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1 High prevalence of *edin-C* encoding RhoA-targeting toxin in clinical isolates of
2 *Staphylococcus aureus*

3 Patrick Munro¹, René Clément¹, Jean-Philippe Lavigne^{4,5}, Céline Pulcini^{2,3}, Emmanuel
4 Lemichez^{1*} and Luce Landraud^{1,6*}

5
6 **Running title:** EDIN exotoxins in *S. aureus* infections

7
8 1 INSERM, U895, C3M, toxines microbiennes dans la relation hôte pathogènes, Université de
9 Nice-Sophia-Antipolis, UFR Médecine, IFR50, Nice, F-06204, France

10 2 Université de Nice-Sophia-Antipolis, UFR Médecine, IFR50, Nice, F-06204, France

11 3 Service d'Infectiologie, Hôpital l'Archet 1, Route Saint Antoine de Ginestière, BP 3079,
12 06202 Nice Cedex 3, France

13 4 INSERM, Espri 26, Université Montpellier 1, UFR de Médecine, Nîmes, France

14 5 Laboratoire de Bactériologie, CHU Caremeau, Nîmes, France

15 6 Laboratoire de Bactériologie, CHU de Nice, Hôpital l'Archet, Nice, France.

16
17
18
19 *** Corresponding authors:**

20 **Luce Landraud**, INSERM, U895, C3M, toxines microbiennes dans la relation hôte
21 pathogènes, Université de Nice-Sophia-Antipolis, UFR Médecine, IFR50, Nice, F-06204,
22 France, and Laboratoire de Bactériologie, CHU de Nice, Hôpital l'Archet, Nice, France.

23 Telephone: 00 33 4 89 06 42 61

24 Fax: 00 33 4 89 06 42 60

25 Mail: landraud.l@chu-nice.fr

26 **Emmanuel Lemichez**, INSERM, U895, C3M, toxines microbiennes dans la relation hôte
27 pathogènes, Université de Nice-Sophia-Antipolis, UFR Médecine, IFR50, Nice, F-06204,
28 France, and Laboratoire de Bactériologie, CHU de Nice, Hôpital l'Archet, Nice, France.

29 Telephone: 00 33 4 89 06 42 61

30 Fax: 00 33 4 89 06 42 60

31 Mail: lemichez@unice.fr

32
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1 **Abstract** *Staphylococcus aureus*, a major causative agent of human infection produces a large
2 array of virulence factor including various toxins. Among them, the host RhoA GTPase ADP-
3 ribosylating EDIN toxins are considered as potential virulence factors. Using the polymerase
4 chain reaction, we analyzed the virulence profile of 256 *S. aureus* isolates from various
5 clinical sites of infections. We developed specific primers to detect the three isoforms of *edin*
6 encoding genes. We found a prevalence of 14% (36 bacteria) of *edin* encoding genes among
7 these clinical isolates. Strikingly, we found that 90% of all *edin*-bearing *S. aureus* isolates
8 carried the type-C allele. Both the *spa* types and the profile of virulence factors of these *edin*-
9 positive isolates are highly variable. Notably, we show for the first time that *edin*-C positive
10 isolates were more frequently recovered from deep-seated infections than other types of
11 infections. Our present work thus strongly suggests that presence of *edin*-C is a risk factor of
12 *S. aureus* dissemination in tissues and thus represents a predictive marker for a pejorative
13 evolution of staphylococcal infections.

14

15

16

17 **Keywords**

18 *Staphylococcus aureus*, EDIN, toxin, ADP-ribosyltransferase, virulence factors, Rho
19 GTPases.

20

1 **Introduction**

2

3 *Staphylococcus aureus* is a common bacterium, which is responsible for a unique variety of
4 infections [1]. Development of peyorative forms of staphylococcal infections involves the
5 combined action of numerous bacterial virulence factors, which corrupt host responses [2].
6 Bacterial virulence factors include specific adhesins, collectively referred as Microbial
7 Surface Components Recognizing endothelial cell Adhesive Matrix Molecules
8 (MSCRAMMs) and a large variety of toxins, such as the exfoliative toxins (ETs), hemolysin,
9 leukocidin, enterotoxins and EDINs (epidermal cell differentiation inhibitors) [3-6].

10 EDINs belong to the family of *Clostridium botulinum* C3 exoenzyme [6, 7]. They are
11 members of a group of major bacterial virulence factors targeting host Rho GTPases [4, 6-9].
12 Rho proteins control essential cellular processes such as cell polarity, movement and
13 phagocytosis, as well as cohesion of intercellular junctions [6, 10, 11]. Recent findings
14 suggest that EDINs might favor bacterial dissemination in tissues, by a haematogenous route,
15 through induction of large transcellular tunnels in endothelial cells named macroapertures
16 [12-14]. Indeed, recent data show that *S. aureus* EDIN toxin promotes formation of infection
17 foci in a mouse model of bacteremia [15]. To date, three isoforms of EDIN have been
18 characterized. These comprise the first discovered EDIN isoform (EDIN-A), isolated from the
19 E-1 strain of *S. aureus* [16], as well as EDIN-B [6, 17] and EDIN-C [18]. The chromosomal
20 gene encoding *edin-B* is located within a pathogenicity island frequently associated with the
21 *etD* gene encoding the exfoliative toxin ET-D [17]. EDIN-C is encoded by the pETB plasmid,
22 which also carries genes encoding ET-B and conferring cadmium resistance [18].

23 A first epidemiological survey, involving staphylococcal strains isolated from patients
24 hospitalized for various infectious diseases demonstrated a higher prevalence of *edin-*
25 encoding genes in this group compared to nasal strains isolated from healthy students [19].
26 Another study shows that *edin-B* is present in 7% of bacteriemic *S. aureus* strains [17].
27 However, most other epidemiological data on *edin* are based on surveys focused on
28 exfoliative toxins or PVL rather than EDIN toxin itself. For example, a genetic association
29 between *etD* and *edin-B* was detected in the emerging ST80 clone Panton-Valentine
30 Leukocidin (PVL)-positive and community-acquired (CA) methicillin-resistant *S. aureus*
31 (MRSA) [20]. This clone is spreading throughout France and Tunisia and is most frequently
32 associated with infections of the skin and soft tissues. Also, two-thirds of the strains
33 belonging to the emerging ST123 epidemic European fusidic acid-resistant impetigo clone

1 (EEFIC) were positive for *etB* and sequence analysis of pETB2 (a close homolog of pETB) in
2 one of these strains suggested that it also bears *edin-C* [21].

3 In this study, we have developed a PCR-based method, to detect EDIN isoforms
4 specifically. We demonstrate that 90% of all *edin*-bearing *S. aureus* isolates carry the type-C
5 allele. We also show that these isolates are more significantly associated with deep-seated soft
6 tissue infections than other types of infections (Fisher's exact test, $p=0.03$).

7

1 **Materials and methods**

2

3 *S. aureus* isolates

4

5 A total of 256 isolates of *S. aureus* belonging to the collection of the Bacteriology department
6 of the Hospital University of Nice were analyzed. These isolates were recovered from
7 randomly consecutive episodes of *S. aureus* infections in patients hospitalized during 2005.
8 These isolates were obtained from various types of clinical samples, comprising blood
9 cultures (28 bacteria); skin infections including chronic ulcers, burns or wounds (83 bacteria);
10 urine samples (41 bacteria); sputum samples (69 bacteria); and various deep-seated soft tissue
11 infections such as subcutaneous or visceral abscesses, spontaneously or post operative soft
12 tissue infections (35 bacteria). For the last group, bacteria were isolated from specimens
13 obtained by guidance radiography needle biopsy or during endoscopic and surgical
14 procedures. All isolates were characterized using routine methods according to each
15 manufacturer's recommendations. All were positive for catalase, DNase production and
16 mannitol fermentation in Chapman medium, and confirmed to be *S. aureus* by specific
17 32rapidStaph (BioMérieux, Marcy-l'Etoile, France).

18

19 Antibiotic susceptibility determinations

20

21 Antimicrobial susceptibility testing was performed on all isolates obtained during the study
22 using the disk diffusion method [22] on Mueller-Hinton medium (Difco Laboratories, Detroit,
23 MI) according to the recommendations of the French Antibigram Committee
24 [<http://www.sfm.asso.fr/nouv/general.php?pa=2>]. Antibiotics tested were penicillin G,
25 oxacillin, erythromycin, clindamycin and fusidic acid to focus on epidemiologic profiles.

26

27 DNA isolation and PCR-based detection of genes

28

29 For *edin* detection, total DNA was isolated from bacterial strains grown overnight at 37°C in
30 BHI medium. Bacteria were lysed in 10 mM TrisHCl pH7.8, 100 mM NaCl, 1mM EDTA, 1%
31 Triton X100. After incubation for 10 minutes at 100°C, DNA was collected and frozen. PCR
32 amplification was used to detect the presence of *edin*-A, B and C using the primers described
33 in Table 1. We have determined optimized thermal cycling conditions for *edin*-A (25 cycles of
34 30s at 94°C, 45s annealing at 58°C and 1 min elongation at 72°C), *edin*-B (25 cycles of 30s at

1 95°C, 1 min annealing at 50°C and 1 min elongation at 72°C) and *edin-C* (30 cycles of 30s at
2 94°C, 45s annealing at 54°C and 1 min elongation at 72°C). For the detection of other
3 virulence genes, total DNA was isolated from bacterial strains grown three hours at 37°C in
4 BHI medium. DNA was subsequently extracted with NucleoSpin Tissue (Macherey-Nagel
5 GmbH, Düren, Germany) according to manufacturer's recommendations. Briefly, bacteria
6 were pelleted by centrifugation at 8,000 ×g for 5 min, resuspended in 180µl of lysis buffer
7 with 33µl of proteinase K (20mg/ml) (Invitrogen Life Technologies, Carlsbad, CA) and 3µl of
8 recombinant lysostaphin (3U/µl) (Sigma-Aldrich, St Louis, MI), and incubated for 60 min at
9 37°C. DNA samples were eluted with 100 µl alkaline elution buffer (BE buffer, NucleoSpin
10 Tissue, Macherey-Nagel). The presence of 30 genes, among the most prevalent virulence-
11 associated genes, was evaluated by PCR as described previously: staphylococcal enterotoxins
12 (*se*) A, B, C, D, E, G, H, I, J, K, and Q, toxic shock syndrome toxin 1 (*tst-1*), exfoliative
13 toxins A, B and D (*etA*, *etB*, *etD*), PVL (*lukS-PV-lukF-PV*), LukDE leukocidin (*lukE*), nine
14 MSCRAMM (*bbp*, *cna*, *ebpS*, *clfA*, *clfB*, *fib*, *fnbA*, *fnbB*, *eno*). The accessory gene regulator
15 (*agr*) allele group was determined by multiplex PCR.

16

17 *spa* sequencing

18

19 *spa* typing was performed as described previously [23], using the *spa* typing website
20 (<http://www.spaserver.ridom.de/>) that is developed by Ridom GmbH and curated by
21 SeqNet.org (<http://www.SeqNet.org/>). Primers are indicated in Table 1.

22

23 Statistical analysis

24

25 The chi-square or Fisher's exact test for categorical variables was used to compare data as
26 appropriate. A *P* value of less than 0.05 was considered significant.

27

1 **Results**

2

3 Detection of *edin* isoforms

4

5 *S. aureus* isolates analyzed in this study were collected at the university hospital of Nice from
6 various infected patients. We designed primers with high sensitivity and specificity in order
7 to detect, by PCR, *edin*-A, B and C alleles in these isolates. This was especially challenging
8 for *edin*-C, which was poorly detected using a previously described pair of primers designed
9 to amplify all *edin* isoforms. This is consistent with the fact that the sequence of *edin*-C has
10 the most substantial sequence variations in regions recognized by these primer sequences
11 (17% and 32% homology with the forward and reverse primers, respectively) (Fig. 1) [19]. As
12 shown in figure 1, the three pairs of primers designed allowed us to amplify specifically a 455
13 bp DNA fragment for *edin*-A and B, and a 320 bp DNA fragment for *edin*-C.

14 We next analyze the 256 clinical isolates of *S. aureus*. We found that 14 % (36) of these
15 isolates were positive for one of the *edin* alleles. Among these 36 isolates, 90% were positive
16 for *edin*-C and 5% were positive for either *edin*-A or B. To confirm the nature of the *edin*
17 isoforms, we performed complete sequence analysis of five *edin*-C encoding genes from
18 randomly selected isolates. We also sequenced *edin*-A and *edin*-B encoding genes. These
19 results confirm the specificity of the new primers used and demonstrate that *edin*-C was more
20 prevalent than other alleles of *edin*.

21

22 Detection of genes encoding virulence factors

23

24 The 36 isolates positive for *edin* genes were next analyzed for the distribution of major
25 MSCRAMMs, various staphylococcal enterotoxins, exfoliative toxins, the toxic shock
26 syndrome toxin 1 gene *tst-I*, as well as leucotoxin family encoding genes. Among the
27 staphylococcal MSCRAMM genes, *eno*, *clfA* and *clfB* were detected in all *edin*-positive
28 isolates (Table 2). *bbp*, *fnbA* and *ebpS* were the less frequently encountered MSCRAMMs
29 among these isolates. However these adhesion factors had no preferential distribution among
30 *S. aureus* isolated from various types of infections. Among the staphylococcal enterotoxins
31 genes the most frequently encountered were *seg*, *sei* and *sea* (Table 2). In addition, 72% of
32 *edin*-positive isolates (26 bacteria) contained a combination of these three genes. One *edin*-C
33 bearing isolate, isolated from a urine sample, had only the *sea* enterotoxin gene. We detected
34 the exfoliative toxin gene *etD* exclusively among the *edin*-B positive isolates. Two *edin*-A

1 positive isolates carried the staphylococcal exfoliative gene *etB*. We found that 25 *edin-C*
2 positive *S. aureus* (78%) were negative for *etA*, *etB* and *etD* exfoliative toxin encoding genes.
3 We observed that only 22% of the *edin-C* positive *S. aureus* (7 out of 32) had at least one of
4 the two isoforms (*etA* or *etB*) of the exfoliative toxin gene. Five of these seven isolates carried
5 both the *etA* and *etB* genes. No significant association was found between the presence of *tst-*
6 *1*, exfoliative toxins or leucotoxin family encoded genes and the types of infection. Finally, a
7 large number of *edin*-positive *S. aureus* belonged to the *agr* group 1 (50%, 18 bacteria), and
8 to a lesser extent, to *agr* groups 2, 3 and 4 (17%, 6 bacteria each) (Table 2).

9 10 *spa*-typing

11
12 Determination of the *spa* type of 26 *edin*-positive isolates [23] allowed us to exclude the
13 clonal origin of all *edin-C* positive *S. aureus* in our survey. Among them, only six isolates
14 could be classified as ST45 (two isolates), ST30 (two isolates), ST59 or ST26. The other 20
15 isolates showed a high variability of their *spa* type (Table 3). For 14 isolates, we determined
16 new repeat sequences including unidentified 24-bp repeats thus defining new *spa* types.
17 Among them, bacteria S7926 and S7262, isolated respectively in deep seated soft tissue and
18 sputum sample from unrelated infected patients, presented the same *spa* type t6956 (Table 3).
19 Together these data excluded the clonal origin of all *edin-C* positive *S. aureus* in our survey.

20 21 Antibiotic susceptibility profiles

22
23 We next investigated whether *edin*-positive isolates were associated with specific antibiotic
24 resistance profiles, such as community-acquired methicillin resistant *S. aureus* ST80 and
25 fusidic acid-resistant impetigo clones [20, 21]. In relation with these studies, we determined
26 the minimum inhibitory concentrations (MICs) of *edin*-positive isolates for the following
27 antibiotics: penicillin G, methicillin, erythromycin, clindamycin and fusidic acid. Results
28 were presented in Table 2. Only one isolate, positive for *edin-C*, showed a methicillin-
29 resistance. Finally, *edin*-positive isolates did not show any specific resistance profile to
30 classical antibiotics used to cure infections by *S. aureus* (Table 2).

31 32 Clinical origin of *edin*-positive *S. aureus*

1 Having shown no link between a high prevalence of *edin-C* within our *S. aureus* isolates and
2 any phenotypic profile or clonal origin, we further analyzed the distribution of *edin*-positive
3 isolates with regard to the infectious sites. We noticed that isolates positive for *edin-C* were
4 recovered at all sites of infection (Table 4). Strikingly, *edin*-positive *S. aureus* were more
5 significantly associated with deep-seated infections of soft tissues than other types of
6 infections (25.7%, Fischer exact test, $p=0.03$). No significant association was detected
7 between *edin*-positive or *edin*-negative isolates and other types of infections (blood, urine,
8 superficial soft tissue and sputum culture).

9
10

1 Discussion

2
3 Our study shows that *edin*-positive *S. aureus* isolates are found in all types of clinical
4 infections included in this study, with a global prevalence of 14%. Moreover, we show that
5 90% of *edin*-positive isolates are positive for *edin-C*. This is consistent with a previous study
6 performed with specific primers, which also reported a higher prevalence of *edin-C* in clinical
7 isolates of *S. aureus* in Japan [24]. This points for the need of using specific primers to detect
8 each *edin* isoform, especially *edin-C*, given its high prevalence. On the contrary, both studies
9 point for a possible underestimation of the prevalence of *edin-C* in pathogenic *S. aureus* when
10 consensus primers were used. Both the analysis of *spa* type and the distribution of various
11 virulence factors among *edin*-positive *S. aureus* show their high genetic variability. Our
12 results on the distribution of MSCRAMMs are consistent with previous findings [25].
13 Classically, *edin* and exfoliative toxin encoding genes are associated in specific lineages
14 responsible for skin infections [17, 21, 24]. Interestingly, here we show that *edin-C* is not
15 strictly associated with genes encoding exfoliative toxin of serotypes A/B/D (7 bacteria *edin-*
16 *C*-positive and *et*-positive, versus 25 bacteria *edin-C*-positive and *et*-negative). The high
17 prevalence of *edin-C*-positive and *et*-negative isolates observed in our study might be
18 explained by the plasticity of the pETB plasmid, which has been previously reported in two
19 different variants [18, 21]. Also, a recent study shows genetic variations in pathogenicity
20 islands encoding EDIN-B [17].

21 Given that *spa* analysis constitutes a good tool for epidemiological typing of *S. aureus*
22 [26], the use of this method allowed us to exclude the clonal origin of *edin-C* positive isolates.
23 The fact that *edin-C* is plasmid born might explain its presence in isolates of various genetic
24 backgrounds.

25 *S. aureus* positive for *edin* are more frequently associated with deep-seated infections of
26 soft tissues. We have previously established that infection of endothelial cells, and other cell
27 types, by EDIN-producing *S. aureus* triggers the formation of transcellular tunnels named
28 macroapertures [12, 13]. Opening of transcellular tunnels in the endothelium suggested that
29 EDIN might favour bacterial extravasation in tissues during bacteremia. In a mouse model of
30 intravascular injection of *S. aureus*, we have observed that EDIN plays no detectable role in
31 the persistence of bacteria in the blood stream [15]. This data is consistent with the absence of
32 a higher prevalence of *edin* positive *S. aureus* recovered from patients associated bacteremia
33 in this study. In contrast, in this model of mouse infection EDIN toxin promotes formation of
34 infection foci [15]. This suggested that EDIN might enhance the invasive capacity of *S.*

1 *aureus*. The hypothesis of a role of EDIN in *S. aureus* infection is also supported by our
2 present findings showing that *S. aureus* associated with deep-seated infections of soft tissues
3 have a higher prevalence of *edin*. However, whether or not *edin*-positive *S. aureus* is
4 preferentially associated with a specific type of deep seated infection remains to be further
5 determined”.

6

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12

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18

1 **Figure legend**

2

3 **Fig. 1** Characterization of the three *edin* alleles. A) PCR amplification of *edin-A*, *edin-B* and
4 *edin-C* from *S. aureus* strains S25*edin-A*(+)[15], S7256*edin-B*(+) and S7475*edin-C*(+) (this
5 study), respectively, using specific oligonucleotides *edinA*, *edinB*, *edinC* (Table 1) and the
6 previously described *edin* oligonucleotides referred as *edinX* [19]. B) Sequence alignments of
7 *edin-A*, *edin-B* and *edin-C* showing sequence homologies and localization of each
8 oligonucleotide (underlined: *edinX*; highlighted: *edinA*, *edinB* and *edinC*).

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1 Table 1: Oligonucleotides primers used in this study

2

| Gene | Primer sequences | Size (kb) | References |
|--------------|---|--------------|------------|
| <i>edinA</i> | Sense 5'-GGAGATATTAATAAGCTAGATTC-3' | 455 | This study |
| | Antisense 5'-ATTTTCTTTTTATCATTGACAATTCT-3' | | |
| <i>edinB</i> | Sense 5'-GGTGACGTGAACAAATTATCCGA-3' | 455 | This study |
| | Antisense 5'-ATCTTTCTTTTGTTATCAGAAAGTTTA-3' | | |
| <i>edinC</i> | Sense 5'-CGCCATTAAGGTCTAGTCAAGG-3' | 320 | This study |
| | Antisense 5'-TAGGTCTTCCAGCTAATGCAGC-3' | | |
| <i>bbp</i> | Sense 5'-AACTACATCTAGTACTCAACAACAG-3' | 575 | [25] |
| | Antisense 5'-ATGTGCTTGAATAACACCATCATCT-3' | | |
| <i>cna</i> | Sense 5'-GTCAAGCAGTTATTAACACCAGAC-3' | 423 | [25] |
| | Antisense 5'-AATCAGTAATTGCACTTTGTCCACTG-3' | | |
| <i>ebpS</i> | Sense 5'-CATCCAGAACCAATCGAAGAC-3' | 186 | [25] |
| | Antisense 5'-CTTAACAGTTACATCATCATGTTTATCTTTG-3' | | |
| <i>fnbA</i> | Sense 5'-GTGAAGTTTTAGAAGGTGGAAAGATTAG -3' | 643 | [25] |
| | Antisense 5'-GCTCTTGTAAGACCATTTTTCTTCAC-3' | | |
| <i>fnbB</i> | Sense 5'-GTAACAGCTAATGGTTCGAATTGATACT-3' | 524 | [25] |
| | Antisense 5'-CAAGTTCGATAGGAGTACTATGTTC-3' | | |
| <i>fib</i> | Sense 5'-CTACA ACTACAATTGCCGTCAACAG-3' | 404 | [25] |
| | Antisense 5'-GCTCTTGTAAGACCATTTTTCTTCAC-3' | | |
| <i>clfA</i> | Sense 5'-ATTGGCGTGGCTTCAGTGCT-3' | 292 | [25] |
| | Antisense 5'-CGTTTCTTCCGTAGTTGCATTTG-3' | | |
| <i>clfB</i> | Sense 5'-ACATCAGTAATAGTAGGGGGCAAC-3' | 205 | [25] |
| | Antisense 5'-TTCGCACTGTTTGTGTTTGCAC-3' | | |
| <i>eno</i> | Sense 5'- ACGTGCAGCAGCTGACT-3' | 302 | [25] |
| | Antisense 5'- CAACAGCATYCTTCAGTACCTTC-3' | | |
| <i>sea</i> | Sense 5'-GCAGGGAACAGCTTTAGGC-3' | 520 | [27] |
| | Antisense 5'-GTTCTGTAGAAGTATGAAACACG-3' | | |
| <i>seb</i> | Sense 5'-ATGTAATTTTGATATTCGCAGTG-3' | 683 | [27] |
| | Antisense 5'-TGCAGGCATCATATCATAACCA-3' | | |
| <i>sec</i> | Sense 5'-CTTGTATGTATGGAGGAATAACAA-3' | 283 | [27] |

| | | | |
|--------------|--|-----|------------|
| | Antisense 5'-TGCAGGCATCATATCATACCA-3' | | |
| <i>sed</i> | Sense 5'-GTGGTGAAATAGATAGGACTGC-3' | 384 | [27] |
| | Antisense 5'-ATATGAAGGTGCTCTGTGG-3' | | |
| <i>see</i> | Sense 5'-TACCAATTA ACTTGTGGATAGAC-3' | 170 | [27] |
| | Antisense 5'-CTCTTTGCACCTTACCGC-3' | | |
| <i>seg</i> | Sense 5'-CGTCTCCACCTGTTGAAGG-3' | 327 | [27] |
| | Antisense 5'-CCAAGTGATTGTCTATTGTCG-3' | | |
| <i>seh</i> | Sense 5'-CAACTGCTGATTTAGCTCAG-3' | 360 | [27] |
| | Antisense 5'-GTCGAATGAGTAATCTCTAGG-3' | | |
| <i>sei</i> | Sense 5'-CAACTCGAATTTTCAACAGGTAC-3' | 465 | [27] |
| | Antisense 5'-CAGGCAGTCCATCTCCTG-3' | | |
| <i>sej</i> | Sense 5'-CATCAGAACTGTTGTTCCGCTAG-3' | 142 | [27] |
| | Antisense 5'-CTGAATTTTACCATCAAAGGTAC-3' | | |
| <i>sek</i> | Sense 5'-ATGGCGGAGTCACAGCTACT-3' | 197 | [27] |
| | Antisense 5'-TGCCGTTATGTCCATAAATGTT-3' | | |
| <i>seq</i> | Sense 5'-GAACCTGAAAAGCTTCAAGGA-3' | 209 | [27] |
| | Antisense 5'-ATTCGCCAACGTAATTCCAC-3' | | |
| <i>eta</i> | Sense 5'-CTAGTGCATTTGTTATTCAA-3' | 119 | [28] |
| | Antisense 5'-TGCATTGACACCATAGTACT-3' | | |
| <i>etb</i> | Sense 5'-ACGGCTATATACATTCAATT-3' | 200 | [28] |
| | Antisense 5'-TCCATCGATAATATACCTAA-3' | | |
| <i>etd</i> | Sense 5'-ATGACTAAAAATATATATAAAAAGTT-3' | 846 | This study |
| | Antisense 5'-CTAATGAGACTGTAATTCAGC-3' | | |
| <i>lukPV</i> | Sense 5'-ATCATTAGGTAAAATGTCTGGACATGATCCA-3' | 433 | [29] |
| | Antisense 5'-GCATCAASTGTATTGGATAGCAAAAAGC-3' | | |
| <i>lukE</i> | Sense 5'-TGAAAAAGGTTCAAAGTTGATACGAG-3' | 269 | [29] |
| | Antisense 5'-TGTATTTCGATAGCAAAAAGCAGTGCA-3' | | |
| <i>tst-1</i> | Sense 5'-GCTTGCGACA ACTGCTACAG-3' | 559 | [27] |
| | Antisense 5'-TGGATCCGTCATTCATTGTTAA-3' | | |
| <i>agr1</i> | Sense 5'-ATGCACATGG TGCACATGC-3' | 439 | [30] |
| | Antisense 5'-GTCACAAGTACTATAAGCTGCGAT-3' | | |
| <i>agr2</i> | Sense 5'-ATGCACATGG TGCACATGC-3' | 572 | [30] |
| | Antisense 5'-TATTACTAATTGAAAAGTGC CATAGC-3' | | |

| | | | |
|-------------|--|-----|------|
| <i>agr3</i> | Sense 5'-ATGCACATGG TGCACATGC-3' | 321 | [30] |
| | Antisense 5'-GTAATGTAATAGCTTGTATAATAATACCCAG-3' | | |
| <i>agr4</i> | Sense 5'-ATGCACATGG TGCACATGC-3' | 657 | [30] |
| | Antisense 5'-CGATAATGCCGTAATACCCG-3' | | |
| <i>spa</i> | Sense5'-TGTA AACGACGGCCAGTTAAAGACGATCCTTCGGTGAGC-3' | | [23] |
| | Antisense5'-CAGGAAACAGCTATGACCCAGCAGTAGTGCCGTTTGCTT-3' | | |

1

Table 2: Virulence profile and antibiotic susceptibility of clinical *edin*-positive *S. aureus* isolates.

| | blood (n=2) | | superficial soft tissue (n=11) | | urine sample (n=4) | | sputum sample (n=10) | | deep-seated soft tissue (n=9) | | total (n=36) | |
|--------------------------------------|--------------------|-----|--------------------------------|-----|--------------------|-----|----------------------|-----|-------------------------------|-----|--------------|-----|
| | n | % | n | % | n | % | n | % | n | % | n | % |
| Virulence profile | | | | | | | | | | | | |
| MSCRAMMs | | | | | | | | | | | | |
| <i>bbp</i> | 0 | 0 | 3 | 27 | 3 | 75 | 3 | 30 | 6 | 67 | 15 | 42 |
| <i>cna</i> | 1 | 50 | 9 | 82 | 4 | 100 | 10 | 100 | 8 | 89 | 32 | 89 |
| <i>ebpS</i> | 0 | 0 | 5 | 45 | 1 | 25 | 6 | 60 | 6 | 67 | 18 | 50 |
| <i>fnbA</i> | 1 | 50 | 7 | 64 | 1 | 25 | 5 | 50 | 5 | 56 | 19 | 53 |
| <i>fnbB</i> | 1 | 50 | 11 | 100 | 4 | 100 | 7 | 70 | 8 | 89 | 31 | 86 |
| <i>fib</i> | 2 | 100 | 10 | 91 | 4 | 100 | 7 | 70 | 9 | 100 | 32 | 89 |
| <i>clfA</i> | 2 | 100 | 11 | 100 | 4 | 100 | 10 | 100 | 9 | 100 | 36 | 100 |
| <i>clfB</i> | 2 | 100 | 11 | 100 | 4 | 100 | 10 | 100 | 9 | 100 | 36 | 100 |
| <i>eno</i> | 2 | 100 | 11 | 100 | 4 | 100 | 10 | 100 | 9 | 100 | 36 | 100 |
| SEs | | | | | | | | | | | | |
| <i>sea</i> | 1 | 50 | 10 | 91 | 3 | 75 | 9 | 90 | 9 | 100 | 32 | 89 |
| <i>seb</i> | 1 | 50 | 7 | 64 | 2 | 50 | 8 | 80 | 6 | 67 | 24 | 67 |
| <i>sec</i> | 1 | 50 | 7 | 64 | 2 | 50 | 10 | 100 | 6 | 67 | 26 | 72 |
| <i>sed</i> | 0 | 0 | 8 | 73 | 3 | 75 | 6 | 60 | 6 | 67 | 23 | 64 |
| <i>see</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>sek</i> | 1 | 50 | 5 | 45 | 1 | 25 | 7 | 70 | 2 | 22 | 16 | 44 |
| <i>seq</i> | 1 | 50 | 3 | 27 | 1 | 25 | 5 | 50 | 5 | 56 | 15 | 42 |
| <i>seg</i> | 2 | 100 | 10 | 91 | 2 | 50 | 10 | 100 | 9 | 100 | 33 | 92 |
| <i>seh</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 11 | 1 | 3 |
| <i>sei</i> | 2 | 100 | 11 | 100 | 2 | 50 | 9 | 90 | 8 | 89 | 32 | 89 |
| <i>sej</i> | 0 | 0 | 1 | 9 | 0 | 0 | 2 | 20 | 1 | 11 | 4 | 11 |
| <i>tst-1</i> | 2 | 100 | 6 | 55 | 1 | 25 | 7 | 70 | 5 | 56 | 21 | 58 |
| <i>etA</i> | 0 | 0 | 2 | 18 | 0 | 0 | 2 | 20 | 2 | 22 | 6 | 17 |
| <i>etB</i> | 1 ^{&} | 50 | 4 [§] | 36 | 0 | 0 | 2 | 20 | 1 | 11 | 8 | 22 |
| <i>etD</i> | 0 | 0 | 0 | 0 | 1 [*] | 25 | 1 [*] | 10 | 0 | 0 | 2 | 6 |
| <i>lukPV</i> | 0 | 0 | 1 | 9 | 1 | 25 | 0 | 0 | 3 | 33 | 5 | 14 |
| <i>lukE</i> | 2 | 100 | 8 | 73 | 3 | 75 | 5 | 50 | 4 | 44 | 24 | 67 |
| agr group | | | | | | | | | | | | |
| <i>agr1</i> | 1 | 50 | 3 | 27 | 3 | 75 | 6 | 60 | 5 | 56 | 18 | 50 |
| <i>agr2</i> | 1 | 50 | 3 | 27 | 1 | 25 | 1 | 10 | 0 | 0 | 6 | 17 |
| <i>agr3</i> | 0 | 0 | 3 | 27 | 0 | 0 | 0 | 0 | 3 | 33 | 6 | 17 |
| <i>agr4</i> | 0 | 0 | 2 | 18 | 0 | 0 | 3 | 30 | 1 | 11 | 6 | 17 |
| antibiotic resistance profile | | | | | | | | | | | | |
| <i>penicillin G</i> | 1 | 50 | 5 | 45 | 2 | 50 | 8 | 80 | 6 | 67 | 22 | 61 |
| <i>Methicillin</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 11 | 1 | 3 |
| <i>Erythromycin</i> | 0 | 0 | 1 | 9 | 0 | 0 | 1 | 10 | 1 | 11 | 3 | 8 |
| <i>Clindamycin</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 11 | 1 | 3 |
| <i>Fusidic acid</i> | 0 | 0 | 0 | 0 | 1 [▣] | 25 | 0 | 0 | 1 [▣] | 11 | 2 | 6 |

* *Edin B* positive strains

§ *Edin A* positive for one strain

& *EdinA* positive strain

▣ Increase in fusidic acid MIC, classified as intermediary sensibility

Table 3 : *spa*-type of 36 *edin*-positive *Staphylococcus aureus* isolates.

| Strains | EDIN type | <i>Spa</i> -type or repeat sequences | MLST |
|--------------------------------|-----------|--------------------------------------|-------|
| Blood | | | |
| S25 | A | t6403 | - |
| S7232 | C | NT ^s | - |
| Deep-seated soft tissue | | | |
| S7272 | C | t6953 | - |
| S7404 | C | t6649 | - |
| S7408 | C | t6484 | - |
| S7466 | C | t012 | ST-30 |
| S7595 | C | NT | - |
| S7600 | C | t6677 | - |
| S7926 | C | t6956 | - |
| S8028* | C | NT | - |
| S8087 | C | t6481 | - |
| Sputum sample | | | |
| S7225 | C | t2726 | - |
| S7259 | C | t2088 | - |
| S7262 | C | t6956 | - |
| S7413 | C | t6483 | - |
| S7535 | C | t2647 | - |
| S7634 | C | t6954 | - |
| S7649 | B | NT | - |
| S7920 | C | NT | - |
| S7965 | C | NT | - |
| S8100 | C | t015 | ST-45 |
| Superficial soft tissue | | | |
| S7181 | C | t6650 | - |
| S7183 | C | NT | - |
| S7436 | C | t6957 | - |
| S7475 | C | t6480 | - |
| S7526 | C | t137 | - |

| | | | |
|---------------------|---|-------|-------|
| S7539 | C | NT | - |
| S7569 | A | t031 | ST-45 |
| S7599 | C | t6482 | - |
| S7932 | C | NT | - |
| S7938 | C | t620 | - |
| S7977 | C | NT | - |
| Urine sample | | | |
| S7256 | B | t078 | ST-26 |
| S7322 | C | t012 | ST-30 |
| S7906 | C | t645 | - |
| S7946 | C | t216 | ST-59 |

* MRSA bacteria

\$ Not typable

Table 4: Presence of *edin* genes in 256 *Staphylococcus aureus* isolates associated with various clinical syndromes.

| Source of isolates (N) | Number of <i>edin</i> | | <i>edin</i> -isoforms | | |
|-------------------------------------|-----------------------|-----|-----------------------|--------------|----------------|
| | isolates | (%) | <i>edinA</i> | <i>edinB</i> | <i>edinC</i> |
| Blood (28) | 2 (7.1) | | 1 | 0 | 1 |
| Urine (41) | 4 (9.8) | | 0 | 1 | 3 |
| Superficial soft tissue (83) | 11 (13.3) | | 1 | 0 | 10 |
| Deep-seated soft tissue (35) | 9 (25.7)* | | 0 | 0 | 9 |
| Sputum (69) | 10 (14.5) | | 0 | 1 | 9 |
| Total (256) | 36 (14) | | 2 (5) | 2 (5) | 32 (90) |

* $p < 0,05$ (Fisher's exact test)

High prevalence of *edin-C* encoding RhoA-targeting toxin in clinical strains of

Staphylococcus aureus

Patrick Munro¹, René Clément¹, Jean-Philippe Lavigne^{4,5}, Céline Pulcini^{2,3}, Emmanuel Lemichez¹ and Luce Landraud^{1,6*}

Running title: EDIN exotoxins in *S. aureus* infections

1 INSERM, U895, C3M, toxines microbiennes dans la relation hôte pathogènes, Université de Nice-Sophia-Antipolis, UFR Médecine, IFR50, Nice, F-06204, France

2 Université de Nice-Sophia-Antipolis, UFR Médecine, IFR50, Nice, F-06204, France

3 Service d'Infectiologie, Hôpital l'Archet 1, Route Saint Antoine de Ginestière, BP 3079, 06202 Nice Cedex 3, France

4 INSERM, Espri 26, Université Montpellier 1, UFR de Médecine, Nîmes, France

5 Laboratoire de Bactériologie, CHU Caremeau, Nîmes, France

6 Laboratoire de Bactériologie, CHU de Nice, Hôpital l'Archet, Nice, France.

*** Corresponding author:**

Luce Landraud, INSERM, U895, C3M, toxines microbiennes dans la relation hôte pathogènes, Université de Nice-Sophia-Antipolis, UFR Médecine, IFR50, Nice, F-06204, France, and Laboratoire de Bactériologie, CHU de Nice, Hôpital l'Archet, Nice, France.

Telephone: 00 33 4 89 06 42 61

Fax: 00 33 4 89 06 42 60

Mail: landraud.l@chu-nice.fr

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Abstract *Staphylococcus aureus*, a major causative agent of human infection produces a large array of virulence factor including various toxins. Among them, the host RhoA GTPase targeting EDIN toxins are considered as potential virulence factors. Using the polymerase chain reaction, we analyzed the virulence profile of 256 *S. aureus* strains isolated from various clinical sites of infections. We developed specific primers to detect the three isoforms of *edin* encoding genes. We found a prevalence of 14% (36 strains) of *edin* encoding genes among these clinical strains. Strikingly, we found that 90% of all *edin*-bearing *S. aureus* strains carried the type-C allele. Both the *spa* types and the profile of virulence factors of these *edin*-positive strains are highly variable. Notably, we show for the first time that *edin*-C positive strains were more frequently recovered from deep-seated infections than other types of infections. Our present work thus strongly suggests that presence of *edin*-C is a risk factor of *S. aureus* dissemination in tissues and thus represents a predictive marker for a pejorative evolution of staphylococcal infections.

Keywords

Staphylococcus aureus, EDIN, toxin, ADP-ribosyltransferase, virulence factors, Rho GTPases.

Introduction

Staphylococcus aureus is a common bacterium, which is responsible for a unique variety of infections [1]. Development of peyorative forms of staphylococcal infections involves the combined action of numerous bacterial virulence factors, which corrupt host responses [2]. Bacterial virulence factors include specific adhesins, collectively referred as Microbial Surface Components Recognizing endothelial cell Adhesive Matrix Molecules (MSCRAMMs) and a large variety of toxins, such as the exfoliative toxins (ETs), hemolysin, leukocidin, enterotoxins and EDINs (epidermal cell differentiation inhibitors) [3-6].

EDINs belong to the family of *Clostridium botulinum* C3 exoenzyme [6, 7]. They are members of a group of major bacterial virulence factors targeting host Rho GTPases [4, 6-9]. Rho proteins control essential cellular processes such as cell polarity, movement and phagocytosis, as well as cohesion of intercellular junctions [6, 10, 11]. Recent findings suggest that EDINs might favor bacterial dissemination in tissues, by a haematogenous route, through induction of large transcellular tunnels in endothelial cells named macroapertures [12-14]. Indeed, recent data show that *S. aureus* EDIN toxin promotes formation of infection foci in a mouse model of bacteremia [15]. To date, three isoforms of EDIN have been characterized. These comprise the first discovered EDIN isoform (EDIN-A), isolated from the E-1 strain of *S. aureus* [16], as well as EDIN-B [6, 17] and EDIN-C [18]. The chromosomal gene encoding *edin-B* is located within a pathogenicity island frequently associated with the *etD* gene encoding the exfoliative toxin ET-D [17]. EDIN-C is encoded by the pETB plasmid, which also carries genes encoding ET-B and conferring cadmium resistance [18].

A first epidemiological survey, involving staphylococcal strains isolated from patients hospitalized for various infectious diseases demonstrated a higher prevalence of *edin*-encoding genes in this group compared to nasal strains isolated from healthy students [19]. Another study shows that *edin-B* is present in 7% of bacteriemic *S. aureus* strains [17]. However, most other epidemiological data on *edin* are based on surveys focused on exfoliative toxins or PVL rather than EDIN toxin itself. For example, a genetic association between *etD* and *edin-B* was detected in the emerging ST80 clone Panton-Valentine Leukocidin (PVL)-positive and community-acquired (CA) methicillin-resistant *S. aureus* (MRSA) [20]. This clone is spreading throughout France and Tunisia and is most frequently associated with infections of the skin and soft tissues. Also, two-thirds of the strains belonging to the emerging ST123 epidemic European fusidic acid-resistant impetigo clone

(EEFIC) were positive for *etB* and sequence analysis of pETB2 (a close homolog of pETB) in one of these strains suggested that it also bears *edin-C* [21].

In this study, we have developed a PCR-based method, to detect EDIN isoforms specifically. We demonstrate that 90% of all *edin*-bearing *S. aureus* strains carry the type-C allele. We also show that these strains are more significantly associated with deep-seated soft tissue infections than other types of infections (Fisher's exact test, $p=0.03$).

Materials and methods

S. aureus isolates

A total of 256 strains of *S. aureus* were retrospectively collected from patients hospitalized at the university hospital of Nice during 2005. These strains were obtained from various types of clinical samples, comprising blood cultures (28 strains); skin infections including chronic ulcers, burns or wounds (83 strains); urine samples (41 strains); sputum samples (69 strains); and various deep-seated soft tissue infections such as subcutaneous or visceral abscesses, spontaneously or post operative soft tissue infections (35 strains). All isolates were characterized using routine methods according to each manufacturer's recommendations. All were positive for catalase, DNase production and mannitol fermentation in Chapman medium, and confirmed to be *S. aureus* by specific 32rapidStaph (BioMérieux, Marcy-l'Etoile, France).

Antibiotic susceptibility determinations

Antimicrobial susceptibility testing was performed on all isolates obtained during the study using the disk diffusion method [22] on Mueller-Hinton medium (Difco Laboratories, Detroit, MI) according to the recommendations of the French Antibigram Committee [<http://www.sfm.asso.fr/nouv/general.php?pa=2>]. Antibiotics tested were penicillin G, oxacillin, erythromycin, clindamycin and fusidic acid to focus on epidemiologic profiles.

DNA isolation and PCR-based detection of genes

For *edin* detection, total DNA was isolated from bacterial strains grown overnight at 37°C in BHI medium. Bacteria were lysed in 10 mM TrisHCl pH7.8, 100 mM NaCl, 1mM EDTA, 1% Triton X100. After incubation for 10 minutes at 100°C, DNA was collected and frozen. PCR amplification was used to detect the presence of *edin-A*, B and C using the primers described in Table 1. We have determined optimized thermal cycling conditions for *edin-A* (25 cycles of 30s at 94°C, 45s annealing at 58°C and 1 min elongation at 72°C), *edin-B* (25 cycles of 30s at 95°C, 1 min annealing at 50°C and 1 min elongation at 72°C) and *edin-C* (30 cycles of 30s at 94°C, 45s annealing at 54°C and 1 min elongation at 72°C). For the detection of other virulence genes, total DNA was isolated from bacterial strains grown three hours at 37°C in

BHI medium. DNA was subsequently extracted with NucleoSpin Tissue (Macherey-Nagel GmbH, Düren, Germany) according to manufacturer's recommendations. Briefly, bacteria were pelleted by centrifugation at $8,000 \times g$ for 5 min, resuspended in 180 μ l of lysis buffer with 33 μ l of proteinase K (20mg/ml) (Invitrogen Life Technologies, Carlsbad, CA) and 3 μ l of recombinant lysostaphin (3U/ μ l) (Sigma-Aldrich, St Louis, MI), and incubated for 60 min at 37°C. DNA samples were eluted with 100 μ l alkaline elution buffer (BE buffer, NucleoSpin Tissue, Macherey-Nagel). The presence of 30 genes, among the most prevalent virulence-associated genes, was evaluated by PCR as described previously: staphylococcal enterotoxins (*se*) A, B, C, D, E, G, H, I, J, K, and Q, toxic shock syndrome toxin 1 (*tst-1*), exfoliative toxins A, B and D (*etA*, *etB*, *etD*), PVL (*lukS-PV-lukF-PV*), LukDE leukocidin (*lukE*), nine MSCRAMM (*bbp*, *cna*, *ebpS*, *clfA*, *clfB*, *fib*, *fnbA*, *fnbB*, *eno*). The accessory gene regulator (*agr*) allele group was determined by multiplex PCR.

spa sequencing

spa typing was performed as described previously [23], using the *spa* typing website (<http://www.spaserver.ridom.de/>) that is developed by Ridom GmbH and curated by SeqNet.org (<http://www.SeqNet.org/>). Primers are indicated in Table 1.

Statistical analysis

The chi-square or Fisher's exact test for categorical variables was used to compare data as appropriate. A *P* value of less than 0.05 was considered significant.

Results

Detection of *edin* isoforms

S. aureus isolates analyzed in this study were collected at the university hospital of Nice from various infected patients. We designed primers with high sensitivity and specificity in order to detect, by PCR, *edin-A*, *B* and *C* alleles in these strains. This was especially challenging for *edin-C*, which was poorly detected using a previously described pair of primers designed to amplify all *edin* isoforms. This is consistent with the fact that the sequence of *edin-C* has the most substantial sequence variations in regions recognized by these primer sequences (17% and 32% homology with the forward and reverse primers, respectively) (Fig. 1) [19]. As shown in figure 1, the three pairs of primers designed allowed us to amplify specifically a 455 bp DNA fragment for *edin-A* and *B*, and a 320 bp DNA fragment for *edin-C*.

We next analyze the 256 clinical strains of *S. aureus*. We found that 14 % (36) of these strains were positive for one of the *edin* alleles. Among these 36 strains, 90% were positive for *edin-C* and 5% were positive for either *edin-A* or *B*. To confirm the nature of the *edin* isoforms, we performed complete sequence analysis of five *edin-C* encoding genes from randomly selected strains. We also sequenced *edin-A* and *edin-B* encoding genes. These results confirm the specificity of the new primers used and unravel that *edin-C* was more prevalent than other alleles of *edin*.

Detection of genes encoding virulence factors

The 36 strains positive for *edin* genes were next analyzed for the distribution of major MSCRAMMs, various staphylococcal enterotoxins, exfoliative toxins, the toxic shock syndrome toxin 1 gene *tst-1*, as well as leucotoxin family encoding genes. Among the staphylococcal MSCRAMM genes, *eno*, *clfA* and *clfB* were detected in all *edin*-positive strains (Table 2). *bbp*, *fnbA* and *ebpS* were the less frequently encountered MSCRAMMs among these strains. However these adhesion factors had no preferential distribution among *S. aureus* isolated from various types of infections. Among the staphylococcal enterotoxins genes the most frequently encountered were *seg*, *sei* and *sea* (Table 2). In addition, 72% of *edin*-positive strains (26 strains) presented a combination of these three genes. One *edin-C* bearing strain, isolated from a urine sample, had only the *sea* enterotoxin gene. We detected the exfoliative toxin gene *etD* exclusively among the *edin-B* positive strains. Two *edin-A*

positive strains carried the staphylococcal exfoliative gene *etB*. We found that 25 *edin-C* positive *S. aureus* (78%) were negative for *etA*, *etB* and *etD* exfoliative toxin encoding genes. We observed that only 22% of the *edin-C* positive *S. aureus* (7 out of 32) had at least one of the two isoforms (*etA* or *etB*) of the exfoliative toxin gene. Five of these seven strains carried both the *etA* and *etB* genes. Finally, a large number of *edin*-positive *S. aureus* belonged to the *agr* group 1 (50%, 18 strains), and to a lesser extent, to *agr* groups 2, 3 and 4 (17%, 6 strains each) (Table 2).

spa-typing

Determination of the *spa* type of 26 *edin*-positive strains [23] allowed us to exclude the clonal origin of all *edin-C* positive *S. aureus* in our survey. Among them, only six strains could be classified as ST45 (two isolates), ST30 (two isolates), ST59 or ST26. The other 20 strains showed a high variability of their *spa* type (Table 3). For 14 strains, we determined new repeat successions including unidentified 24-bp repeats thus defining new *spa* types. Among them, strains S7926 and S7262, isolated respectively in deep seated soft tissue and sputum sample from unrelated infected patients, presented the same *spa* type t6956 (Table 3). Together these data excluded the clonal origin of all *edin-C* positive *S. aureus* in our survey.

Antibiotic susceptibility profiles

We next investigated whether *edin*-positive strains were associated with specific antibiotic resistance profiles, such as community-acquired methicillin resistant *S. aureus* ST80 and fusidic acid-resistant impetigo clones [20, 21]. In relation with these studies, we determined the minimum inhibitory concentrations (MICs) of *edin*-positive strains for the following antibiotics: penicillin G, methicillin, erythromycin, clindamycin and fusidic acid. Fourteen *edin*-positive strains were susceptible to all tested antimicrobial molecules (41.6 %), except fusidic acid. Indeed, one of 14 isolates tested presented only an increase in fusidic acid MIC, classified as intermediary sensibility. Twenty two isolates were resistant to penicillin G. Among them, 19 were susceptible to all other antimicrobial molecules tested (83%) and two strains also presented erythromycin resistance (8.7%). Only one strain, positive for *edin-C*, showed a methicillin-resistance associated to additional resistance, i.e. erythromycin and clindamycin as well as an increase of MIC to fusidic acid. This strain was negative for *etA*

and *etB*. Finally, *edin*-positive strains did not show any specific resistance profile to classical antibiotics used to cure infections by *S. aureus* (Table 2).

Clinical origin of *edin*-positive *S. aureus*

Having shown no link between a high prevalence of *edin-C* within our *S. aureus* strains and any phenotypic profile or clonal origin, we further analyzed the distribution of *edin*-positive strains with regard to the infectious sites. We noticed that strains positive for *edin-C* were recovered at all sites of infection (Table 4). Strikingly, *edin*-positive *S. aureus* were more significantly associated with deep-seated infections of soft tissues than other types of infections (25.7%, Fischer exact test, $p=0.03$). No significant association was detected between *edin*-positive or *edin*-negative strains and other types of infections (blood, urine, superficial soft tissue and sputum culture).

Discussion

Our study shows that *edin*-positive *S. aureus* strains are found in all types of clinical infections included in this study, with a global prevalence of 14%. Moreover, we show that 90% of *edin*-positive strains are positive for *edin-C*. This is consistent with a previous study performed with specific primers, which also reported a higher prevalence of *edin-C* in clinical strains of *S. aureus* in Japan [24]. Both studies point for a possible underestimation of the prevalence of *edin-C* in pathogenic *S. aureus*. This points for the need of using specific primers to detect each *edin* isoform, especially *edin-C*, given its high prevalence.

Both the analysis of *spa* type and the distribution of various virulence factors among *edin*-positive *S. aureus* show their high genetic variability. Our results on the distribution of MSCRAMMs are consistent with previous findings [25]. Classically, *edin* and exfoliative toxin encoding genes are associated in specific lineages responsible for skin infections [17, 21, 24]. Interestingly, here we show that *edin-C* is not strictly associated with genes encoding exfoliative toxin of serotypes A/B/D (7 strains *edin-C*-positive and *et*-positive, versus 25 strains *edin-C*-positive and *et*-negative). The high prevalence of *edin-C*-positive and *et*-negative strains observed in our study might be explained by the plasticity of the pETB plasmid, which has been previously reported in two different variants [18, 21]. Also, a recent study shows genetic variations in pathogenicity islands encoding EDIN-B [17].

Given that *spa* analysis constitutes a good tool for epidemiological typing of *S. aureus* [26], the use of this method allowed us to exclude the clonal origin of *edin-C* positive strains. The fact that *edin-C* is plasmid born might explain its presence in strains of various genetic backgrounds.

S. aureus positive for *edin* are more frequently associated with deep-seated infections of soft tissues. Infection of endothelial cells, and other cell types, by EDIN-producing *S. aureus* triggers the formation of transcellular tunnels named macroapertures [12, 13]. Moreover, we recently reported that *S. aureus* EDIN toxin promotes formation of infection foci in a mouse model of bacteremia [15]. Together with the present study, this suggests that EDIN might enhance the invasive capacity of *S. aureus*. However, whether or not *edin*-positive *S. aureus* is preferentially associated with a specific type of deep seated infection remains to be further determined.

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Figure legend

Fig. 1 Characterization of the three *edin* alleles. A) PCR amplification of *edin-A*, *edin-B* and *edin-C* from *S. aureus* strains S25*edin-A*(+)[15], S7256*edin-B*(+) and S7475*edin-C*(+) (this study), respectively, using specific oligonucleotides *edinA*, *edinB*, *edinC* (Table 1) and the previously described *edin* oligonucleotides referred as *edinX* [19]. B) Sequence alignments of *edin-A*, *edin-B* and *edin-C* showing sequence homologies and localization of each oligonucleotide (underlined: *edinX*; highlighted: *edinA*, *edinB* and *edinC*).

Table 1: Oligonucleotides primers used in this study

| Gene | Primer sequences | Size (kb) | References |
|--------------|---|--------------|------------|
| <i>edinA</i> | Sense 5'-GGAGATATTAATAAGCTAGATTC-3' | 455 | This study |
| | Antisense 5'-ATTTTCTTTTTATCATTGACAATTCT-3' | | |
| <i>edinB</i> | Sense 5'-GGTGACGTGAACAAATTATCCGA-3' | 455 | This study |
| | Antisense 5'-ATCTTTCTTTTGTTATCAGAAAGTTTA-3' | | |
| <i>edinC</i> | Sense 5'-CGCCATTAAGGTCTAGTCAAGG-3' | 320 | This study |
| | Antisense 5'-TAGGTCTTCCAGCTAATGCAGC-3' | | |
| <i>bbp</i> | Sense 5'-AACTACATCTAGTACTCAACAACAG-3' | 575 | [25] |
| | Antisense 5'-ATGTGCTTGAATAACACCATCATCT-3' | | |
| <i>cna</i> | Sense 5'-GTCAAGCAGTTATTAACACCAGAC-3' | 423 | [25] |
| | Antisense 5'-AATCAGTAATTGCACTTTGTCCACTG-3' | | |
| <i>ebpS</i> | Sense 5'-CATCCAGAACCAATCGAAGAC-3' | 186 | [25] |
| | Antisense 5'-CTTAACAGTTACATCATCATGTTTATCTTTG-3' | | |
| <i>fnbA</i> | Sense 5'-GTGAAGTTTTAGAAGGTGGAAAGATTAG -3' | 643 | [25] |
| | Antisense 5'-GCTCTTGTAAGACCATTTTTCTTCAC-3' | | |
| <i>fnbB</i> | Sense 5'-GTAACAGCTAATGGTTCGAATTGATACT-3' | 524 | [25] |
| | Antisense 5'-CAAGTTCGATAGGAGTACTATGTTC-3' | | |
| <i>fib</i> | Sense 5'-CTACA ACTACAATTGCCGTCAACAG-3' | 404 | [25] |
| | Antisense 5'-GCTCTTGTAAGACCATTTTTCTTCAC-3' | | |
| <i>clfA</i> | Sense 5'-ATTGGCGTGGCTTCAGTGCT-3' | 292 | [25] |
| | Antisense 5'-CGTTTCTTCCGTAGTTGCATTTG-3' | | |
| <i>clfB</i> | Sense 5'-ACATCAGTAATAGTAGGGGGCAAC-3' | 205 | [25] |
| | Antisense 5'-TTCGCACTGTTTGTGTTTGCAC-3' | | |
| <i>eno</i> | Sense 5'- ACGTGCAGCAGCTGACT-3' | 302 | [25] |
| | Antisense 5'- CAACAGCATYCTTCAGTACCTTC-3' | | |
| <i>sea</i> | Sense 5'-GCAGGGAACAGCTTTAGGC-3' | 520 | [27] |
| | Antisense 5'-GTTCTGTAGAAGTATGAAACACG-3' | | |
| <i>seb</i> | Sense 5'-ATGTAATTTTGATATTCGCAGTG-3' | 683 | [27] |
| | Antisense 5'-TGCAGGCATCATATCATAACCA-3' | | |
| <i>sec</i> | Sense 5'-CTTGTATGTATGGAGGAATAACAA-3' | 283 | [27] |

| | | | |
|--------------|--|-----|------------|
| | Antisense 5'-TGCAGGCATCATATCATACCA-3' | | |
| <i>sed</i> | Sense 5'-GTGGTGAAATAGATAGGACTGC-3' | 384 | [27] |
| | Antisense 5'-ATATGAAGGTGCTCTGTGG-3' | | |
| <i>see</i> | Sense 5'-TACCAATTA ACTTGTGGATAGAC-3' | 170 | [27] |
| | Antisense 5'-CTCTTTGCACCTTACCGC-3' | | |
| <i>seg</i> | Sense 5'-CGTCTCCACCTGTTGAAGG-3' | 327 | [27] |
| | Antisense 5'-CCAAGTGATTGTCTATTGTCG-3' | | |
| <i>seh</i> | Sense 5'-CAACTGCTGATTTAGCTCAG-3' | 360 | [27] |
| | Antisense 5'-GTCGAATGAGTAATCTCTAGG-3' | | |
| <i>sei</i> | Sense 5'-CAACTCGAATTTTCAACAGGTAC-3' | 465 | [27] |
| | Antisense 5'-CAGGCAGTCCATCTCCTG-3' | | |
| <i>sej</i> | Sense 5'-CATCAGAACTGTTGTTCCGCTAG-3' | 142 | [27] |
| | Antisense 5'-CTGAATTTTACCATCAAAGGTAC-3' | | |
| <i>sek</i> | Sense 5'-ATGGCGGAGTCACAGCTACT-3' | 197 | [27] |
| | Antisense 5'-TGCCGTTATGTCCATAAATGTT-3' | | |
| <i>seq</i> | Sense 5'-GAACCTGAAAAGCTTCAAGGA-3' | 209 | [27] |
| | Antisense 5'-ATTCGCCAACGTAATTCCAC-3' | | |
| <i>eta</i> | Sense 5'-CTAGTGCATTTGTTATTCAA-3' | 119 | [28] |
| | Antisense 5'-TGCATTGACACCATAGTACT-3' | | |
| <i>etb</i> | Sense 5'-ACGGCTATATACATTCAATT-3' | 200 | [28] |
| | Antisense 5'-TCCATCGATAATATACCTAA-3' | | |
| <i>etd</i> | Sense 5'-ATGACTAAAAATATATATAAAAAGTT-3' | 846 | This study |
| | Antisense 5'-CTAATGAGACTGTAATTCAGC-3' | | |
| <i>lukPV</i> | Sense 5'-ATCATTAGGTAAAATGTCTGGACATGATCCA-3' | 433 | [29] |
| | Antisense 5'-GCATCAASTGTATTGGATAGCAAAAAGC-3' | | |
| <i>lukE</i> | Sense 5'-TGAAAAAGGTTCAAAGTTGATACGAG-3' | 269 | [29] |
| | Antisense 5'-TGTATTTCGATAGCAAAAAGCAGTGCA-3' | | |
| <i>tst-1</i> | Sense 5'-GCTTGCGACA ACTGCTACAG-3' | 559 | [27] |
| | Antisense 5'-TGGATCCGTCATTCATTGTTAA-3' | | |
| <i>agr1</i> | Sense 5'-ATGCACATGG TGCACATGC-3' | 439 | [30] |
| | Antisense 5'-GTCACAAGTACTATAAGCTGCGAT-3' | | |
| <i>agr2</i> | Sense 5'-ATGCACATGG TGCACATGC-3' | 572 | [30] |
| | Antisense 5'-TATTACTAATTGAAAAGTGC CATAGC-3' | | |

| | | | |
|-------------|--|-----|------|
| <i>agr3</i> | Sense 5'-ATGCACATGG TGCACATGC-3' | 321 | [30] |
| | Antisense 5'-GTAATGTAATAGCTTGTATAATAATACCCAG-3' | | |
| <i>agr4</i> | Sense 5'-ATGCACATGG TGCACATGC-3' | 657 | [30] |
| | Antisense 5'-CGATAATGCCGTAATACCCG-3' | | |
| <i>spa</i> | Sense5'-TGTA AACGACGGCCAGTTAAAGACGATCCTTCGGTGAGC-3' | | [23] |
| | Antisense5'-CAGGAAACAGCTATGACCCAGCAGTAGTGCCGTTTGCTT-3' | | |

Table 2: Virulence profile and antibiotic susceptibility of clinical *edin*-positive *S. aureus* strains.

| | blood (n=2) | | superficial soft tissue (n=11) | | urine sample (n=4) | | sputum sample (n=10) | | deep-seated soft tissue (n=9) | | total (n=36) | |
|--------------------------------------|--------------------|-----|--------------------------------|-----|--------------------|-----|----------------------|-----|-------------------------------|-----|--------------|-----|
| | n | % | n | % | n | % | n | % | n | % | n | % |
| Virulence profile | | | | | | | | | | | | |
| MSCRAMMs | | | | | | | | | | | | |
| <i>bbp</i> | 0 | 0 | 3 | 27 | 3 | 75 | 3 | 30 | 6 | 67 | 15 | 42 |
| <i>cna</i> | 1 | 50 | 9 | 82 | 4 | 100 | 10 | 100 | 8 | 89 | 32 | 89 |
| <i>ebpS</i> | 0 | 0 | 5 | 45 | 1 | 25 | 6 | 60 | 6 | 67 | 18 | 50 |
| <i>fnbA</i> | 1 | 50 | 7 | 64 | 1 | 25 | 5 | 50 | 5 | 56 | 19 | 53 |
| <i>fnbB</i> | 1 | 50 | 11 | 100 | 4 | 100 | 7 | 70 | 8 | 89 | 31 | 86 |
| <i>fib</i> | 2 | 100 | 10 | 91 | 4 | 100 | 7 | 70 | 9 | 100 | 32 | 89 |
| <i>clfA</i> | 2 | 100 | 11 | 100 | 4 | 100 | 10 | 100 | 9 | 100 | 36 | 100 |
| <i>clfB</i> | 2 | 100 | 11 | 100 | 4 | 100 | 10 | 100 | 9 | 100 | 36 | 100 |
| <i>eno</i> | 2 | 100 | 11 | 100 | 4 | 100 | 10 | 100 | 9 | 100 | 36 | 100 |
| SEs | | | | | | | | | | | | |
| <i>sea</i> | 1 | 50 | 10 | 91 | 3 | 75 | 9 | 90 | 9 | 100 | 32 | 89 |
| <i>seb</i> | 1 | 50 | 7 | 64 | 2 | 50 | 8 | 80 | 6 | 67 | 24 | 67 |
| <i>sec</i> | 1 | 50 | 7 | 64 | 2 | 50 | 10 | 100 | 6 | 67 | 26 | 72 |
| <i>sed</i> | 0 | 0 | 8 | 73 | 3 | 75 | 6 | 60 | 6 | 67 | 23 | 64 |
| <i>see</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>sek</i> | 1 | 50 | 5 | 45 | 1 | 25 | 7 | 70 | 2 | 22 | 16 | 44 |
| <i>seq</i> | 1 | 50 | 3 | 27 | 1 | 25 | 5 | 50 | 5 | 56 | 15 | 42 |
| <i>seg</i> | 2 | 100 | 10 | 91 | 2 | 50 | 10 | 100 | 9 | 100 | 33 | 92 |
| <i>seh</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 11 | 1 | 3 |
| <i>sei</i> | 2 | 100 | 11 | 100 | 2 | 50 | 9 | 90 | 8 | 89 | 32 | 89 |
| <i>sej</i> | 0 | 0 | 1 | 9 | 0 | 0 | 2 | 20 | 1 | 11 | 4 | 11 |
| <i>tst-1</i> | 2 | 100 | 6 | 55 | 1 | 25 | 7 | 70 | 5 | 56 | 21 | 58 |
| <i>etA</i> | 0 | 0 | 2 | 18 | 0 | 0 | 2 | 20 | 2 | 22 | 6 | 17 |
| <i>etB</i> | 1 ^{&} | 50 | 4 [§] | 36 | 0 | 0 | 2 | 20 | 1 | 11 | 8 | 22 |
| <i>etD</i> | 0 | 0 | 0 | 0 | 1 [*] | 25 | 1 [*] | 10 | 0 | 0 | 2 | 6 |
| <i>lukPV</i> | 0 | 0 | 1 | 9 | 1 | 25 | 0 | 0 | 3 | 33 | 5 | 14 |
| <i>lukE</i> | 2 | 100 | 8 | 73 | 3 | 75 | 5 | 50 | 4 | 44 | 24 | 67 |
| agr group | | | | | | | | | | | | |
| <i>agr1</i> | 1 | 50 | 3 | 27 | 3 | 75 | 6 | 60 | 5 | 56 | 18 | 50 |
| <i>agr2</i> | 1 | 50 | 3 | 27 | 1 | 25 | 1 | 10 | 0 | 0 | 6 | 17 |
| <i>agr3</i> | 0 | 0 | 3 | 27 | 0 | 0 | 0 | 0 | 3 | 33 | 6 | 17 |
| <i>agr4</i> | 0 | 0 | 2 | 18 | 0 | 0 | 3 | 30 | 1 | 11 | 6 | 17 |
| antibiotic resistance profile | | | | | | | | | | | | |
| <i>penicillin G</i> | 1 | 50 | 5 | 45 | 2 | 50 | 8 | 80 | 6 | 67 | 22 | 61 |
| <i>Methicillin</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 11 | 1 | 3 |
| <i>Erythromycin</i> | 0 | 0 | 1 | 9 | 0 | 0 | 1 | 10 | 1 | 11 | 3 | 8 |
| <i>Clindamycin</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 11 | 1 | 3 |
| <i>Fusidic acid</i> | 0 | 0 | 0 | 0 | 1 [⊠] | 25 | 0 | 0 | 1 [⊠] | 11 | 2 | 6 |

* *Edin B* positive strains

§ *Edin A* positive for one strain

& *EdinA* positive strain

⊠ Increase in fusidic acid MIC, classified as intermediary sensibility

Table 3 : *spa*-type of 36 *edin*-positive *Staphylococcus aureus* isolates.

| Strains | EDIN type | <i>Spa</i> -type or repeat sequences | MLST |
|--------------------------------|-----------|--------------------------------------|-------|
| Blood | | | |
| S25 | A | t6403 | - |
| S7232 | C | NT ^s | - |
| Deep-seated soft tissue | | | |
| S7272 | C | t6953 | - |
| S7404 | C | t6649 | - |
| S7408 | C | t6484 | - |
| S7466 | C | t012 | ST-30 |
| S7595 | C | NT | - |
| S7600 | C | t6677 | - |
| S7926 | C | t6956 | - |
| S8028* | C | NT | - |
| S8087 | C | t6481 | - |
| Sputum sample | | | |
| S7225 | C | t2726 | - |
| S7259 | C | t2088 | - |
| S7262 | C | t6956 | - |
| S7413 | C | t6483 | - |
| S7535 | C | t2647 | - |
| S7634 | C | t6954 | - |
| S7649 | B | NT | - |
| S7920 | C | NT | - |
| S7965 | C | NT | - |
| S8100 | C | t015 | ST-45 |
| Superficial soft tissue | | | |
| S7181 | C | t6650 | - |
| S7183 | C | NT | - |
| S7436 | C | t6957 | - |
| S7475 | C | t6480 | - |
| S7526 | C | t137 | - |

| | | | |
|---------------------|---|-------|-------|
| S7539 | C | NT | - |
| S7569 | A | t031 | ST-45 |
| S7599 | C | t6482 | - |
| S7932 | C | NT | - |
| S7938 | C | t620 | - |
| S7977 | C | NT | - |
| Urine sample | | | |
| S7256 | B | t078 | ST-26 |
| S7322 | C | t012 | ST-30 |
| S7906 | C | t645 | - |
| S7946 | C | t216 | ST-59 |

* MRSA strain

\$ Not typable

Table 4: Presence of *edin* genes in 256 *Staphylococcus aureus* strains associated with various clinical syndromes.

| Source of isolates (N) | Number of <i>edin</i> | | <i>edin</i> -isoforms | | |
|-------------------------------------|-----------------------|-----|-----------------------|--------------|----------------|
| | isolates | (%) | <i>edinA</i> | <i>edinB</i> | <i>edinC</i> |
| Blood (28) | 2 (7.1) | | 1 | 0 | 1 |
| Urine (41) | 4 (9.8) | | 0 | 1 | 3 |
| Superficial soft tissue (83) | 11 (13.3) | | 1 | 0 | 10 |
| Deep-seated soft tissue (35) | 9 (25.7)* | | 0 | 0 | 9 |
| Sputum (69) | 10 (14.5) | | 0 | 1 | 9 |
| Total (256) | 36 (14) | | 2 (5) | 2 (5) | 32 (90) |

* $p < 0,05$ (Fisher's exact test)

Figure 1

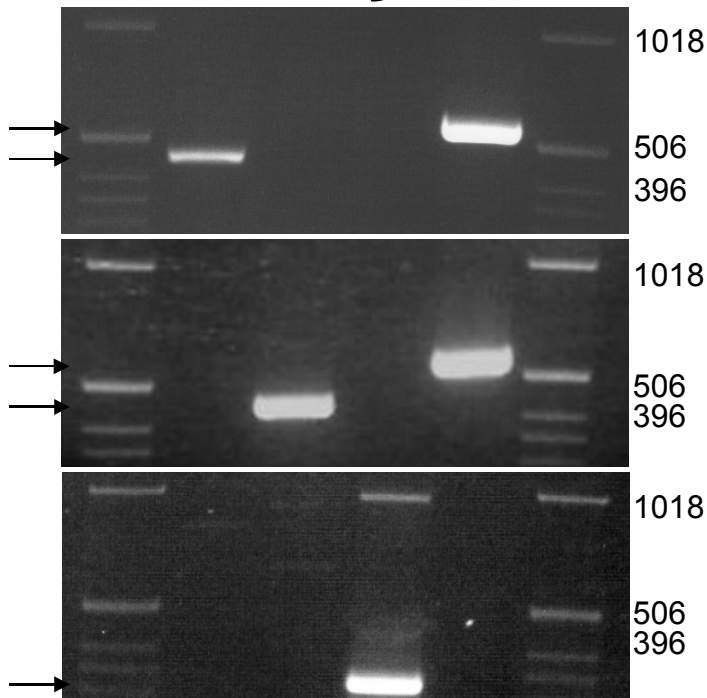
A

Specific primers

(*edinA*) (*edinB*) (*edinC*) (*edinX*)

Strains

MW



B

```

ED IN-A   ATGAAAAACAAATTA CTTTTTAAAAATTTTTTGAGTTTATC TTTAGCATT
ED IN-C   ATGAAAAGAAAATTA TTTTTTAAAAATTTTTTGTTTATC TTTAGTATT
ED IN-B   ATGAAAAGATA---CAATTGTAAAAATTTCTATCAGCATTCTCTGTGATTT
***** * * * * * * * * * * * * * * * * * * * * * * * * * * * *

ED IN-A   AAGCGTTTATTCAATTAATGA----TAAAAATC ATAGAAGTATCTAATACT
ED IN-C   AAGCATTCATTCAATTAATGA----CAGAACTACAGAGTTATCAAACATT
ED IN-B   AAGTATTAGTTTGTAGATAGATACATC TTTTAGCTCTAAATATAATAAAA-ATC
*** ** * * * * * * * * * * * * * * * * * * * * * * * * * * * *

ED IN-A   TCTTTAGCAGCTGATGTTAAAAATTTCACTGATTTAGATGAGGCAACTAA
ED IN-C   GC TTTAGCAGATGATGTTAAAAATTTTACC GA TTTAACTGAAGCAACTAA
ED IN-B   TCAA TAGCTGCCGAGACTAAAAATTTACAGACTTAGTTGAAGCTACTAA
* * * * * * * * * * * * * * * * * * * * * * * * * * * * *

ED IN-A   ATGGGGGAATAAACCTTATAAAAACAAGCTAAGTATAGTTCGGATGATAAAA
ED IN-C   CTGGGGTAATAAGCTTATAAAAACAAGCTAA TTAGCTCAAAGACAAAAG
ED IN-B   ATGGGGAACTCA TTAATAAAGTCAGCCAA GTATTCTTCTAAGATAAGA
***** * * * * * * * * * * * * * * * * * * * * * *

ED IN-A   TAGCTCTATACGAATATACAAAAGATAGTTCTAAGATAAAATGGTCCATTA
ED IN-C   AAGCTATTTATAAATTATACAAAATATAGCTCGCCTATAAATA CGGCATTA
ED IN-B   TGGCTATTTATAA TTTATACAAAATAAGTTTCA CCCCATAAATACTCTCTCTA
*** * * * * * * * * * * * * * * * * * * * * * * * * * * * *

ED IN-A   AGACTCGCAGGTGGAGA TATTTAATAAGTC TTAGATTCAA CAAC TCAA GACAAA
ED IN-C   AGGTCTAGTCAAGGTGATATAAAGTAA TTTTTC TGCAGATTTACAAGAAA
ED IN-B   AGATCAGCAAATGGTGA CGTGAACAAATTTATCGGAAAACATTC AAGAGCA
** * * * * * * * * * * * * * * * * * * * * * * * * * * * *

ED IN-A   AGTAAGAAGATTAGATTTCATCTATTTCTAAATCTACTACTCTCTGAATCTG
ED IN-C   AATACTTC GATTAGATAGACTCATAAGCAAATCAAGTACTAGTGA TTC TG
ED IN-B   GGTTAGACAA TTAGACTCAACGATATCTAAATCTGTAACAC CAGATTCAG
* * * * * * * * * * * * * * * * * * * * * * * * * * * * *

ED IN-A   TATACGTTTATAGACTTTTTAAATTTAGATTATTGACAAAGTATCGTTGGA
ED IN-C   TATATGTTTATAGATTGCTAAATCTGACTATTTATC CAGTGTAAAGGT
ED IN-B   TCTATGTATATAGATTA TTAATTTAGACTACTTATCAAGTATAA CTGGC
* * * * * * * * * * * * * * * * * * * * * * * * * * * * *

ED IN-A   TTTACAAA TGAAATTTATATAAAA TTACAA CAGACCAATTAATGGCCAGTA
ED IN-C   TTTTCTTC TGAAGATTTGGAAATTA TTATACAAAACAGAAAA TTGGTAAAGTA
ED IN-B   TTTACGCGAGAAGATTTACATATGCTACAA CAAC TAACAA TGGTCAATA
*** * * * * * * * * * * * * * * * * * * * * * * * * * * * *

ED IN-A   TGATGAAAATCTAGTTAGAAAAGCTTAAATAACGTTATGAAATGAGAGAATAT
ED IN-C   TAATGAAGAA TTA GTTA AAAA ACTTAATAATA TTA TGAAATG TAAAATTT
ED IN-B   TGATGAAGCGCTTGTGTCAAAACTAAATAATC TTA TGAAATAGTAGAATTT
* * * * * * * * * * * * * * * * * * * * * * * * * * * * *

ED IN-A   ATAGAGAAGACGGATACTCTAGTACACAATTAGTTAGTGGAGCAGCTGTA
ED IN-C   ATAC TGAGTACGGTTAATCTAGCACTCAATTAGTTAAAGGAGCTGCATTA
ED IN-B   ACAGAGAAA TGGCTACTCTAGTACACAAC TAGTTAGTGGTGCAGCAC TA
* * * * * * * * * * * * * * * * * * * * * * * * * * * * *

ED IN-A   GGTGGTAGACCTATTGAATTAAGGTTAGAA TTACC AAAAGGGACTAAAGC
ED IN-C   GC TGGAAAGACCTATTGAATTTGAAA TTACAA TTACAAAAGGTACTAAAAGC
ED IN-B   GCAGGTAGGCCAA TTTGAATTA AAAATTAGAA TTACC TAAAAGGTACTAAAAGC
* * * * * * * * * * * * * * * * * * * * * * * * * * * * *

ED IN-A   TGGCTATCTTAAATCTA AAAAGATTTTAACTGCTTACTATGGTCAAC AAGAAG
ED IN-C   TGCC TATATCGATTCTTAAAATCTTACTGCTATATCCGGACAA C AAGAAG
ED IN-B   AGCA TATATTTGATCTTAAAAGATTAACAGCATACC CAGGTC AAC AAGAAG
** * * * * * * * * * * * * * * * * * * * * * * * * * * * *

ED IN-A   TTTTATTACC TAGAGGCACAGAATACGC TGTGGAAAGTGTAGAATTGTCA
ED IN-C   TATTGTTGCC TAGAGGAACAGACTCAC TATAAATACAGTCAAAC TTTTCA
ED IN-B   TTCTTTTGCC TAGAGGGACAGAATATGC TGTAGGCAGTGT TAAACTTTCT
* * * * * * * * * * * * * * * * * * * * * * * * * * * * *

ED IN-A   AATGATAAAAAGAAAATCATAATAACAGCTATTGTTTTTAAAAAATAG
ED IN-C   GATGATCA TAAAAGAA TTTTAAATC GAAGGTATCGTTTTTCAAAGATA
ED IN-B   GATAACAAAAGAAAGATA TTAATAAC TGCTGTAGTTTTTAAAAAATAA
** * * * * * * * * * * * * * * * * * * * * * * * * * * * *

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Figure 1

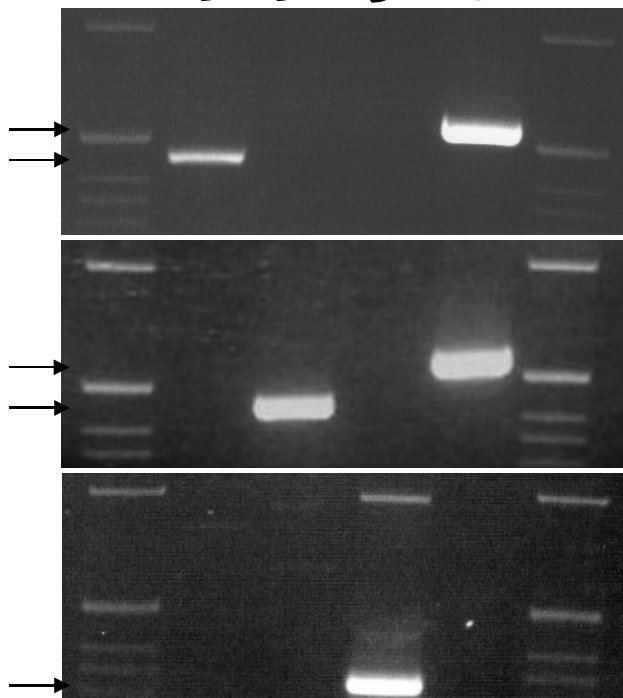
A

Specific primers

(*edinA*) (*edinB*) (*edinC*) (*edinX*)

Strains

MW



B

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ED IN-A    ATGAAAAACAAATTACTTTTAAAATTTTGTGAGTTTATCTTTAGCATT
ED IN-C    ATGAAAAGAAAATTA TTTTAAAATTA TTTTGT TTTATCTTTAGTATT
ED IN-B    ATGAAAAGATA--CAATTGTAAAATTTCTATCAGCATTCCTGTGTATT
***** *  * * * * * * * * * * * * * * * * * * * * * *
ED IN-A    AAGCGTTTATTCAAATTAATGA---TAAAAATCATAGAAGTATCTAATACT
ED IN-C    AAGCATTCATTCAAATTAATGA---CAGAACTACAGAGTTATCAAACATT
ED IN-B    AAGTATTAGTTGTAGATAGATACATCTTTTGTCTCTAAATAAATAA-AATC
***** * * * * * * * * * * * * * * * * * * * * * *
ED IN-A    TCTTTAGCAGCTGATGTAAAAATTTCACTGATTTAGATGAGGCAACTAA
ED IN-C    GCTTTAGCAGATGATGTAAAAATTTTACCAGATTTAACTGAAGCAACTAA
ED IN-B    TCAA TAGCTGCCGAGACTAAAAATTTACAGACTTAGTTGAAGCTACTAA
* * * * * * * * * * * * * * * * * * * * * * * * * * *
ED IN-A    ATGGGGGAATAAACTTA TAAAAACAAGCTAAGTATAGTTCGGATGATAAAA
ED IN-C    CTGGGGTAATAAGCTTA TAAAAACAAGCTAA TTACAGTTCAAAAGACAAAAG
ED IN-B    ATGGGGAACTCA TTAATAAAGTCAGCCAA GTATTCTTCAAGATAAGA
***** * * * * * * * * * * * * * * * * * * * * * *
ED IN-A    TAGCTCTATACGAATATACAAAAGATAGTTCTAAGATAAAATGGTCCATTA
ED IN-C    AAGCTATTATAAATTAATACAAAATATAGCTCGCCTATAAATACGCCATTA
ED IN-B    TGGC TATTTATAATTATACAAAAATAGTTACCCATAAAATCTCTCTTA
***** * * * * * * * * * * * * * * * * * * * * * *
ED IN-A    AGACTCGCAGGTGGAGA TATTAATAAGTCGATTTCAA CAAC TCAAGACAA
ED IN-C    AGGTCTAGTCAAGGTGATATAAGTAA TTTTTC TGCAGATTTACAAGAAA
ED IN-B    AGATCAGCAAATGGTGAACGTCGAACAAATTA TCCGAAAACATTC AAGAGCA
** * * * * * * * * * * * * * * * * * * * * * * *
ED IN-A    AGTAAGAAGATTAGATTCATCTATTTCTAAATCTACTACTCTCTGAATCTG
ED IN-C    AATACTTCGATTAGATAGACTCATAAGCAAATCAAGTACTAGTGA TTC TG
ED IN-B    GGTTAGACAATTAGACTCAACGATATCTAAATCTGTAACACCAAGATTCAG
* * * * * * * * * * * * * * * * * * * * * * * * * * *
ED IN-A    TATACGTTTATAGACTTTTAAATTTAGATTATTGACAAAGTATCGTTGGA
ED IN-C    TATATGTTTTATAGATTGCTAAATCTGACTATTTATCTCAGTGTAAAGGT
ED IN-B    TCTATGTATATAGATTA TTAATTTAGACTACTTATCAAGTATAACTGGC
* * * * * * * * * * * * * * * * * * * * * * * * * * *
ED IN-A    TTTCAAA TGAAGATTTATATAAA TTACAA CAGACCAATAA TGGCCAGTA
ED IN-C    TTTTCTTC TGAAGATTTGGAATTA TTATACAAAACAGAAAA TTGGTAAGTA
ED IN-B    TTTACGCGAGAAGATTTACATATGCTACAA CAAC TAACAA TGGTCAATA
*** * * * * * * * * * * * * * * * * * * * * * * * *
ED IN-A    TGATGAAAATCTAGTTAGAAAAGCTTAATAACGTTATGAA TAGCAGAATAT
ED IN-C    TAATGAAGAA TTA GTTAAAACCTAATAA TATTA TGAATAGTAAAATTT
ED IN-B    TGATGAAGCGCTTGTGTCAAAACTAAATAA TCTTATGAA TAGTAGAATTT
* * * * * * * * * * * * * * * * * * * * * * * * * * *
ED IN-A    ATAGAGAAGACGGATACTCTAGTACACAATTAGTTAGTGGAGCAGCTGTGA
ED IN-C    ATAC TGAGTACGTTTATCTAGCACTCAATTAGTTAAAGGA GCCTGCATTA
ED IN-B    ACAGAGAAAA TGGCTACTCTAGTACACAAC TAGTTAGTGGTGCAGCAC TA
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ED IN-A    GGTGGTAGACCTATTGAATTAAAGTTAGAA TTACC AAAAGGGACTAAAGC
ED IN-C    GC TGGGAAGACCTA TTGAATTGAAA TTACAA TTACAAAAGGTA CTAAGGC
ED IN-B    GCAGGTAGCCCAA TTGAATTAAAA TTAGAA TTACCTAAAAGGTA CTAAGGC
* * * * * * * * * * * * * * * * * * * * * * * * * * *
ED IN-A    TGGCTATCTTAATCTTAAAGATTTAACTGCTTACTATGGTCAACAAAGAAG
ED IN-C    TGCC TATATCGATTCTTAAAA TCTTACTGCATATCCGGACAACAAGAAA
ED IN-B    AGCATATATTTGATCTTAAAGATTAACAGCATACCAGGTCACAAGAAG
** * * * * * * * * * * * * * * * * * * * * * * *
ED IN-A    TTTTATTACC TAGAGCCACAGAATACGC TGTGGAAAGTGTAGAATTGTCA
ED IN-C    TATTGTTGCC TAGAGGAACAGACTACATATAAATACAGTCAAAC TTTCA
ED IN-B    TTC TTTTGCCTAGAGGGACAGAATATGCTGTAGGCAGTGTAAACTTTTCT
* * * * * * * * * * * * * * * * * * * * * * * * * * *
ED IN-A    AATGATAAAAAGAAAAT CATAATAACAGCTATTGTTTTTAAAAAATAG
ED IN-C    GATGATCA TAAAAGAA TTTTAAATC GAAGGTATCGTTTTTCAAAAAGTAA
ED IN-B    GATAACAAAAGAAAGATA TTATAACTGCTGTAGTTTTTAAAAAATAA
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