Veterinary Microbiology 200 (2017) 95–100

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Complete sequence of a plasmid from a bovine methicillin-resistant *Staphylococcus aureus* harbouring a novel *ica*-like gene cluster in addition to antimicrobial and heavy metal resistance genes

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ARTICLE INFO

Article history:
Received 17 February 2016
Received in revised form 7 July 2016
Accepted 8 July 2016

Keywords:
MRSA
Multiresistance plasmid
Biofilm formation

ABSTRACT

The multiresistance plasmid pAFS11, obtained from a bovine methicillin-resistant *Staphylococcus aureus* (MRSA) isolate, was completely sequenced and analysed for its structure and organisation. Moreover, the susceptibility to the heavy metals cadmium and copper was determined by broth macrodilution. The 49,189-bp plasmid harboured the apramycin resistance gene *apmA*, two copies of the macrolide/lincosamide/streptogramin B resistance gene *erm*(B) (both located on remnants of a truncated transposon Tn*917*), the kanamycin/neomycin resistance gene *aadD*, the tetracycline resistance gene *tet*(L) and the trimethoprim resistance gene *dfrK*. The latter three genes were part of a 7,284-bp segment which was bracketed by two copies of IS*431*. In addition, the cadmium resistance operon *cadDX* as well as the copper resistance genes *copA* and *mco* were located on the plasmid and mediated a reduced susceptibility to cadmium and copper. Moreover, a complete novel *ica*-like gene cluster of so far unknown genetic origin was detected on this plasmid. The *ica*-like gene cluster comprised four different genes whose products showed 64.4–76.9% homology to the Ica proteins known to be involved in biofilm formation of the *S. aureus* strains Mu50, Mu3 and N315. However, 96.2–99.4% homology was seen to proteins from *S. sciuri* NS1 indicating an *S. sciuri* origin. The finding of five different antibiotic resistance genes co-located on a plasmid with heavy metal resistance genes and an *ica*-like gene cluster is alarming. With the acquisition of this plasmid, antimicrobial multiresistance, heavy metal resistances and potential virulence properties may be co-selected and spread via a single horizontal gene transfer event.

1. Introduction

Staphylococcus aureus including methicillin-resistant *S. aureus* (MRSA) are major pathogens in human and veterinary medicine and show substantial differences regarding their resistance and virulence properties (Kadlec et al., 2009; Feßler et al., 2010, 2011a; Monecke et al., 2011; Fitzgerald, 2012; Rasigade and Vandenesch, 2014). In this regard, the development and spread of resistance and virulence properties via horizontal gene transfer is a major cause of concern. During recent years, several novel resistance genes have been described in *S. aureus* and other staphylococci, which are mainly located on mobile genetic elements (Kadlec et al., 2012b; Schwarz et al., 2015; Wipf et al., 2014, 2015; Wendlandt et al., 2015). Plasmids can undergo interplasmid recombinations resulting in novel plasmids that carry segments of each ancestor plasmid. Moreover, small plasmids are known to be integrated in part or in toto into larger plasmids. Finally, plasmids can also act as vectors for non-conjugative and conjugative transposons (Schwarz et al., 2011, 2015). As such, plasmids play a key role in the horizontal transfer of resistance genes.

The *apmA* gene, which has been described as the first and so far only apramycin resistance gene in staphylococci, was found to be

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http://dx.doi.org/10.1016/j.vetmic.2016.07.010
0378-1135/

located on larger multiresistance plasmids (Feßler et al., 2011b) or on the small 4,809-bp plasmid pKKS49 from porcine MRSA (Kadlec et al., 2012a). The *apmA*-carrying multiresistance plasmids were found in MRSA isolates from bovine mastitis, diseased pigs (Feßler et al., 2011b) and food of poultry origin (Feßler et al., 2011a). The presence of the *apmA* gene on multiresistance plasmids enables a co-selection of different resistance genes under the selective pressure imposed by the use of a single antimicrobial agent. To better understand where the resistance genes found on multiresistance plasmids come from and whether there are other genes that play a role in the co-selection of the resistance genes, a detailed analysis of these multiresistance plasmids is necessary.

The aim of the present study was to sequence one of these *apmA*-carrying multiresistance plasmids completely and analyse it for its structure and organisation.

2. Materials and methods

2.1. Bacterial isolates and heavy metal susceptibility testing

For this study, the multiresistance plasmid pAFS11, obtained from the MRSA ST398 isolate Rd11 from a case of bovine mastitis, obtained in 2009 in Germany, has been chosen. Of this plasmid, an 11,312 bp EcoRI fragment, that carried the *apmA* gene, had been already sequenced (Feßler et al., 2011b). In this previous study, the plasmid has also been transformed into *S. aureus* RN4220 and the transformant shown to mediate resistance to tetracycline, erythromycin, clindamycin, apramycin, trimethoprim, kanamycin and neomycin. Moreover, the plasmid is known to carry the genes *tet*(L), *erm*(B), *apmA*, *dfrK* and *aadD* (Feßler et al., 2011b). In the present study, the bovine MRSA isolate harbouring plasmid pAFS11 and the transformant *S. aureus* RN4220 harbouring pAFS11 were tested for their ability to confer elevated MICs to cadmium sulphate ($CdSO_4$) and copper sulphate ($CoSO_4$). Heavy metal susceptibility testing was performed by broth macrodilution and 2-fold dilution series comprising the concentration ranges of 0.001–2 mmol/L for $CdSO_4$ and 0.125–128 mmol/L for $CuSO_4$ were used. In addition, the concentration 6 mmol/L for $CuSO_4$ was tested. Results were read after 20 h incubation at $35 \pm 2\,^{\circ}C$ in a rotary shaker at 60 rpm. The recipient strain *S. aureus* RN4220 was included in these experiments for comparison.

2.2. Sequence analysis of plasmid pAFS11

Plasmid DNA was extracted using the QIAGEN Plasmid Midi Kit (Qiagen, Hilden, Germany), the quantity and quality of extracted plasmid were determined using a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA) and agarose gel electrophoresis. Samples of the prepared plasmid DNA were sent to the Berry Genomic Company (Beijing, China) for library construction and sequencing. Approximately 3 μg of the plasmid DNA was used for shotgun library construction. Subsequently, whole plasmid sequencing was performed with the HiSeq 2500 sequencing platform, which produced 150-bp paired-end reads. A draft assembly of the sequences was conducted using the CLC Genomics Workbench 5.0 (CLC Bio, Aarhus, Denmark). The closure of gaps between the different contigs was performed by PCR assays followed by sequencing of the respective amplicons. The sequence of the plasmid was analysed using the ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) tools from the National Center for Biotechnology Information (NCBI) as well as the Artemis program from the Welcome Trust Sanger Institute (Rutherford et al., 2000). The plasmid sequence has been submitted to the EBI database and assigned accession no. FN806789.

2.3. Biofilm formation assays

Semiquantitative measurements of biofilm formation were performed in 96-well flat bottom polystyrene cell culture microtitre plates (Greiner Bio-One, Frickenhausen, Germany), based on the method of Christensen et al. (1985), with the following modifications. Bacterial suspensions with an initial OD_{600} of 0.05 were prepared in trypticase soy broth (TSB) medium or TSB supplemented with either 2.0% glucose, 3.0% NaCl, 0.5% ethanol, 0.06% urea or subinhibitory concentrations of quinupristin/dalfopristin (30, 60 and 90 ng/μl). After grown at 37 °C for 18 h, the plates were washed three times with 1× phosphate-buffered saline (PBS, pH 7.2) to remove unattached bacteria. Adherent cells were dried at 65 °C for 1 h, prior to staining for 2 min with a crystal violet solution. After removing the staining solution and removal of excess staining by washing with distilled water, absorbance at 492 nm was determined. Each strain was tested at least three times in technical quadruples, and average results with standard deviations are presented. The well-characterized biofilm-producing strain *S. epidermidis* RP62A (ATCC® 35984) and the biofilm-negative strains *S. epidermidis* ATCC® 12228 and *S. carnosus* TM300 were used as controls.

3. Results and discussion

The plasmid pAFS11 had a total size of 49,189 bp. It harboured 40 reading frames for proteins of >100 amino acids (aa), three of which appeared to be truncated and one to be disrupted (Fig. 1). The plasmid showed a mosaic structure with segments known to originate from other previously known plasmids and transposons. Plasmid pAFS11 harboured the apramycin resistance gene *apmA*, two copies of the macrolide/lincosamide/streptogramin B resistance gene *erm*(B), the kanamycin/neomycin resistance gene *aadD*, the tetracycline resistance gene *tet*(L) and the trimethoprim resistance gene *dfrK* (Fig. 1). The results of the antimicrobial susceptibility testing of the pAFS11-carrying transformant in *S. aureus* RN4220 compared to the empty recipient strain confirmed the functionality of the antimicrobial resistance genes (Feßler et al., 2011b). Moreover, pAFS11 carried a cadmium and a copper resistance operon and a novel type of an *ica* gene cluster.

3.1. The apmA and erm(B) gene region

The *apmA* gene was located upstream of one *erm*(B) copy. A comparison with the *apmA* gene of the small apramycin resistance plasmid pKKS49 from a porcine MRSA isolate revealed 94.4% nucleotide sequence identity in a segment which comprised the *apmA* gene as well as 72 bp upstream and 64 bp downstream of this gene (Kadlec et al., 2012a). When comparing the aa sequences deduced from the *apmA* genes of the two plasmids, a 12 aa difference was observed (Kadlec et al., 2012a). Based on the observed MICs, these aa alterations did not have an impact on the functionality of the ApmA protein. The differences might be explained by naturally occurring mutations or a simultaneous development of the two genes. Since there is homology in the flanking regions of the two *apmA* genes, mutations are more likely than the parallel development of two different *apmA* gene variants.

The two *erm*(B) genes were located in the same orientation, but 5,790 bp apart from each other. In both cases, the *erm*(B) gene was part of a truncated transposon Tn*917*. The first Tn*917* relic, located immediately upstream of the *apmA* gene, was 1,401 bp in size and comprised the entire left-hand terminus of Tn*917*, including the 73-bp terminal repeat, the *erm*(B) leader peptide, and the complete *erm*(B) gene. Similarity to Tn*917* stopped 38 bp after the *erm*(B) translational stop codon. The second Tn*917* relic was 1,395 bp in size and had virtually the same content. However, similarity to