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Isolation and characterization of *Staphylococcus aureus* strains from a Paso del Norte dairy[1]

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Abstract

The primary purpose of this study was to determine if methicillin-resistant *Staphylococcus aureus* (MRSA) strains could be identified in the milk of dairy cattle in a Paso del Norte region dairy of the United States. Using physiological and PCR-based identification schemes, a total of 40 *Staph. aureus* strains were isolated from 29 raw milk samples of 133 total samples analyzed. Pulsed-field gel electrophoresis after digestion with the *Sma*I enzyme revealed that the 40 confirmed strains were represented by 5 pulsed-field types, which each contained 3 or more strains. Of 7 hospital strains isolated from cows undergoing antibiotic therapy, 3 demonstrated resistance to 3 or more antimicrobial classes and displayed similar pulsed-field gel electrophoresis patterns. A secondary purpose of this study was to elucidate the evolutionary relationships of strains isolated in this study to genomically characterized *Staph. aureus* strains. Therefore, Roche 454 GS (Roche Diagnostics Corp., Dallas, TX) pyrosequencing was used to produce draft genome sequences of an MRSA raw milk isolate (H29) and a methicillin-susceptible *Staph. aureus* (PB32). Analysis using the BLASTn database (http://blast.ncbi.nlm.nih.gov/) demonstrated that the H29 draft genome was highly homologous to the human MRSA strain JH1, yet the β-lactamase plasmid carried by H29 was different from that carried by JH1. Genomic analysis of H29 also clearly explained the multidrug resistance phenotype of this raw milk isolate. Analysis of the PB32 draft genome (using BLASTn) demonstrated that this raw milk isolate was most related to human MRSA strain 04-02981. Although PB32 is not a MRSA, the PB32 draft genome did reveal the presence of a unique staphylococcal cassette *mec* (SCC*mec*) remnant. In addition, the PB32 draft genome revealed the presence of a novel bovine staphylococcal pathogenicity island, SaPIbovPB32. This study demonstrates the presence of clones closely related to human and (or) bovine *Staph. aureus* strains circulating in a dairy herd.

[1]This Whole Genome Shotgun project has been deposited at the DNA Data Bank of Japan/European Bioinformatics Institute/GenBank (DDBJ/EMBL/GenBank) under the accessions PRJNA179361 (strain H29) and PRJNA179544 (strain PB32). The versions described in this paper are the first versions, ANHW01000000.

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Keywords

Staphylococcus aureus; methicillin-resistant; mastitis; genomics

INTRODUCTION

Staphylococcus aureus is responsible for life-threatening human infections acquired in hospitals and within the community (Richards et al., 2001; Moran et al., 2006). The first methicillin-resistant *Staph. aureus* (**MRSA**) infection was described in 1961 (Jevons, 1961) and since then, human infections caused by multi-drug-resistant MRSA have become common (Waness, 2010). The MRSA phenotype results from the acquisition of the staphylococcal cassette chromosome *mec* (**SCC*mec***), which harbors the *mecA* gene that encodes the low-affinity penicillin-binding protein 2a (PBP2a) (Katayama et al., 2000). The structure of numerous SCC*mec* elements reveals that this element is following multiple evolutionary trajectories [for review, see Ito et al. (2001), Li et al. (2011), and Shore et al. (2011)].

Staphylococcus aureus is also a major cause of bovine mastitis worldwide, which results in substantial economic losses for the dairy industry. Methicillin-resistant *Staph. aureus*-causing bovine mastitis was first reported in 1972 (Devriese et al., 1972) and MRSA transmission appears to occur between animals, and from animals to humans (Lee, 2003; Voss et al., 2005; Lozano et al., 2011a). Carriage of MRSA by livestock has been shown to correlate with the MRSA colonization of farmers and farm families, veterinarians, and health-care workers (Voss et al., 2005; Juhász-Kaszanyitzky et al., 2007; Wulf et al., 2008). Sequencing of 7 housekeeping gene amplicons has been used to group MRSA isolates into clonal complexes (**CC**) using “based upon related sequence types,” or BURST, analysis (Enright et al., 2000). These analyses have revealed that certain strains, referred to as livestock-associated MRSA, belong to a small number of CC, with the most prevalent being CC398, and it is known that CC398 strains also cause infections in humans (Voss et al., 2005; Salmenlinna et al., 2010; Lozano et al., 2011b). A recent report suggests that CC398 strains emerged from a human evolved methicillin-susceptible *Staph. aureus* (**MSSA**) strain and that livestock-associated MRSA sublineages arose during antimicrobial selection in livestock (Price et al., 2012).

The aim of this study was primarily to characterize *Staph. aureus* strains isolated from raw milk samples of mastitic and healthy dairy cattle from a Paso del Norte region dairy, in an effort to identify the presence of MRSA. Using the draft genomes of 1 raw milk MRSA isolate and 1 MSSA isolate, we also report the evolutionary relatedness of these strains to previously sequenced *Staph. aureus* strains.

MATERIALS AND METHODS

Isolation and Characterization of Staph. aureus Strains

Samples of raw milk were collected from 33 hospital cows, some receiving antibiotic treatment, and 100 healthy cows in 2 milking parlors (A and B) at a local dairy. The

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isolation of *Staph. aureus* strains was carried out as follows: briefly, 100 μL of PBS-diluted milk samples were spread onto Baird-Parker agar (Becton Dickinson and Co., Sparks, MD) plates and incubated at 37°C for 48 h. Resulting colonies were then subjected to Gram staining, a catalase test with 3% hydrogen peroxide, and a coagulase test using rabbit plasma (Becton Dickinson and Co.). Presumptive *Staph. aureus* isolates were further scrutinized on mannitol salt agar (Acumedia Manufacturers Inc., Lansing, MI) plates. Luria broth (**LB**; Becton Dickinson and Co.) cultures of all presumptive isolates were then grown overnight and glycerol (20% vol/vol) was added to aliquots, which were then stored at −80°C. Working cultures of these isolates were maintained on LB agar plates at 4°C. Total DNA from 20-mL LB overnight cultures of 40 suspected *Staph. aureus* milk isolates, 2 positive-control *Staph. aureus* laboratory strains (COL and ATCC 25923), and a negative control culture (*Staphylococcus epidermidis* ATCC 12228) were isolated as previously described (Riordan et al., 2006). These DNA samples were then scrutinized by a PCR protocol designed to detect the *Staph. aureus*-specific *nucA* nuclease gene or for *mecA* as previously described (Murakami et al., 1991; Brakstad et al., 1992).

Antimicrobial Susceptibility

Antimicrobial susceptibility was determined using the disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI, 2008). Inducible clindamycin resistance was determined as previously described (McDougal et al., 2003). Vancomycin MIC were determined using E-test strips (AB Biodisk North America Inc., Piscataway, NJ) according to the manufacturer's recommendations.

Pulsed-Field Gel Electrophoresis

Overnight cell suspensions were embedded in 1.5% (wt/vol) low-melting point agarose (Sigma-Aldrich, St. Louis, MO) plugs, treated with lysostaphin and proteinase K (Sigma-Aldrich), and were then digested with *Sma*I (New England BioLabs Inc., Ipswich, MA). The plugs were electrophoresed in a 1% (wt/vol) agarose gel for 23 h at 6 V/cm, with an initial switch time of 1 s and final switch time of 30 s at 11.3°C using the CHEF DR III system (Bio-Rad Laboratories Inc., Hercules, CA). Band patterns were compared using the Fingerprinting II Informatix software (Bio-Rad Laboratories Inc.) to determine the clonal relatedness (O'Brien et al., 2005). Strains with 80% or greater pattern similarity were considered clonal (Tenover et al., 1995). Strain NCTC 8325 (NCTC, National Collection of Type Cultures) chromosomal DNA digested with *Sma*I was used as the size marker (Figure 1).

Genome Sequencing

Initially, total DNA was isolated from overnight LB cultures of MRSA strain H29 and MSSA strain PB32. The genomes of both isolates were sequenced (>20× overall coverage) using Roche 454 GS (FLX titanium; Roche Diagnostics Corp., Dallas, TX) pyrosequencing according to the manufacturer's instructions. All reads were assembled into contigs using the Newbler assembler 2.3 (454 Life Sciences Corp., Branford, CT). The draft genomes were then uploaded into the Rapid Annotations using Subsystems Technology (RAST) server for annotation (Aziz et al., 2008). Contigs for both genomes were then compared with

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other genomes in the databases using BLASTn (http://blast.ncbi.nlm.nih.gov/) and EvoPrinter (http://evoprinter.ninds.nih.gov/). Sequence types (**ST**) and allele numbers were determined with the *Staph. aureus* Multi Locus Sequence Typing (**MLST**) website (http://saureus.mlst.net), using sequences obtained from 454 sequencing.

RESULTS AND DISCUSSION

Growth on Baird Parker and mannitol salt agar, Gram stain, and catalase reaction presumptively identified 40 *Staph. aureus* isolates from 29 milk samples. More than one isolated colony was chosen from samples that showed variable reactions on the selective media above (designated by strain number followed by a, b, or c). All 40 of these isolates were coagulase and *nucA* positive. These isolates included 7 from hospital cows (**H** strains), 18 from parlor A cows (**PA** strains), and 15 from parlor B cows (**PB** strains). None of the strains investigated demonstrated resistance to vancomycin by disk diffusion. All H strains except H15c were oxacillin resistant, yet all 7 H strains were *mecA* positive. One H strain (H30) also demonstrated erythromycin resistance, whereas 3 H strains (H24, H26a, H26b) demonstrated resistance to ciprofloxacin and erythromycin. Strain H29 demonstrated resistance to both of these antimicrobials, as well as tetracycline and imipenem, and inducible clindamycin resistance. None of the PA and PB strains were methicillin resistant or *mecA* positive. Strain PA18, however, demonstrated ciprofloxacin and erythromycin resistance, whereas PB26b demonstrated resistance to fusidic acid.

Pulsed-field gel electrophoresis of *Sma*I-digested DNA separated the 40 strains into multiple pulsed-field types (**PFT**). Pulsed-field type A was the most frequently isolated PFT, representing 37.5% (n = 15) of all isolates (Figure 1). Pulsed-field type B contained the largest number of H strains (n = 5) and PA18, all of which demonstrated resistance to 2 or more of the antimicrobials investigated. Pulsed-field type C, D, and E all contained 2 or more strains isolated from a single milk sample, which indicates the clonality of the colonies isolated from these milk samples. In 2 instances where more than a single *Staph. aureus* colony was chosen from 1 milk sample (PA8a and PA8b; PA10a and PA10b), pulsed-field gel electrophoresis analysis revealed that the *Sma*I restriction fragment length polymorphisms were different (Figure 1). Both PA8b and PA10b clustered within the PFT-A strains, whereas PA8a and PA10a clustered close to PFT-A strains (Figure 1). This suggests that more than 1 *Staph. aureus* clone was present in each of these milk samples.

We next chose 2 strains for 454 sequencing. One was H29, which was a representative of the most widely distributed MRSA clone (PFT-B) and the other was PB32, which was an MSSA clone from PFT-C that was most related to the PFT-B MRSA clones identified in this study.

The draft genome of MRSA strain H29, derived from 119 contigs (all >200 bp in length), consists of 2,844,315 bp, which encodes for 2,664 protein-coding genes. The MLST website revealed that strain H29 is ST5 (1,4,1,4,12,1,10), an ST that contains human MRSA isolates and is found within CC5. The presence of ST5 MRSA strains circulating in Paso del Norte area hospitals located near the dairy investigated has previously been reported (O'Brien et al., 2005; Delgado et al., 2007).

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Overall, the H29 draft genome (accession no. PRJ-NA179361) proved to be 100% identical over 97% of the human MRSA strain JH1 genome (accession no. NC009632) by BLASTn comparisons. *Staphylococcus aureus* JH1 is also an ST5 strain that was isolated from a patient with endocarditis and the JH lineage is capable of developing intermediate resistance to vancomycin (Sieradzki et al., 2003; Mwangi et al., 2007). A vancomycin E-test demonstrated that H29 does not demonstrate vancomycin-intermediate resistance.

Contig 1 (27,139 bp) of the H29 draft genome represents a β-lactamase plasmid that had only 42% nucleotide similarity to plasmid pSJH101 (accession no. NC009619) found in JH1, yet was 99.5% identical to plasmid SAP048A (27,268 bp; accession no. GQ900406) found in *Staph. aureus* strain NE 3809, a human clinical blood isolate from Nebraska. Plasmid SAP048A is a representative of pIB485-like plasmids that carry ΔTn552 encoding β-lactamase and a cluster of staphylococcal enterotoxin genes (*sed*, *sej*, and *ser*) that have contributed to outbreaks of *Staph. aureus* foodborne illness [for review, see Gustafson and Wilkinson (2005) and Shearer et al. (2011)]. In addition, contig 34 of H29 demonstrated 97% nucleotide identity to a previously described enterotoxin gene cluster (*egc*) operon that encodes 5 enterotoxin genes (*seo*, *sem*, *sei*, *sen*, and *seg*) and 2 pseudogenes (*ϕent1* and *ϕent2*) located on the *ν*Saβ genomic island (Jarraud et al., 2001; Kuroda et al., 2001). The H29 draft genome also contains the SC-C*mec* ST II, which harbors a erythromycin resistance gene (*erm*; Ito et al., 2001), a Tn916-like transposon containing the tetracycline resistance gene *tetM* (de Vries et al., 2009), and a mutation in the DNA gyrase gene (*gyrA*) that leads to a ^{84}S →^{84}L alteration in GyrA that has previously been tied to ciprofloxacin resistance (Schmitz et al., 1998).

The draft genome of MSSA strain PB32 (accession no. PRJNA179544) derived from 80 contigs consists of 2,808,519 bp, which encodes 2,632 protein-coding genes. The MLST sequences derived from the draft genome confirmed PB32 as an ST124 (3,1,1,37,1,5,3) strain of CC97, which is represented by bovine as well as shared bovine/human strains (Smith et al., 2005). There do not appear to be any readily identifiable plasmid sequences in the PB32 draft genome.

Overall, the PB32 draft genome (accession no. PRJ-NA179544) proved to be 100% identical over 94% of the human MRSA strain 04-02981 genome (accession no. CP001844.2) by BLASTn comparisons. Strain 04-02981 is a plasmidless ST225 (1,4,1,4,12,25,10) multidrug-resistant SCC*mec* ST II strain that was isolated in Köln (Germany) in 2004 (Nübel et al., 2010). Sequence type ST225, which was first described in the 1990s in the United States, represents a single locus variant of ST5 strains and the genome of 04-02981 is also collinear with the JH1 genome (Nübel et al., 2010).

Staphylococcus aureus SCC*mec* insertion is carried out by site-specific recombination between the *attB* site on the chromosome and the *attS* site on SCC*mec* (Ito et al., 1999; Katayama et al., 2000). This recombination event results in copies of the *att* sites at each end of SC-C*mec*, with *attR* within *orfX* and *attL* at the other end of the SCC*mec* (Wang and Archer, 2010). A 27,082-bp segment within contig 15 (215,480 bp) contains several genes previously described in SCC*mec* elements as well as the *attL* attachment site (Figure 2). These genes include the recombinase genes *ccrA* and *ccrB*, *pbp4*, and an arsenic resistance

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operon (*arsA-D*, *arsR*). It has been reported that all ST225 and JH strains carry the same *ccrB* gene (Nübel et al., 2010). The *ccrB* gene of PB32, however, only demonstrated 93% nucleotide identity to the *ccrB* gene of both strains 04-02981 and JH1. The *mecA* gene and the *mecA* controlling genes (*mecI* and *mecRI*) were not present in the draft genome of PB32. In the PB32 draft genome, *orfX* is found on one end of contig 75 (130,864 bp) with *attR* attachment site (Figure 2), suggesting that contig 15 and contig 75 belong together. *Staphylococcus aureus* strains that possess chromosomally located remnants of SCC*mec* determinants, including some with a *pbp4* gene, have been reported (Luong et al., 2002; Corkill et al., 2004; Donnio et al., 2007; Shore et al., 2008; Wong et al., 2010; Lindqvist et al., 2012) and we suspect that sequences within contigs 15 and 75 described here represent a novel SCC*mec* remnant. It is of interest to note that similar to PB32, ST225 MSSA isolates can also carry SCC*mec* remnants (Nübel et al., 2010).

Strain PB32 contains another mobile genetic element, a novel bovine staphylococcal pathogenicity island (**SaPIbov**) located within contig 66 (127,041 bp), which will be referred to as **SaPIbovPB32**. Sequence comparison of SaPIbovPB32 (nucleotide 90,549–104,940) with other SaPIbov sequences demonstrated the greatest nucleotide identity to SaPIbov5 (accession no. HM228919) at 79% identity, followed by 66% identity to SaPIbov4 (accession no. HM211303; Viana et al., 2010). The SaPIbov5 (13,526 bp) was described in a *Staph. aureus* ST398 clone (Viana et al., 2010). *Staphylococcus aureus* strains that contain SaPIbov5 have been isolated from different animals including cows, sheep, and goats (Viana et al., 2010). The SaPIbovPB32 is 14,391 kb in length and is flanked by 21 bp direct repeats that are found in other SaPIbov sequences (Figure 3). Of the 18 open reading frames found in SaPIbov5, 11 were shared with SaPIbovPB32, which included the integrase-, excisionase-, and primase-like genes as well as a von Willebrand factor-binding gene (*vwb*). Strain PB32 also has a second *vwb* variant located on contig 12. Similar to PB32, it has been previously been reported that ruminant-associated *Staph. aureus* strains possess 2 variants of *vwb*, one located on SaPIbov and another located elsewhere on the chromosome (Guinane et al., 2010; Viana et al., 2010). The *vwb* gene contained within the SaPIbov is responsible for coagulation of ruminant-specific plasma and is suggested to play an important role in host adaptation (Guinane et al., 2010; Viana et al., 2010). The genome of strain 04-02981 does not demonstrate the presence of a SaPIBov sequence. Lastly, a gene encoding enterotoxin A (*sea*) that produces a product with 91% amino acid identity to the *Staph. aureus* strain COL SeaA (Gill et al., 2005) was located on contig 22 of the PB32 draft genome.

CONCLUSIONS

Pulsed-field gel electrophoresis analysis revealed clonally related *Staph. aureus* strains circulating within the dairy herd investigated. Strains represented by PFT-A, which included 13 MSSA strains and 2 *mecA*-positive strains (H15a and H15c), were the most widely distributed clones found within this herd. The presence of *mecA*-positive strains within PFT-A, including one that was also oxacillin resistant (H15a), suggests that a PFT-A clone has either gained or lost the *mecA* determinant. Collectively, genomic analysis corroborates the multidrug resistance phenotype displayed by H29 and revealed the presence of both a novel SaPIbov sequence and SCC*mec* remnant in PB32. Genome analysis also demonstrated that H29 carried enterotoxin genes on both plasmid and chromosome locations, whereas PB32

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harbored a chromosomally located *sea* gene. This finding suggests that these strains are capable of causing staphylococcal food poisoning. In addition, although the draft genome of PB32 was most related to MRSA strain 04-02981, based on differences in SCC*mec* and SaPIbov content, ST, and *ccrB* sequences, PB32 and 04-02981 represent clearly diverged clones. Overall, our data demonstrates that H29 and PB32 represent novel clones of human and (or) bovine-related strains of *Staph. aureus*. We speculate that the unique genomic features of these strains possibly reflect the evolutionary trajectory they have taken in the dairy herd examined.

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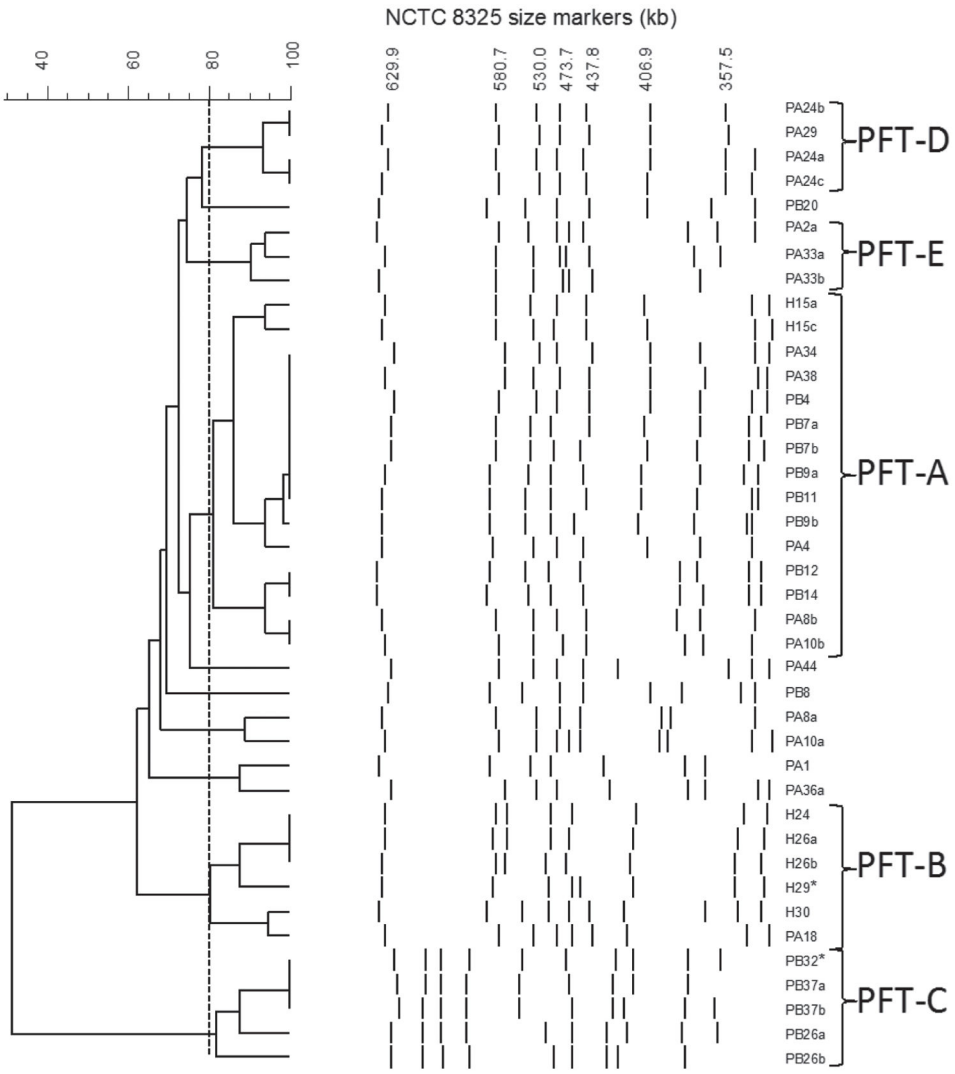

Figure 1.

Pulsed-field gel electrophoresis (PFGE) patterns of *Sma*I-digested chromosomal DNA of *Staphylococcus aureus* strains investigated and dendrogram of percent relatedness derived from the patterns. Asterisks represent the 2 strains that were sequenced. NCTC = National Collection of Type Cultures; PFT = pulsed-field type.

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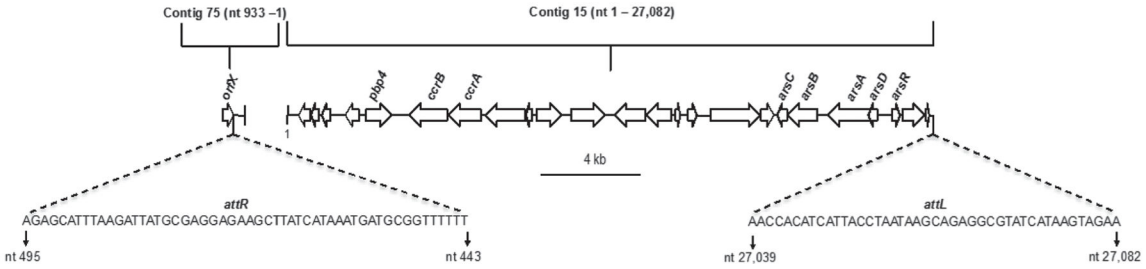

Figure 2.

Cartoon representing open reading frames (ORF) of the staphylococcal cassette chromosome *mec* (SCC*mec*) remnant found within contigs 15 and 75 of *Staphylococcus aureus* strain PB32. Direction of the arrows represents the orientation of the ORF; attachment sites and their respective nucleotide sequences and positions are indicated. nt = nucleotide.

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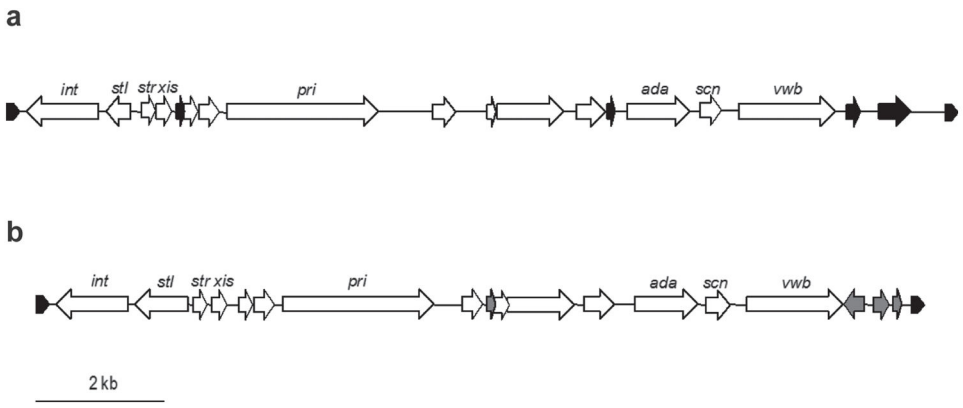

Figure 3.

Comparison of staphylococcal pathogenicity island PB32 (SaPIbovPB32; a) with SaPIbov5 (b). Direction of the arrows represents the orientation of the open reading frames (ORF). Open reading frames shared between SaPIbov5 and SaPIbovPB32 are indicated by white arrows. Black arrows in (a) indicate ORF found only in SaPIbovPB32 but not in the SaPIbov5, whereas the gray arrows in (b) indicate ORF found only in SaPIbov5. The hatched arrows at the ends of both sequences indicate the position of the 21-bp direct repeats (GAGTGGGAATAATTATATATA).