Chromosomal integration of the novel plasmid pUR3912 from methicillin-susceptible *Staphylococcus aureus* ST398 of human origin

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Abstract

The novel *erm*(T)-*cadDX*-carrying plasmid pUR3912 has recently been described in the methicillin-susceptible *Staphylococcus aureus* ST398-t571 strain C3912 from a healthy human in Spain. Structural analysis revealed that pUR3912 belongs to the pC194 replicon family, replicates via a rolling circle mechanism and harbours putative double-strand (*dso*) and single-strand (*sso*) origins of replication. Besides its plasmid location, a copy of pUR3912 was also found in the chromosomal DNA of strain C3912. Two IS*431* copies, which flank the plasmid, most probably mediated its chromosomal integration. Its ability to not only exist extrachromosomally, but also to integrate into the chromosomal DNA ensures persistence and dissemination of pUR3912.

Keywords: Co-integrate, *erm*(T)-*cadDX*, IS*431*, pC194, rolling-circle replication
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Recently, plasmid pUR3912, which carries the macrolide-lincosamide-streptogramin B resistance gene *erm*(T), the cadmium resistance operon *cadDX* and two IS*431* copies, has been identified and completely sequenced (see Supplementary material, Fig. S1) [1]. This plasmid originated from the methicillin-susceptible *Staphylococcus aureus* (MSSA) strain C3912, which has the sequence-type (ST) 398 and *spa*-type t571 and was obtained from a healthy human [1]. The genetic content and part of the gene organization of pUR3912 resembled a chromosomal segment of MSSA ST398-t571 strain ST398NM01, the whole genome sequence of which has been recently published [2]. In the present study, we investigated plasmid pUR3912 for its ability to integrate into the chromosomal DNA of strain C3912 and analysed its reorganized chromosomal structure.

Structural analysis of pUR3912 identified this plasmid as a rolling circle replication plasmid of the pC194 family [3,4]. Many rolling circle replication plasmids carry antimicrobial and heavy metal resistance genes and/or insertion sequences and transposons [3]. Upstream of the *rep* gene in pUR3912, a putative *dso* with homology to that of pC194 was observed (Fig. S1). An additional sequence involved in initiation of the synthesis of the lagging-strand, the single-strand origin of replication (*sso*) [3–5] was detected 80 bp upstream of the cadmium resistance gene *cadD* in pUR3912 (Fig. S1). Due to the great similarity between pUR3912 and the corresponding chromosomal segment of strain ST398NM01, strains C3912, *S. aureus* RN4220 and an *S. aureus* RN4220 protoplast transformant carrying pUR3912 (RN4220/pUR3912) [1] were subjected to Southern blot analysis to evaluate the possible location of an additional pUR3912 copy in the chromosomal DNA. After digestion of genomic DNA with I-CeuI (New England Biolabs, Ipswich, MA, USA), pulsed-field gel electrophoresis was conducted [6]. I-CeuI digests were transferred to a nylon-membrane and hybridization and detection with specific probes comprising the complete pUR3912, and with a 16S rDNA probe, were conducted according to the manufacturer's recommendations (Roche, Basel, Switzerland). Southern blot analysis confirmed the presence of pUR3912 also in the chromosomal DNA of strain C3912, but not in RN4220 and RN4220/pUR3912.

Assuming that pUR3912 is located in the chromosomal DNA of C3912 at the same integration site as in strain ST398NM01, i.e. within the *tnp* transposase gene of an IS*712G*-like insertion sequence (IS) [2], long-range PCR with primers tnp-fw (5′-CCAAATTATGCTGAGCTTGGTC-3′) and tnp-rv (5′-ACCGGGATTAGTTTCTACGC-3′), located in the upstream and downstream regions of this potential integration site, and LA *Taq* DNA polymerase (TaKaRa, Shiga, Japan) were employed. Two amplicons of 6551 and 312 bp were obtained (Fig. 1). This observation indicated that (i) strain C3912 harbours more than one copy of this IS element, and (ii) the integration of a 6239-bp fragment has occurred within at least one of the present copies of this IS element. Complete sequence analysis of the 6551 bp chromosomal

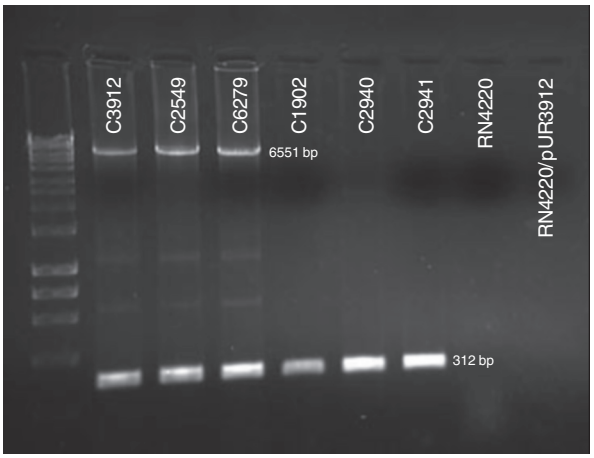

FIG. 1. Gel electrophoresis profile (running conditions, 1 h at 96 V) of the PCR products obtained from the three pUR3912-carrying ST398 methicillin-susceptible *Staphylococcus aureus* (MSSA) strains C3912, C2549 and C2679, three ST398 methicillin-resistant *S. aureus* (MRSA) strains (C1902, C2940 and C2941), the pUR3912-recipient *S. aureus* RN4220 strain and its protoplast transformant (RN4220/pUR3912) using primers tnp-fw and tnp-rv. M, HyperLadder™ I (Bioline, London, UK); band sizes: 0.2, 0.4, 0.6, 0.8, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8 and 10 kb. The faint bands detected in C3912, C2549 and C2679 are unspecific PCR products.

fragment of C3912 after amplicon purification using a QIAquick Gel extraction kit (Qiagen, Hilden, Germany) revealed two identical IS*431* copies located in the same orientation that flank an *erm*(T)-*cadDX* segment closely related to that of pUR3912 (Fig. 2).

It is known that IS*431* and similar ISs, such as IS*257*, are able not only to integrate small plasmids into larger plasmids, but also small plasmids into the chromosomal DNA [7,8]. This process requires the presence of one copy of the IS element on the small plasmid and another copy in the structure in which the small plasmid will be integrated, followed by a recombination between the two IS elements. To explain the findings obtained from strain C3912, the following model has been generated based on the sequence of plasmid pUR3912 [1] and the knowledge of IS*431*/IS*257*-mediated co-integrate formation [7] (Fig. 2). In a first step, either the IS431_L of pUR3912, which based on its 8-bp direct repeats upstream and downstream of its integration site—seems to have independently integrated into a pUR3912 precursor, or a free IS*431* copy has integrated into the *tnp* gene of a chromosomal IS*712G*-like element and thereby produced the 8-bp direct repeats (5′-CCTTTTTC-3′) (Fig. 2). In a second step, this chromosomal IS*431*, has undergone homologous recombination with the IS431_R of a pUR3912-like plasmid resulting in the integration of pUR3912 into the chromosomal DNA flanked by both recombined IS*431* copies. This chromosomal structure is highly similar to the corresponding chromosomal region of strain ST398NM01. However, a 700-bp segment (with identity to plasmid pSSP1 of *Staphylococcus saprophyticus* ATCC15305) was present in both, pUR3912 and the chromosomal co-integrate in strain C3912, but was absent in ST398NM01 [1]. Two very similar sequences, 5′-TAAAAATT-3′ and 5′-TAAAAAATT-3′, that flank this 0.7-kb segment were detected and may have played a role in its acquisition. The only difference between the free pUR3912 and its co-integrate was the presence of a 64-bp duplication within the *dso* of the integrated pUR3912.

Two unrelated *erm*(T)-positive MSSA ST398 strains of human origin from our collection (strains C2549 with *spa* type t571 and C2679 with t1451) [9], which, based on the expected size and gene content (*erm*(T) and *cadDX*), carried plasmid pUR3912 [1], and harboured an integrated copy of pUR3912 within the same genetic context (Fig. 1). On the other hand, this segment was not present in three additional recently described multi-resistant *erm*(T)-*cadDX*-carrying plasmids from animal-associated methicillin-resistant *S. aureus* (MRSA) ST398 of porcine and human origin (Fig. 1) [10]. Chromosomal integration within this IS element in unrelated ST398-t571 and ST398-t1451 MSSA strains points towards a preferable integration site. Moreover, a recent study that analysed a collection of different *S. aureus* ST398 isolates revealed that 95% of human-associated MSSA ST398 carried the *cadDX* operon, in contrast to only 25% of pig-associated MRSA ST398 isolates investigated [11]. Unfortunately, the location of *cadDX* in these isolates was not investigated. The same study reported ϕ3 bacteriophage, which carries the human-specific immune evasion cluster genes *scn* and *chp*, as the best genetic marker of the human-specific ST398 clade. PCR analysis of the immune evasion cluster genes [12] revealed the presence of both *scn* and *chp* in the three isolates. As plasmid pUR3912 is neither conjugative nor does it carry mobilization genes, co-resident plasmids that carry the respective genes for horizontal transfer are needed. Such plasmids have so far not yet been detected in the strains that carry pUR3912-like plasmids. This may point towards limited options for horizontal transfer of pUR3912 to other MRSA/MSSA lineages, including the animal-associated ones, under the current conditions. Further epidemiological studies are warranted to elucidate whether pUR3912 and/or its chromosomal co-integrate are preferentially present in the animal-independent ST398 MSSA clade.

The ability of pUR3912 to integrate into the chromosomal DNA of different MSSA ST398 strains via ISs, as well as its co-localization as an extrachromosomal element within the same cell, ensure its persistence and dissemination. The

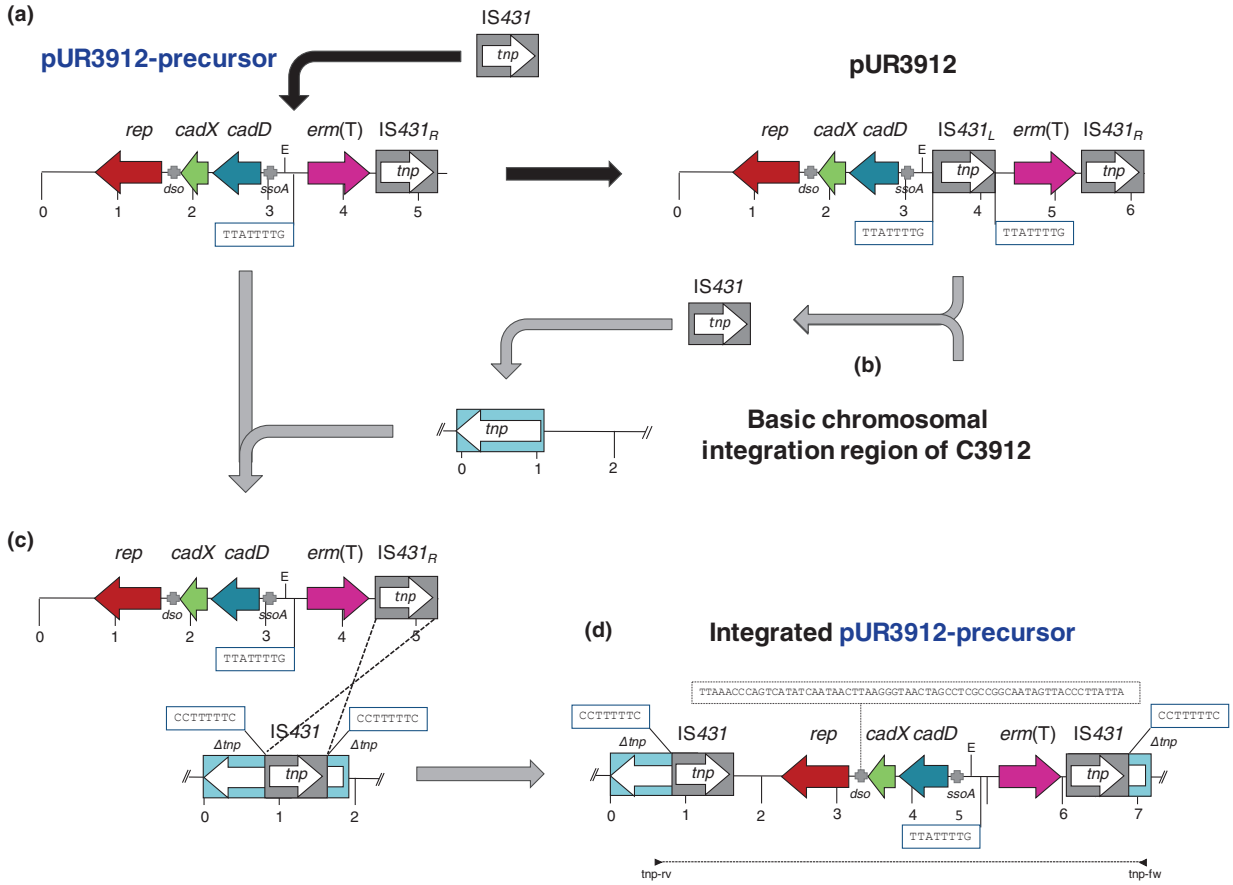

FIG. 2. Schematic representation of the insertion sequence IS*431*-mediated integration of plasmid pUR3912 (accession no. HE805623; the plasmid display was adjusted for this figure, the database entry starts with the *Eco*RI (E) cleavage site) into the chromosomal DNA of strain C3912. The different steps include (a) the integration of an IS*431* copy into a pUR3912 precursor with one IS*431* to generate pUR3912 with two IS*431* copies, IS*431*$_L$ and IS*431*$_R$, (b) the integration of IS*431*$_L$ or a free IS*431* copy into the chromosomal *tnp* gene followed by (c) reciprocal homologous recombination of the integrated IS*431* with the IS*431*$_R$ of the pUR3912 precursor. This latter process led to (d) the integration of the pUR3912 precursor into the chromosome of strain C3912. The positions of primers used for the detection of the putative chromosomal location of pUR3912 are labelled tnp-fw and tnp-rv and indicated by arrowheads with the joining line representing the extension of the amplified fragment. The arrows indicate the extents and directions of transcription of the genes *erm*(T) (resistance to macrolides-lincosamides-streptogramin B), *rep* (plasmid replication), *cadX* (translational regulator) and *cadD* (P-ATPase metal efflux). The *dso* and the *ssoA*, involved in plasmid replication, are indicated. The IS*431* and IS*1216*-like elements are shown as grey and blue boxes, respectively, with the white arrow indicating the transposase gene (*tnp*). The 8-bp direct repeats at the IS*431* integration sites within the chromosomal DNA (in *tnp*) as well as the 8-bp direct repeats of IS*431*$_L$ at its integration sites within pUR3912 are shown in boxes. The 64-bp duplication within the *dso* in the integrated pUR3912 is shown within a dashed box. A size scale in kb is given below each map.

observations made in this study underline the role of insertion sequences in the acquisition, maintenance and dissemination of antimicrobial and/or metal resistance genes.

Nucleotide Sequence Accession Number

The 6551-bp nucleotide sequence of the chromosomal integrated plasmid pUR3912 of C3912 has been deposited in the EMBL database (accession number HF677199).

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Transparency Declaration

The authors declare no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Schematic presentation of the circular free copy of pUR3912 and the *dso* and *ssoA* detected.

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