ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 2006, p. 3920–3922 Vol. 50, No. 11
0066-4804/06/$08.00+0 doi:10.1128/AAC.00223-06

Novel Streptomycin Resistance Gene from *Mycobacterium fortuitum*▽

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Received 21 February 2006/Returned for modification 2 June 2006/Accepted 30 August 2006

We have isolated the *aph*(*3″*)-*Ic* gene, encoding an aminoglycoside 3″-*O*-phosphotransferase [APH(3″)-Ic], from a genomic library of an environmental *Mycobacterium fortuitum* strain, selecting for streptomycin resistance. APH(3″)-Ic phosphorylates and inactivates streptomycin. Similar genes have been described in *Streptomyces griseus* and plasmid RSF1010. It is also present in some *M. fortuitum* clinical isolates.

Streptomycin (STR) resistance in *Mycobacterium*, a genus that includes important human pathogens, such as *Mycobacterium tuberculosis*, along with fast-growing opportunistic species, such as *Mycobacterium fortuitum*, is due to several mechanisms. Mutations in either the S12 ribosomal protein or the 16S rRNA confer high and moderate levels of STR resistance (21). We have previously characterized an efflux pump from *M. fortuitum* and *M. tuberculosis* that transports and confers decreased susceptibility to STR (1). In the present work, we describe the isolation and characterization of a novel aminoglycoside phosphotransferase gene from *M. fortuitum* which can confer STR resistance in mycobacteria.

Isolation of the *M. fortuitum aph*(*3″*)-*Ic* gene. We constructed a plasmid library of genomic DNA from *M. fortuitum* strain FC1, a natural isolate which shows resistance to 50 μg/ml of STR (2), and transformed the library into *M. smegmatis* mc^{2}155 (16) (STR MIC, 0.5 μg/ml) by electroporation as described previously (13). Six colonies were selected that showed a 64-fold increase in the STR MIC, as determined by the microtiter assay using resazurin (12), whereas transformation of vector pSUM36 (2) did not produced such an increase. Among the recombinant plasmids from STR-resistant colonies, pAC5 and pAC6 were found, and they overlapped in a common 2.5-kb fragment (Fig. 1). This fragment was subcloned in pSUM36, resulting in pSAN19, which was also capable of increasing the STR MIC for *M. smegmatis* mc^{2}155 (Table 1).

Sequencing and FramePlot analysis (http://www.nih.go.jp/~jun/cgi-bin/frameplot.pl) (10) revealed three complete and two incomplete open reading frames (Fig. 1). *orfA* and *orfB* are similar to Rv0513 and Rv0514 from *M. tuberculosis* (possible transmembrane proteins; 65.4 and 46% amino acid identity, respectively). *orfC* is similar to *aph*(*3″*)-*Ia* from *Streptomyces griseus* and *aph*(*3″*)-*Ib* from plasmid RSF1010 (streptomycin phosphotransferases; 53.3 and 46.5% amino acid identity, respectively). *orfD* is similar to Rv0519c (possible conserved membrane protein; 56.5% amino acid identity) and Rv0774c (possible conserved exported protein; 53.4% amino acid identity) from *M. tuberculosis*; *orfE* is similar to Rv0826 and Rv1645c from *M. tuberculosis* (conserved hypothetical proteins; 37.8 and 44.7% amino acid identity, respectively).

Since the protein encoded by *orfC* shows similarity to APH(3″) enzymes, it is putatively involved in the STR resistance phenotype. A 1-kb fragment containing only *orfC* (plasmid pSAN26; Fig. 1) also conferred STR resistance to *M. smegmatis* mc^{2}155 (Table 1). Introduction of pSAN19 or pSAN26 by electroporation into slow-growing *M. bovis* BCG-Pasteur conferred a 16-fold increase in the STR MICs (Table 1). Plasmid pSAN26 did not confer any increase in the MICs of the aminoglycosides spectinomycin, gentamicin, amikacin, 2′-*N*-ethylnetilmicin, and 6′-*N*-ethylnetilmicin to *M. smegmatis* mc^{2}155 and *M. bovis* BCG-Pasteur (Table 1); therefore, STR seems to be the only substrate of the APH(3″)-like enzyme from *M. fortuitum* FC1.

As mentioned above, the closest relatives of the APH(3″)-like enzyme from *M. fortuitum* FC1 are the APH(3″)-Ia enzyme from STR-producing *S. griseus* strains (8, 15, 17) and APH(3″)-Ib, encoded by the broad-host-range plasmid RSF1010 (19) (Fig. 2). According to Shaw et al. (14), the enzyme from *M. fortuitum* was named APH(3″)-Ic.

Enzymatic activity of the APH(3″)-Ic enzyme. Previously, we reported STR phosphotransferase activity in some *M. fortuitum* isolates (R. Gómez-Lus, J. Timm, C. Martín, J. Davies, and B. Gicquel, Abstr. 29th Intersci. Conf. Antimicrob. Agents

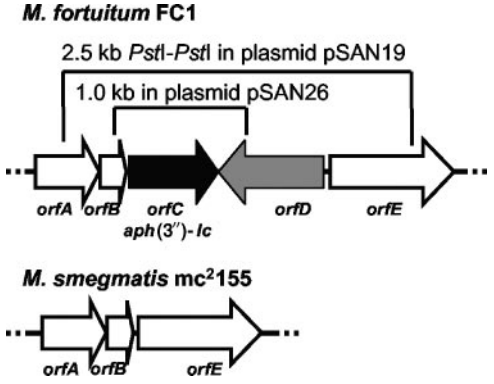

FIG. 1. Open reading frames present in the cloned fragments conferring STR resistance from *M. fortuitum* FC1 and comparison with a similar locus in *M. smegmatis* mc^{2}155. *orfA*, *orfB*, and *orfE* are found in the loci of both *M. fortuitum* and *M. smegmatis*; *orfC* and *orfD* are found only in the locus of *M. fortuitum*.

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▽ Published ahead of print on 5 September 2006.

TABLE 1. MICs of aminoglycosides for *M. smegmatis* and *M. bovis* BCG strains containing or lacking the *aph*(3″)-*Ic* gene

Strain	Plasmid	Presence of *aph*(3″)-*Ic* gene[d]	MIC (μg/ml) for aminoglycoside[a]:					
			STR	SPT	GEN	AMK	2′-NeNET	6′-NeNET
M. smegmatis mc²155	pSUM36[b]	−	0.5	128	1	32	16	8
	pSAN19[c]	+	32	128	1	32	16	8
	pSAN26[c]	+	32	128	1	32	16	8
M. bovis BCG Pasteur	pSUM36[b]	−	0.2	1	1	0.12	1	2
	pSAN19[c]	+	3.2	1	1	0.12	1	2
	pSAN26[c]	+	3.2	1	1	0.12	1	2

[a] Streptomycin, STR; spectinomycin, SPT; gentamicin, GEN; amikacin, AMK; 2′-*N*-ethylnetilmicin, 2′-NeNET; 6′-*N*-ethylnetilmicin, 6′-NeNET.
[b] pSUM36, an *E. coli*-mycobacterium shuttle cloning vector; kanamycin resistant.
[c] pSAN19 and pSAN26 are pSUM36 derivatives containing, respectively, the *aph*(3″)-*Ic* gene from *M. fortuitum* FC1 cloned in 2.5- and 1-kb fragments (Fig. 1).
[d] The presence (+) or absence (−) of the *aph*(3″)-*Ic* gene is indicated.

Chemother., abstr. 156, 1989). Since the antimicrobial activity of STR is lost when isolates are phosphorylated by APH(3″) enzymes, we tested the ability of crude extracts of *M. smegmatis* strains [producing or not producing APH(3″)-Ic] to inactivate STR. For this, we first obtained crude extracts from *M. smegmatis* strains by sonication (Bioruptor; Cosmo Bio Co., Ltd.). Next, 4 mg/ml of protein from the crude extracts was mixed with ATP and STR as described previously (7). After 1, 2, and 4 h of incubation at 37°C, 5 μl from each reaction was taken in triplicate, added to paper discs, and dried. Finally, discs were assayed against STR-susceptible *Escherichia coli* ATCC 25922. We estimated the load of STR on the discs by comparing the size of the inhibition zones with that of a series of discs containing known amounts of STR.

No loss of antimicrobial activity of STR was observed when crude extracts of either *M. smegmatis* mc²155 (wild type) or *M. smegmatis* strain SUM36 (containing only the cloning vector) was used, since there was no change in the size of inhibition zones in comparison with negative control reactions. Crude extracts from *M. smegmatis* SAN19 [which produces APH(3″)-Ic] reduced the size of the inhibition zone, indicating that the antimicrobial activity of the STR solution had been reduced by approximately 50%. This confirms that APH(3″)-Ic is capable of inactivating STR, providing these strains with an effective STR resistance mechanism. It should be noted that the possibility of acquiring STR resistance through 16S rRNA mutations in *M. fortuitum* (which has two rRNA operons) would be much less than that in *M. tuberculosis* (which has only one). Also, APH(3″)-Ic could have an additional role in protein phosphorylation or other metabolic processes, as has been suggested for APH enzymes (4, 18).

Distribution of the *aph*(3″)-*Ic* gene in *M. fortuitum* and other mycobacteria. The aminoglycoside-2′-*N*-acetyltransferase gene from *M. fortuitum* is universally present in this species (3), and

FIG. 2. Comparison of APH(3″) enzymes from *S. griseus*, *M. fortuitum*, and plasmid RSF1010. The motif $HGD(X)_4N$, characteristic of this family of proteins (20), has been located within the histidine domain II between residues 172 and 179 of the mycobacterial APH(3″). Other catalytic motifs described by Wright and Thompson (20) and Heinzel et al. (8) are boxed, and the significant residues are highlighted in black.

most mycobacterial species have similar genes. Using Southern blot hybridization, we detected the *aph*(*3″*)-*Ic* gene in 4 out of 22 *M. fortuitum* clinical isolates from our culture collection. Those isolates containing the *aph*(*3″*)-*Ic* gene were resistant to STR, three of them showing STR MICs of 50 μg/ml, which is the highest level of STR resistance we have detected in our collection of *M. fortuitum* isolates. However, some *M. fortuitum* isolates lacking this gene were also resistant to STR. We can therefore conclude that APH(3″)-Ic contributes to STR resistance, but most likely there could be many other yet unknown mechanisms that also contribute to STR resistance.

Homologues of the *aph*(*3″*)-*Ic* gene could not be found in databases of mycobacterial genomes such as *M. tuberculosis* H37Rv and *M. leprae* (both at http://genolist.pasteur.fr/) or *M. smegmatis* (http://www.tigr.org/). This gene therefore seems to be exclusive to some *M. fortuitum* strains. We can speculate that these strains might have a common *M. fortuitum* ancestor that acquired the *aph*(*3″*)-*Ic* gene from plasmid RSF1010, since under laboratory conditions this plasmid and its derivatives can replicate in mycobacteria (6, 9), perhaps facilitated by the high GC content of RSF1010 (60.9%), which is similar to that of mycobacterial genomes.

Physical location of the *M. fortuitum aph*(*3″*)-*Ic* gene. Genes encoding aminoglycoside-modifying enzymes can be located either in plasmids or in the bacterial chromosome. Plasmids are frequently found in *M. fortuitum* isolates (11), but a chromosomally located aminoglycoside-*N*-acetyltransferase also has been characterized (3). We assume the *aph*(*3″*)-*Ic* gene is located in the chromosome of *M. fortuitum* for two reasons. First, using PCR, the *aph*(*3″*)-*Ic* gene could not be amplified from a 16-kb plasmid identified previously in the *M. fortuitum* FC1 strain (5). Second, genes flanking it are similar to chromosomal genes from *M. tuberculosis*, and this region is very similar to a locus in the chromosome of *M. smegmatis* (Fig. 1).

Nucleotide sequence accession number. The nucleotide sequence determined in this work has been deposited in the GenBank, EMBL, and DDBJ databases under the accession number DQ336355.

This work was supported by CIBER grant CB06/06/0020 from the Spanish Ministerio de Sanidad y Consumo, EU grant QLK2-CT-2000-01761, and Spanish Ministerio de Educación y Ciencia (MEC) grants BIO2002-01297 and BIO 2005-01801. J.A.A. is a recipient of a "Ramon y Cajal" fellowship. S.R.-G. holds a fellowship from MEC (AP2001-1114).

We acknowledge P. Goñi for critical reading of the manuscript.

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