterogeneity. The stability of *S. epidermidis* enterotoxin genes was also assessed following freezing at −80 °C in 30% glycerol. In addition, enterotoxigenic *S. epidermidis* 4S isolate was subjected to 10 consecutive passages in liquid microbiological medium. In précis, 100 μL of 24-hour *S. epidermidis* planktonic culture was transferred everyday into 5 mL of fresh BHI broth and grown at 37 °C with agitation (230 rpm). Each day 500 μL of 24-hour bacterial culture was harvested and centrifuged, and then the pellet was used for DNA isolation. PCR for respective enterotoxin genes was performed on the DNA from each bacterial passage. The same procedure was performed in altered temperature or shaking velocity (20 °C and 80 rpm, respectively).

2.5. Genome sequencing and analysis of S. epidermidis 4S isolate

An indexed, paired-end sequencing library was prepared for *S. epidermidis* 4S isolate using a Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, CA, USA) and cleaned with AMPure XP beads (Agencourt Bioscience, Beverly, MA, USA). The library was sequenced on an IlluminaMiSeq instrument using a 600 cycle Reagent Kit v3 (Illumina), which yielded 2.47 million paired-end reads for this isolate. CLC Genomics Workbench v7 was used to assemble the reads de novo after trimming and filtering for base quality of Q13 (equivalent to base error probability of ≤0.05), number of ambiguities ≤2, and length ≥ 15 bp. The assembly resulted in 321 contigs ranging in size from 500 bp to 70,462 bp with an N50 of 14,093 bp. Multilocus sequence type (ST) was determined from the genome sequence by extracting the appropriate gene fragments and comparing with the sequences deposited in the international MLST database (sepidermidis.mlst.net). *S. epidermidis* 4S contigs were also aligned against *S. epidermidis* FRI909 SePI sequence (WGS accession number AENR00000000). The sequence gaps between contigs comprising SePI were filled by Sanger dideoxy sequencing, and the resulting SePI sequence was submitted to GenBank and given accession number KT845956.

2.6. Cloning, expression, and purification of rSEC from S. epidermidis 4S isolate

The region encoding mature SEC was PCR-amplified from *S. epidermidis* 4S DNA using the Prime STAR HS DNA Polymerase (Takara Bio Inc., Otsu, Shiga, Japan) according to the protocol: 98 °C for 2 min; followed by 30 cycles at 98 °C for 10 s; 55 °C for 10 s; 72 °C for 7 s. The forward cloning primer CATGCCATGGGAGAGAGTCAACCAGACC and the reverse cloning primer CGGCTCGAGTCCATTCTTTGTTGTAAG carried the restriction sites for NcoI and XhoI. The product was purified from agarose gel, digested with XhoI and NcoI (Thermo Fisher Scientific Inc.), ligated into the pET-22b plasmid vector (Merck, Kenilworth, NJ, USA)., and introduced using calcium chloride into NovaBlue *Escherichia coli* cells (Merck). The plasmid was purified using NucleoSpin Plasmid (Macherey-Nagel, Düren, Germany) and sequenced. The plasmid containing the intact sequence of the respective region of *sec* was transformed into *E. coli* Rosetta cells (Merck). Expression was performed using the IPTG induction protocol. Briefly, 500 mL of freshly inoculated bacterial culture in LB medium was incubated in 37 °C, at 230 rpm, until $OD_{600}$of 0.7, then IPTG (Sigma-Aldrich, St. Louis, MO, USA) was added to a final concentration of 0.5 mM. After 3 h of incubation bacterial cells were harvested by centrifugation for 10 min at 5000 ×*g* in 4 °C.