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Characterization of a Genetic Element Carrying the Macrolide Efflux Gene *mef*(A) in *Streptococcus pneumoniae*

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The *mef*(A) gene from a clinical isolate of *Streptococcus pneumoniae* exhibiting the M-type resistance to macrolides was found to be part of the 7,244-bp chromosomal element Tn*1207.1*, which contained 8 open reading frames. *orf2* encodes a resolvase/invertase, and *orf5* is a homolog of the macrolide-streptogramin B resistance gene *msr*(SA).

A new mechanism of resistance to macrolides based on an efflux system has recently emerged among clinical isolates of *Streptococcus pyogenes* and *Streptococcus pneumoniae* (35, 37). It is due to the presence of macrolide efflux (*mef*) genes conferring the M phenotype, which is characterized by resistance to macrolides and sensitivity to lincosamides and streptogramin B antibiotics (37). *mef*(A), originally described in *S. pyogenes* (8), and *mef*(E), originally described in *S. pneumoniae* (38), are 90% identical and were assigned to the same *mef*(A) class of macrolide resistance determinants (32). Genes of the *mef*(A) class (i) were found in different gram-positive genera including *Corynebacterium*, *Enterococcus*, *Micrococcus*, and *Streptococcus*, (ii) were never found associated with extrachromosomal plasmid DNA, and (iii) in some instances were shown to be transferred by conjugation (3–5, 10, 14, 15, 17, 26, 40). Both *mef*(E) and *mef*(A) have been reported to occur in pneumococci from Italy (19, 20, 25). Here we report the first characterization of a *mef*-carrying genetic element. The recently proposed nomenclature for macrolide resistance determinants (32) was adopted throughout this paper.

Transfer of *mef*(A). To isolate and characterize the genetic element that carries the macrolide efflux gene of *S. pneumoniae* STR174b, *mef*(A) was transferred from STR174b to Rx1 (34), a rough (unencapsulated) laboratory strain of pneumococcus. Clinical strain STR174b, isolated from sputum and exhibiting the M-type resistance to macrolides, was used as donor in conjugation and transformation experiments (Table 1). The *mef*(A) gene could not be transferred from STR174b by conjugation ($<3.4 \times 10^{-8}$ transconjugants per donor), in filter matings performed as described by Smith and Guild (36), while successful transfer to Rx1 was accomplished by transformation (Table 1). Competent cells of Rx1 were prepared as previously described (28); transforming DNA of STR174b was added at 1 μg per ml of competent cells, and erythromycin-resistant transformants were isolated at a frequency of 4.7×10^{-4} per recipient (Table 1). MF4, a representative transformant, was kept for further analysis. MF4 showed the same resistance phenotype as the parent clinical strain, being resistant to erythromycin (MIC, 8 μg/ml) and sensitive to lincosamides and streptogramin B antibiotics. Again, *mef*(A) could not be transferred by conjugation in matings where MF4 was the donor and *S. pneumoniae*, *S. pyogenes*, and *Streptococcus gordonii* were used as recipients (Table 1).

Sequencing and sequence analysis. The nucleotide sequence of the DNA adjacent to *mef*(A) was determined in both STR174b and MF4, using templates obtained by ligation-mediated and/or inverse PCR (24, 31). Direct automated sequencing of long PCR fragments was performed as previously described (11, 12). The software BLAST (2) was used to conduct homology searches of the GenBank database and the microbial genome databases available at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/) and at the WIT Computational Biology Group at Argonne National Laboratory website (http://wit.mcs.anl.gov./WIT2/CGI). Sequence analysis was carried out at the Baylor College of Medicine Search Launcher server (http://dot.imgen.bcm.tmc.edu:9331/index.htlm). RNA structure prediction was done using RNA structure 2.5 (21).

Nucleotide sequence of the *mef*(A) element. Both in STR174b and in MF4 the *mef* gene was shown to be 100% identical to the already described *mef*(A) gene (8) and was found to be part of a chromosomal element which was designated Tn*1207.1*. Tn*1207.1* was found to be 7,244 bp in size (Fig. 1), and its nucleotide sequence was identical in both strains. Sequence data analysis showed the presence of 8 open reading frames (ORFs), of which the first 5 have the same direction of transcription, while *orf6*, *orf7*, and *orf8* are oriented opposite to the others

TABLE 1. Transfer of Tn*1207.1*

Streptococcus pneumoniae donor[a]	Recipient[b]	Frequency[c]
Transformation		
STR174b (clinical isolate)	*S. pneumoniae* Rx1 (34)	4.7×10^{-4}
Conjugation		
STR174b (clinical isolate)	*S. pneumoniae* DP1002 (34)	$<3.4 \times 10^{-8}$
MF4 (transformant of Rx1, this work)	*S. pneumoniae* DP1002 (34)	$<1.1 \times 10^{-8}$
MF4 (transformant of Rx1, this work)	*S. pyogenes* D471 (33)	$<1.4 \times 10^{-8}$
MF4 (transformant of Rx1, this work)	*S. gordonii* GP204 (29, 30)	$<1.6 \times 10^{-8}$

[a] The origin is shown in parentheses.
[b] The reference(s) are shown in parentheses.
[c] Conjugation frequency is expressed as CFU of transconjugants per CFU of donor, whereas transformation frequency is expressed as CFU of transformants per CFU of recipient.

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TABLE 2. Homologies of the ORFs of Tn*1207.1*

ORF[a]	Homologous gene[b]	Origin[c]	Proposed function of gene product	E value	Amino acid identity	Amino acid similarity
orf1 (218)	None					
orf2 (370)	*ccrB* (AB014438)	*mec* DNA (*S. aureus*) (13)	Site-specific recombinase	4e-18	93/294 (31%)	144/294 (48%)
	cisA (M29040)	*skin* element (*B. subtilis*) (39)	Site-specific recombinase	2e-14	79/304 (25%)	139/304 (44%)
	int (AJ242593)	Bacteriophage A118 (*L. monocytogenes*) (16)	Integrase	6e-12	59/196 (30%)	104/196 (52%)
	tnpX (U15027)	Transposon Tn*4451* (*C. perfringens*) (6)	Site-specific recombinase	1e-11	78/312 (25%)	132/312 (42%)
orf3 (138)	None					
mefA (405)	*mef*(A) (U70055)	(*S. pyogenes*) (8)	Macrolide-efflux protein	0	405/405 (100%)	
orf5 (487)	*msr*(SA) (AB016613)	Plasmid pEP2104 (*S. aureus*) (22)	Macrolide-resistance protein	6e-91	180/471 (38%)	285/471 (60%)
	vga(A) (M90056)	Plasmid pIP524 (*S. aureus*) (1)	Streptogramin A-resistance protein	2e-89	183/485 (37%)	283/485 (57%)
orf6 (99)	*orf11* (L29324)	Conjugative transposon Tn*5252* (*S. pneumoniae*) (23)	Unknown	2e-22	45/76 (59%)	61/76 (80%)
orf7 (122)	*orf12* (L29324)	Conjugative transposon Tn*5252* (*S. pneumoniae*) (23)	Unknown	3e-06	30/96 (31%)	46/96 (47%)
orf8 (117)	*umuC* (L29324)	Conjugative transposon Tn*5252* (*S. pneumoniae*) (23)	UV resistance protein	6e-36	70/116 (60%)	89/116 (76%)

[a] The number of amino acids is shown in parentheses.
[b] The accession number is shown in parentheses.
[c] The number in parentheses is the reference.

(Fig. 1). Between *orf3* and *mef*(A) there is an intergenic region with a high potential for the formation of hairpins (21). Palindromes indicative of terminators can be detected downstream of *orf1* and *orf3*. Terminal repeats could not be detected.

Chromosomal insertion site. Tn*1207.1* was found integrated at a specific site in the chromosome of *S. pneumoniae*, downstream of the A nucleotide in position 1676 of the *celB* gene (27). Both in clinical isolate STR174b and in transformant MF4, the integration of Tn*1207.1* caused a 1,947-bp deletion in the pneumococcal genome (nucleotides 1393 to 3339 of contig 4139) (The Institute for Genomics Research [http://www.tigr.org]) involving the 3′ end of *celB* (nucleotides 2775 to 3339 of contig 4139) and the 5′ end of *orf436* (nucleotides 1393 to 1784 of contig 4139) (WIT website [see above]).

ORFs of Tn*1207.1*. To identify genes homologous to the ORFs of Tn*1207.1*, homology searches were done at a protein level, using the BLAST software (2). The E value, which is considered a very reliable statistical scoring measure (7), is reported for each homolog detected by the search (Table 2). No homolog was found for *orf1* and *orf3*, while *orf2* was found to be homologous to site-specific recombinases of genetic elements of gram-positive bacteria. These elements included chromosomal elements of *Staphylococcus aureus* (13) and *Bacillus subtilis* (39), a bacteriophage of *Listeria monocytogenes* (16), and a transposon of *Clostridium perfringens* (6) (Table 2). An additional ORF potentially involved in determining resistance to macrolides and streptogramin antibiotics was found adjacent to *mef*(A). *orf5*, whose coding sequence starts 119-bp downstream of the *mef*(A) stop codon (Fig. 1), was found to be homologous to *msr*(SA) (22) and *vga*(A) (1). *msr*(SA) confers resistance to macrolides and streptogramin B, and *vga*(A) confers resistance to streptogramin A antibiotics. Both genes are putative members of the ABC transporter superfamily and mediate resistance by encoding an antibiotic-specific efflux pump (32). *orf6*, *orf7*, and *orf8* are homologous to 3 ORFs of the pneumococcal conjugative transposon Tn*5252* (23). The 5′ end of *orf8* appears truncated since its gene product is homologous to the C-terminal portion of UmuC, an UV-resistance protein encoded by Tn*5252* (Table 2). No function is known for the gene products of *orf6* and *orf7* and the Tn*5252* homologs. Even if no significant homology can be detected at the nucleotide level, *orf6* to *orf8* and their Tn*5252* homologs show a similar organization characterized by overlapping ORFs. *orf6* and *orf7* overlapped by 14 bp, and *orf7* and *orf8* overlapped by 4 bp.

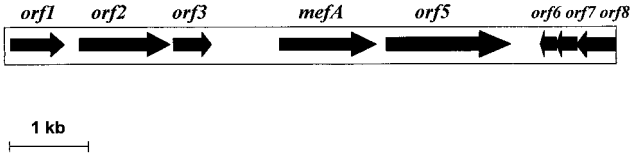

FIG. 1. Structure of the chromosomal genetic element Tn*1207.1*, which is 7,244-bp long.

Conclusions. In this work we characterized a genetic element that carries the macrolide efflux gene *mef*(A) in a clinical isolate of *S. pneumoniae* exhibiting the M-type resistance to macrolides. *mef*(A) was found to be part of a chromosomal genetic element designated Tn*1207.1*. Tn*1207.1* should be considered a defective transposon, since it terminates with a truncated ORF at the right side, while in different clinical isolates it is integrated at the same chromosomal site and has the same overall genetic organization but is larger in size at its right side (Santagati and colleagues, unpublished data). The presence of an ORF potentially encoding a site-specific recombinase of the resolvase/invertase family resembles clostridial transposons Tn*4451*, Tn*4453*, and Tn*5397*, in which the TnpX and TndX resolvases are involved in the transposon excision process (6, 9, 18, 41). Although it has been reported that *mef* genes from *S. pneumoniae* and group C streptococci can be transferred by conjugation (15, 17), Tn*1207.1* appeared to be nonconjugative in nature.

Nucleotide sequence accession number. The complete nucleotide sequence of Tn*1207.1* was assigned GenBank accession no. AF227520.

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