Identification of a novel plasmid-associated spectinomycin adenyltransferase gene spd in methicillin-resistant Staphylococcus aureus ST398 isolated from animal and human sources

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Objectives: Previously described methicillin-resistant Staphylococcus aureus (MRSA) ST398 strains revealed a high frequency of phenotypic resistance to spectinomycin. However, only a few were found to carry the spc resistance determinant. The aim of this study was to identify the genetic mechanism of spectinomycin resistance among spc-negative MRSA ST398 strains.

Methods: Nine spectinomycin-resistant, but spc-negative, MRSA ST398 strains were analysed. The strains were screened for carriage of the spw gene and tested for the presence of transferrable spectinomycin resistance. Plasmid DNA was isolated from all strains and used in transformation assays. The plasmid identified as mediating resistance to spectinomycin was fully sequenced. The function of the novel spectinomycin resistance gene was confirmed by restriction digest inactivation and its distribution was determined using a PCR assay.

Results: A single MRSA ST398 strain was spw positive. The remaining strains carried a plasmid that mediated resistance to spectinomycin. Sequence analysis of a single plasmid, termed pDJ91S, revealed that it was 3928 bp in size and contained three open reading frames: a novel spectinomycin resistance gene, designated spd, as well as a repN gene and a rec gene. The XmnI digest inactivation of the spd gene resulted in a 4-fold decrease in spectinomycin MIC. The spd gene was detected in seven other spectinomycin-resistant MRSA ST398 strains that carried a plasmid comparable in size to pDJ91S.

Conclusions: A novel gene, designated spd, that confers resistance to spectinomycin has been identified on a small plasmid in MRSA ST398.

Keywords: MRSA, antimicrobial resistance, aminoglycosides

Introduction

Methicillin-resistant Staphylococcus aureus (MRSA) ST398 emerged as a livestock-associated MRSA lineage that was first identified among pig herds, followed by its detection in other livestock animals such as cattle, horses and chickens.1–3 MRSA ST398 isolates frequently display a diverse antimicrobial resistance genotype, carrying determinants that confer resistance to a heterogeneous group of antimicrobial compounds.4 Spectinomycin resistance in MRSA ST398 isolates has previously been described and was associated with the carriage of the spc gene (also known as ant9-Ia or aad9).5

In our previous work, we analysed antimicrobial resistance genotypes and phenotypes among a panel of MRSA ST398 strains and reported that >50% were resistant to spectinomycin.6 However, only 2 of 11 spectinomycin-resistant strains carried the spc resistance determinant. Recently, a novel spectinomycin resistance gene, designated spd, has been identified.7 The work presented here describes further analysis of the spc-negative spectinomycin-resistant MRSA ST398 strains, which involved screening for spw carriage and identification of a novel plasmid-associated spectinomycin adenyltransferase gene.

Materials and methods

Bacterial strains

The analysed MRSA ST398 panel (n=9) consisted of previously described strains 91, 92, 93, 95, 99, 102, 107, 109 and 110,8 which had comparatively elevated spectinomycin MICs (>1024 mg/L), but were spc negative. The strains were isolated in Belgium, from various sources including chicken, horse, rat, cattle, pig and human. The strains had previously...
been genotyped and each represented a unique PFGE fingerprint (Figure S1, available as Supplementary data at JAC Online).

**Analysis of spw carriage**

Isolates were screened for carriage of the spw gene using a previously developed PCR assay, as described by Wendlandt et al.1

**Plasmid DNA isolation, transformation, cloning and sequence analysis**

Plasmid DNA was isolated using a commercial kit QIAprep (Qiagen) following the manufacturer’s instructions with minor modifications. An overnight culture in tryptic soy broth was pelleted by centrifugation and washed with 0.1 M PBS. The QIAprep protocol was then followed, beginning with step 1: resuspension in Buffer P1. When isolating plasmid DNA from S. aureus, Buffer P1 was supplemented with 10 mg/mL lysozyme and 50 μg/mL lysostaphin, and the cell suspension was incubated for 1 h at 37°C.

Plasmid DNA was transformed into electrocompetent S. aureus RN4220 by electroporation, as described by McNamara.8 Transformants were selected on tryptic soy agar plates containing 200 mg/L spectinomycin and screened for antimicrobial susceptibility and plasmid carriage.

Plasmid DNA from a selected transformant was digested with XbaI, which generated two fragments of ~3.8 kb and ~0.2 kb in size. The fragments were ligated into the pUC19 vector. The recombinant vector was transformed into chemically competent Escherichia coli TOP10F as described by Sambrook and Russell.9 The transformants were selected on tryptic soy agar plates containing 200 mg/L spectinomycin and screened for antimicrobial susceptibility and plasmid carriage.

Plasmid DNA was isolated from the transformant 91 SPE, with the remaining MRSA ST398 strains. All transformants demonstrated a spectinomycin MIC of >1024 mg/L and were susceptible to all other agents tested (Table S1, available as Supplementary data at JAC Online). All carried a plasmid of ~4 kb.

A single transformant, 91 SPE, was selected for plasmid sequence analysis. The plasmid was designated pDJ91S.

**Transformation assays**

Spectinomycin-resistant transformants were obtained with plasmid DNA derived from all spw-negative (n=8) MRSA ST398 strains. All transformants demonstrated a spectinomycin MIC of >1024 mg/L and were susceptible to all other agents tested (Table S1, available as Supplementary data at JAC Online). All carried a plasmid of ~4 kb.

**Detection of spw**

Carriage of the spw gene was analysed using a PCR assay (spw_F: 5’-CATGAAATGAAAATGGCTTATCC-3’ and spw_R: 5’-CCTGTTTCCATAAGTTTAGATC-3’, annealing temperature 60°C), which amplified an spw internal fragment of 317 bp.

**Susceptibility testing**

The antimicrobial susceptibility of the transformants was determined using the broth microdilution method, as described previously.6

**Results**

**Analysis of spw carriage**

A single MRSA ST398 strain was spw positive, with the remaining eight strains carrying an unknown mechanism of spectinomycin resistance.

**Nucleotide sequence analysis of pDJ91S**

The pDJ91S plasmid was 3928 bp in length and contained three open reading frames (Figure 1). A putative repN gene encoded a 322 amino acid protein that shared 86% sequence identity with the replication initiation protein from the 3.8 kb chloramphenicol resistance plasmid pSBK203 from S. aureus (GenBank accession number AAA26474). Downstream of the repN sequence was located a novel spectinomycin adenyltransferase gene, which was designated spd. The gene encoded a novel 257 amino acid protein, named Spd, which shared 47% identity with the

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spectinomycin adenyltransferase Aad9 from Enterococcus faecalis (GenBank accession number AAA16527). The Spd protein also shared 45% sequence identity with the recently described Spw protein from S. aureus (GenBank accession number AFU35063). Downstream of the spd gene was located a rec gene, which encoded a putative 405 amino acid recombination protein that shared 73% identity with the recombination determinant from the 4.1 kb chloramphenicol resistance plasmid pKH7 from S. aureus (GenBank accession number NP_052168).

The Spd protein sequence was further analysed by constructing an amino acid sequence homology tree with previously described spectinomycin adenyltransferases (Figure 2). The novel Spd protein co-clustered only with the Aad9 enzyme from E. faecalis at a 65% identity. The homologous region of the Spd enzyme accounted for around 80% of the protein sequence, which consisted of the conserved nucleotidyltransferase NT_KNTase_like and DUF4111 domains.

As shown in Figure 1, the entire structure of pDJ91S resembled two other S. aureus plasmids: pS8K203 and pKH7. The structural similarity was accompanied by a level of nucleotide sequence homology involving the rep and rec elements as well as parts of non-coding regions.

**Confirmation of spd as a resistance determinant**

Spectinomycin MIC analysis of plasmid-free E. coli TOP10F and the transformant carrying the pUC19 vector with the 3.8 kb XbaI fragment of pDJ91S showed an increase in spectinomycin MIC from 8 to 128 mg/L. The E. coli TOP10F transformant carrying the pUC19 vector with XmnI-digested pDJ91S demonstrated a spectinomycin MIC of 8 mg/L, which was a 4-fold drop by comparison with the transformant carrying the 3.8 kb XbaI fragment of pDJ91S.

**Distribution of spd among MRSA ST398 isolates**

The spd gene was subsequently detected by PCR in the remaining seven spectinomycin-resistant MRSA ST398 strains that carried a 4 kb plasmid.

**Discussion**

Spectinomycin is authorized by the European Medicines Agency for the treatment of enteric and respiratory infections in cattle, sheep, pigs and poultry. Resistance to this compound in Gram-positive species is often mediated by the Aad(9) spectinomycin adenyltransferases. As demonstrated in Figure 2, a number of distinct Aad9 enzymes with an overall protein sequence homology below 60% have been previously described. The Spd enzyme identified in this study also demonstrated a low protein sequence similarity with the other spectinomycin adenyltransferases, and was most closely related to the Aad9 from E. faecalis. However, the low percentage of sequence homology might suggest that the common ancestor of the novel Spd protein and the Aad9 reported from E. faecalis is quite distant.

The identification of the spd gene reported here follows other studies that have described novel or unknown mechanisms of spectinomycin resistance in MRSA isolates. Wendlandt et al. recently reported the identification of the novel spw spectinomycin resistance gene, located within a multiresistance gene cluster of the pV7037 plasmid, derived from a porcine MRSA. A recent report on the prevalence of MRSA in slaughter pigs in Switzerland revealed a 70% frequency of spectinomycin resistance among MRSA isolates, with three isolates assigned to ST398 carrying an unidentified mechanism of resistance (spc negative). The emerging diversity of spectinomycin resistance genes might suggest a significant antimicrobial selective pressure on the MRSA population associated with animals.

The high prevalence of the novel spd resistance determinant among the described MRSA ST398 strains, particularly in comparison with the distribution of the spc gene, might suggest high rates of horizontal transfer of the associated plasmid. The spd gene might thus be particularly prone to dissemination among staphylococci. This notion can be further supported by the demonstrated structural similarity between pDJ91S and other S. aureus-associated plasmids. Small plasmids carrying a single resistance determinant are common mobile genetic elements among S. aureus isolates and have played an important role in the dissemination of antimicrobial resistance among staphylococci.

To conclude, the reported findings provide further evidence that the MRSA ST398 lineage constitutes a significant reservoir of novel or previously unreported resistance genes. Surveillance for the presence of the spd gene would need to be conducted on a larger panel of animal-associated staphylococci to determine its distribution and wider significance. Retrospective analyses of spectinomycin-resistant, but spc- and spw-negative, S. aureus isolates could reveal whether the spd gene has emerged only recently or has been disseminating unrecognized over a period of time.

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**Transparency declarations**

None to declare.

**Supplementary data**

Figure S1 and Table S1 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

**References**

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