RESEARCH LETTER

Conjugal mobilization of the mega element carrying *mef*(E) from *Streptococcus salivarius* to *Streptococcus pneumoniae*

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Abstract

We report the isolation and characterization of an unusual strain of *Streptococcus salivarius*, 3C30, displaying both the macrolide–lincosamide–streptogramin B and the tetracycline resistance phenotypes. It harbours the *mef*(E), *erm*(B), and *tet*(M) genes carried by different genetic elements. The genetic element carrying *mef*(E), named mega, was investigated by long PCR and sequencing, while the presence of the Tn3872-like element, carrying *tet*(M) and *erm*(B), was demonstrated by sequencing of both the int-xis-Tn and the fragment between the two resistance genes. In strain 3C30 the mega element is 5388 bp in size and its nucleotide sequence is identical to that of the element described previously in *S. salivarius*, with the exception of a 912 bp deletion at the left end. The composite Tn3872-like element appeared to be nonconjugative while the mega element was transferred by conjugation to *Streptococcus pneumoniae*. It was, however, impossible to transfer it again from these transconjugants to other strains. In addition, only in the 3C30 strain did mega form circular structures, as identified by real-time PCR. In conclusion, we found a clinical strain of *S. salivarius* carrying both mega and Tn3872-like genetic elements. Mega is transferable by conjugation to *S. pneumoniae* but it is not transferable again from the transconjugants, suggesting a possible mobilization by recombinases of the coresident Tn3872-like transposon.

Introduction

M-type resistance, referring to a phenotype of low-level resistance to 14- and 15-membered macrolides only, is essentially based on an active efflux system associated with the *mef* genes, having emerged in recent years in *Streptococcus pneumoniae* and *Streptococcus pyogenes* (Clancy *et al*., 1996; Sutcliffe *et al*., 1996). The *mef* gene, which was originally considered the necessary determinant of the efflux mechanism, appears to be a component of a more complex system that includes *mef* and an *msr*(A) homologue, recently renamed *mrs*(D) (Daly *et al*., 2004; Ambrose *et al*., 2005). The *mrs*(D) gene encodes an ATP-binding protein, likely constituting an efflux transport system of the ATP-binding cassette superfamily (Iannelli *et al*., 2004).

Several variant *mef* genes, *mef*(A), *mef*(E), and *mef*(I) (Santagati *et al*., 2000; Gay & Stephens, 2001; Mingoia *et al*., 2007), showed high similarity at the nucleotide sequence level and all have been disseminated differently; to date, they have been found in five streptococcal species, whereas *mef*(E) alone has been identified in five more streptococcal species and in nine additional microbial species (Luna *et al*., 2000; Klaassen & Mouton, 2005).

The *mef*(A) and *mef*(E) genes, in spite of their identity at the DNA level (90%) – which led to a proposal of inclusion in a single class named *mef*(A) (Roberts *et al*., 1999) – have a number of marked differences (Klaassen & Mouton, 2005), including the structure of the genetic elements carrying them and the fact that they also behave quite differently.

The *mef*(A)-element, originally characterized in *S. pneumoniae* as a nonconjugative transposon of 7.2 kb, Tn*1207.1* (Santagati *et al*., 2000), was also found in *S. pyogenes* as part of a longer conjugative transposon of 52.4 kb, Tn*1207.3* or a 58.8 kb element (Φ10394.4), or a larger mobile *mef*(A)-*tet*(O)-element of 60 kb (Banks *et al*., 2003; Santagati *et al*., 2003).

The *mef*(E) gene was shown to be carried by a chromosomal element called mega, 5.5 kb in size, similar to Tn*1207.1*, but lacking the upstream *mef* region. This element is prevalent among clinical strains of *S. pneumoniae* (Gay &

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Stephens, 2001; Amezaga *et al.*, 2002; Del Grosso *et al.*, 2002, 2004; Montanari *et al.*, 2003), but was found in other bacteria such as *Streptococcus salivarius* (Stadler & Teuber, 2002), *Streptococcus agalactiae* (Marimon *et al.*, 2005), *Neisseria* spp. (Cousin *et al.*, 2003), and viridans streptococci (Jonsson & Swedberg, 2006). Conjugal transfer of mega has never been observed in streptococcal species other than in viridans streptococci (Jonsson & Swedberg, 2006). Because of the lack of recombinase systems, mega has only been transferred by transformation (Del Grosso *et al.*, 2006).

Even though several sites of mega integration have been described and classified, this element has a preferential genomic site for integration, proving to be a very 'social' element able to realize cooperation, especially with Tn*916*, carrying the *tet*(M) gene responsible for tetracycline resistance, considered as a precursor in the evolution of larger composite elements (Gawron-Burke & Clewell, 1982; Clewell & Gawron-Burke, 1986; Christie *et al.*, 1987; Oggioni *et al.*, 1996; Daly *et al.*, 2004).

In fact, Tn*2009* and Tn*2010* are composite elements where mega is inserted into the *orf*6 of Tn*916*. They possess a similar structure, but Tn*2010* has, in addition, an *erm*(B) genetic element, located in *orf*20 (Del Grosso *et al.*, 2007). Both composite *mef(*E) elements are not conjugative.

As is well known, MLS_B-mediated resistance by *erm* genes, such as *erm*(B) and *erm*(A) subclass *erm*(TR), confers high-level resistance to macrolides, lincosamides, and streptogramine B by ribosomal modification, and is prevalent in streptococci (Cascone *et al.*, 2002).

The description of Tn*3872* further confirms the 'social' role of Tn*916*, as it is composed of Tn*917* carrying the *erm(*B) gene in the *orf*9 of Tn*916* (McDougal *et al.*, 1998).

In this study, we characterized a macrolide- and tetracycline-resistant *S. salivarius* strain 3C30 isolated from the throat of a child with pharyngo-tonsillitis, which had erroneously been identified as *S. pyogenes* due to its unusual β-haemolysis, bacitracin, and penicillin susceptibility, possessing *mef*(E), *erm*(B), and *tet*(M) genes. The study was undertaken to characterize the genetic elements carrying these genes and their capability to transfer by conjugation.

Materials and methods

Growth conditions

The erythromycin- and tetracycline-resistant strain of *S. salivarius* 3C30, isolated from the throat of a child with pharyngo-tonsillitis, was examined. The strain was identified by PCR and sequencing of the 16S and *sod*A genes (Hoshino *et al.*, 2005).

All organisms used in this study were grown on Mueller–Hinton agar (Oxoid) supplemented with 5% defibrinated horse blood (Oxoid). For broth cultures, the strains were grown on liquid media containing Brain Heart Infusion (Oxoid). The unusual β-haemolysis of our strain was confirmed by the use of horse blood in a base containing starch medium (Saunders & Ball, 1980).

Antibiotic susceptibility testing and resistance genes

Susceptibility to erythromycin, azithromycin, clindamycin and tetracycline was performed by the broth dilution method, as recommended by the Clinical and Laboratory Standards Institute (Standard, 2006). The resistance genes – *erm*, *mef*, and *tet* – were studied by PCR using previously published primers and protocols, followed by sequencing (Borbone *et al.*, 2007).

Pulsed-field gel electrophoresis (PFGE)

Total bacterial DNA was extracted in agarose plugs as described previously (Cascone *et al.*, 2002). After digestion with the SacII enzyme (TaKaRa BIO), macrorestriction fragments were resolved in a 1% agarose gel using 0.5 × tris-borate-ethylenediaminetetraacetic acid buffer (TBE) (BioRad) at 14 °C. The CHEF DR PFGE (BioRad) system was used and switch and run times were 1–15 s for 20 h, with a voltage gradient of $6\,V\,cm^{-2}$. The macrorestriction fragments were visualized by a blue-light trans-illuminator (Safe Imager Invitrogen) after staining with 1 × SYBR Green (SYBR Safe DNA gel staining Invitrogen) in TBE 0.5 ×. The macrorestriction fragments were transferred from the gel to a nylon membrane (Hybond N, Amersham) in a downward direction using a Vacuum blotter 785 (BioRad) and denaturing solutions (NaOH 0.5 M/NaCl 1.5 M). DNA fragments were immobilized by UV radiation (Ultraviolet Crosslinker Amersham). The hybridization assays were performed using the 'ECL Direct Nucleic Acid Labelling and Detection Systems' (RPN 3000 Amersham) and following the protocol provided with the kit.

Long and inverse PCR assays

To investigate genetic elements related to resistance determinants and to clarify gene resistance linkage, long PCR assays were performed. In particular, MS146 and MS147 primers (Cascone *et al.*, 2005) were used for the detection of the genetic element related to the *mef*(E) gene, mega; the linkages between *erm*(B) and *tet*(M) were investigated using Tet(M)up (5′-ATATGTGTGTGACGAACTTTACC-3′) and MS6 (5′-TTCCCTTTAGTAACGTGTAACTT-3′) primers and *int*-Tn primers as described previously (Doherty *et al.*, 2000). The mixture for long PCR assays contained 2.5 mM $MgCl_2$, 1 × LaPCR Buffer II (Mg^{2+} free), 2.5 mM dNTPs, 2.5 U of TaKaRa LA Taq, and 1 μL of genomic DNA

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extracted from the 3C30 strain. Inverse PCR was performed as described previously (Santagati *et al*., 2003).

Sequencing and sequence analysis

All amplification products were purified using the QIAquick PCR gel extraction Kit (Qiagen) and sequenced with a LICOR DNA 4000L sequencer. The DNA sequence was analysed by the GAPPED BLAST software (Altschul *et al*., 1997).

Conjugation assays

Mating experiments were carried out as described previously (Smith & Guild, 1980), using the 3C30 strain as donor and a novabiocin-resistant DP1002 *S. pneumoniae* strain as a recipient (Shoemaker & Guild, 1974). The selection of transconjugants was performed with erythromycin (1 μg mL^{-1}) or tetracycline (5 μg mL^{-1}) and novobiocin (10 μg mL^{-1}) (Smith & Guild, 1980). The transfer of resistance genes in the transconjugants was confirmed by PCR.

Real-time PCR assays

Real-time PCR (MX 3000P® 27 Stratagene) assays were performed using an SYBR® Green PCR Master Mix kit following the manufacturer's instructions. Reactions were primed by MS146 rev/MS147 rev synthetic oligonucleotides, complementary to MS146 and MS147, respectively.

Results and discussion

Resistance genes and genetic elements

Streptococcus salivarius 3C30, identified by sequencing the 16S and *sod*A genes, displayed cross-resistance to erythromycin, azithromycin, and clindamycin and resistance to tetracycline with the following minimal inhibitory concentrations (MICs): erythromycin ≥512 μg mL^{-1}; azithromycin ≥512 μg mL^{-1}; clindamycin = 256 μg mL^{-1}; and tetracycline = 512 μg mL^{-1}. PCR analysis and sequencing showed that *S. salivarius* 3C30 harboured the *erm*(B), *mef*(E), and *tet*(M) genes. A link between *erm*(B) and *tet*(M) was demonstrated by the SacII genome macrorestriction (PFGE) analysis and hybridization using a specific probe for each resistance gene (Fig. 1). The PFGE patterns showed that *erm*(B) and *tet*(M) are inserted on the SacII fragment of 194 kb, suggesting a colocalization of the two genes on the same chromosomal fragment, while *mef*(E) hybridized alone in a single band of 242.5 kb. No link between *mef*(E) and *tet*(M) or *erm*(B) was found. On the basis of these results, the association between *erm*(B) and *tet*(M) was investigated by long PCR using tetM-up/MS6 primers located, respectively, on the *tet*(M) and *erm*(B) genes. The amplicon, in the 3C30 strain, was *c*. 3 kb in size. Its DNA sequence analysis

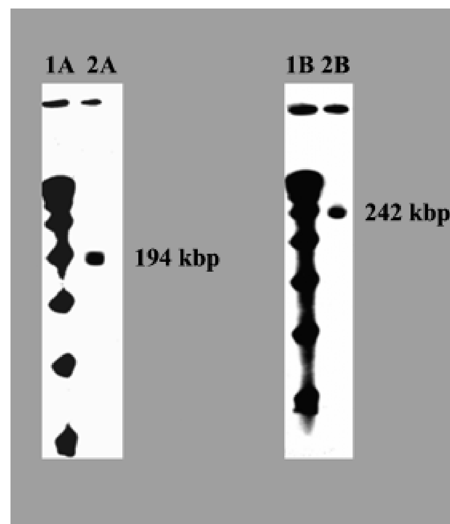

Fig. 1. Hybridization of *erm*(B), *tet*(M), and *mef*(E) probes on PFGE/SacII macrorestriction profile of *Streptococcus salivarius* 3C30. Lanes 1A and 1B, PFGE λ ladder; lane 2A, hybridization band obtained with *erm*(B) and *tet*(M); and lane 2B, hybridization band obtained with *mef*(E).

demonstrated the presence of Tn*3872*-like, a composite transposon in which Tn*917*, carrying *erm*(B), was inserted into *orf*9 of Tn*916*-like carrying *tet*(M), at base 14 525 of Tn*916*-like (GenBank accession no. U09422) (Poyart *et al*., 2000).

The entire element carrying *mef*(E) was amplified by long PCR using MS146/MS147 primers designed on the nucleotide sequence of mega ends (Cascone *et al*., 2005). Sequence analysis demonstrated that mega of strain 3C30 is 5388 bp long and is 100% identical to the mega element described previously in *S. salivarius* (GenBank accession no. AJ318993) from position 912 to 6299 (Fig. 2). Therefore, in strain 3C30 mega has a 912 bp deletion at the left end that includes an already described *orf*(a). Additional orfs, namely *orf*(b), (c), and (d), present at the right end of mega of *S. salivarius* (GenBank accession no. AJ318993) but not present in the mega element of *S. pneumoniae* (GenBank accession no. AF274302), are also not present in strain 3C30 (Fig. 2).

In addition, the mega of 3C30 was found to be 100% identical to the mega described in *S. pneumoniae* by Gay and Stephens (GenBank accession no. AF274302) with a deletion of 131 bp at the left end. *orfs 3–7* of the mega of 3C30 *S. salivarius* are homologous to *orfs 3–7* of the mega of *S. salivarius* (GenBank accession no. AJ318993) and of the mega of *S. pneumonie* (GenBank accession no. AF274302), described previously.

Inverse PCR that was performed to determine the integration site of mega in *S. salivarius* 3C30 showed that the *mef*(E)-element is inserted into *str0653* at 615 802 bp, which

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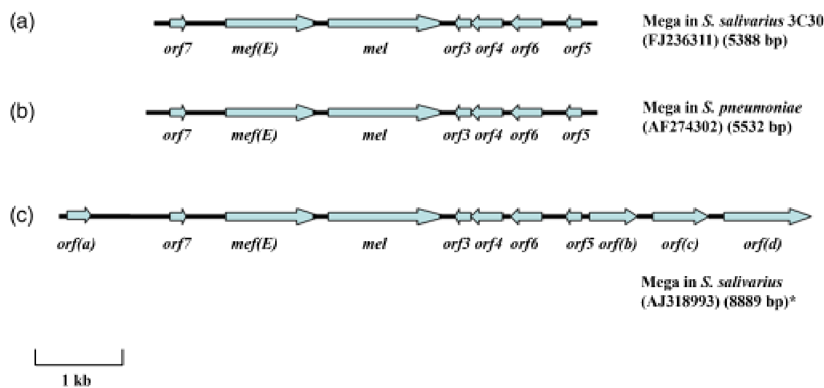

Fig. 2. Organization of the mega element in *Streptococcus salivarius* 3C30 (a), compared with the mega of *Streptococcus pneumoniae* (b) and the mega of *S. salivarius* (c). *See also reference Stadler & Teuber (2002).

Table 1. Conjugal transfer of the mega element

Donor	Recipient*	Frequency†	Resistance gene
S. salivarius 3C30	*S. pneumoniae* DP1002	7.3 ± 10^{-7}	*mef*(E)

*Conjugation frequency was expressed as CFU of transconjugants per CFU donor.
†Values represent the mean of three different experiments ± the standard error of the mean.

left end **right end**

CACACGTG ***GAG*** TGTGTAGC
GTGTGCAC ***CTC*** ACACATCG

Fig. 3. Sequence of the joint fragments obtained by excision assay of the mega in *Streptococcus salivarius* 3C30. The 3 bp overlaps are in bold.

encoded tRNA methyltransferases, part of the TrmA family of *Streptococcus thermophilus* CNRZ1066. In addition, the junction fragment between the left end element and the genome overlapped by 18 bp, namely GAGTGTGTAGC TTTGCTC.

Conjugal transfer of genetic elements and circular forms

The conjugal transfer was tested by filter mating experiments in the presence of DNAse, using the 3C30 strain as a donor and *S. pneumoniae* DP1002 resistant to novobiocin, as a recipient. Only the *mef*(E)-element was able to be transferred at the frequency of 7.3×10^{-7} (CFU of transconjugants per CFU donor) as shown in Table 1. All transconjugants exhibited the M-type resistance to macrolides, with a MIC of $8\,\mu g\,mL^{-1}$ for erythromycin and susceptible to tetracycline. The presence of *mef*(E) was confirmed by PCR.

The insertion site of mega in two representative transconjugants of *S. pneumoniae* was obtained by inverse PCR. The mega element was found to be inserted into *spr1451*, which encoded tRNA pseudouridine synthase A in R6 *S. pneumoniae* and can be considered a novel insertion site, adjacent to the gene for a putative phosphomethylpyrimidin kinase. It is interesting to note that it was impossible to transfer the *mef*(E)-element again from representative transconjugants to other strains.

The presence of circular structures was assayed by real-time PCR using chromosomal DNA as a substrate and synthetic oligonucleotides MS146rev and MS147rev as divergent primers. Amplicons of *c.* 300 bp were obtained and sequenced. Sequence analysis indicated that they were circular forms resulting from the interaction between the ends of the *mef*(E) element with an overlap of GAG, as shown in Fig. 3. These intermediate forms were observed when chromosomal DNA of strain 3C30, but not of any of the other transconjugants, was used as a template of the amplification reactions.

In summary, we found a strain of *S. salivarius* 3C30 displaying the MLS_B phenotype that contains two different genetic elements: mega, carrying *mef*(E), and Tn*3872*-like, in which *erm*(B) carried by Tn*917* is inserted into Tn*916*. Sequence analysis of the former element is identical to the mega found in different species of streptococci described previously, namely *S. pneumoniae* and *S. salivarius*, showing a typical structure and organization.

Several studies described different mega insertion sites in the streptococcal chromosome.

In the 3C30 strain, mega is inserted into the *str0653* of the *S. thermophilus* CNRZ1066 genome. The conjugal transfer of mega and the presence of circular structures, demonstrated here for the first time, suggest another and new mode of mega transfer that has usually been associated with transformation, due to the lack of transposases or recombinases in the mega element. These results suggest that the *mef*(E)-element could be mobilized by a coresident conjugative transposon such as Tn*3872*-like or by host factors required for excision, demonstrating the role of foreign recombinases in the promotion of the excision and mobility. In conclusion, the conjugal mobilization of mega could be

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responsible for the diffusion of this element even in non-transformable organisms, providing a contribution for the spread of antibiotic resistance genes.

Nucleotide sequence accession no.

The sequence of the mega element in *S. salivarius* 3C30 has been assigned GenBank accession no. FJ236311.

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