

Aph(3′)-IIc, an Aminoglycoside Resistance Determinant from *Stenotrophomonas maltophilia*[∇]

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We report the characterization of an intrinsic, chromosomally carried *aph(3′)-IIc* gene from *Stenotrophomonas maltophilia* clinical isolate K279a, encoding an aminoglycoside phosphotransferase enzyme that significantly increases MICs of kanamycin, neomycin, butirosin, and paromomycin when expressed in *Escherichia coli*. Disruption of *aph(3′)-IIc* in K279a results in decreased MICs of these drugs.

Stenotrophomonas maltophilia is an emerging nosocomial pathogen. Isolates display intrinsic resistance to many commonly prescribed antimicrobials, particularly β -lactams and aminoglycosides. They can also evolve broad-spectrum resistance to a cross section of other drugs that have been used to treat infections (6). Intrinsic β -lactam resistance in *S. maltophilia* is due to two β -lactamase enzymes, L1 and L2, which are produced at higher levels following β -lactam challenge (3, 4, 7). Aminoglycoside resistance in *S. maltophilia* is less well understood. An aminoglycoside acetyltransferase, AAC(6′)-Iz, is produced by some isolates, conferring reduced susceptibility to amikacin, netilmicin, sisomicin, and tobramycin (11, 12). However, isolates are also frequently resistant to aminoglycosides known to be substrates of aminoglycoside phosphotransferase (APH) enzymes, for example, kanamycin and neomycin, suggesting that an *aph* gene might be present in the *S. maltophilia* genome (12). In order to test this possibility, we used the APH(3′)-IIa amino acid sequence encoded on Tn5 (5) to search for genes encoding similar proteins in the genome of *S. maltophilia* clinical isolate K279a, which has been extensively characterized in this laboratory (2–4, 7, 8). tBLASTn searches (1) of the K279a genome sequence (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/s_maltophilia) revealed a single significant hit, and the amino acid sequence of the putative protein was used to perform a BLASTp search (1) against the NCBI database. The protein falls into the APH(3′)-II class according to sequence similarity, being 50% identical to both the Tn5 APH(3′)-IIa and the *Pseudomonas aeruginosa* chromosomally encoded APH(3′)-IIb (9). The putative *S. maltophilia* APH(3′)-II shares >45% identity with putative APH(3′)-II proteins encoded on the chromosomes of various environmental organisms, including *Burkholderia* spp. (though not pathogenic species), *Rhizobium etli*, *Mesorhizobium loti*, and *Sinorhizobium meliloti*. In contrast, it is only 34% identical to the archetypal APH(3′)-I encoded on Tn903 (13).

The *S. maltophilia* K279a *aph(3′)-II* gene was PCR amplified

using the PCR protocol described previously (2) and the primers *aph-F* (5′-ATGGAAGCACCCAATCC-3′) and *aph-R* (5′-TGCTGGCGTGGGACAACA-3′). The 1,102-bp amplicon was TA cloned into the pBAD-TOPO expression vector (Invitrogen, Leek, Holland) according to the manufacturer's instructions, and recombinant plasmid carrying correctly oriented *aph* was transformed into *Escherichia coli* DH5 α (Invitrogen). As a negative control, DH5 α ::pBAD was used. MICs of a variety of aminoglycosides were determined against the recombinant *E. coli* strains in the presence and absence of arabinose (1%, wt/vol), which induces expression of the cloned gene (Table 1). Clearly, of those aminoglycosides tested, K279a *aph* expression confers greatly reduced susceptibility to kanamycin, neomycin, butirosin, and paromomycin, as is expected given its predicted molecular type (4).

MICs of all aminoglycosides tested, including APH(3′)-II substrates, against *S. maltophilia* isolate K279a are high. We confirmed by PCR using the primers previously defined (12) that isolate K279a carries an *S. maltophilia aac(6′)-Iz* gene (11, 12) encoding resistance to (of those aminoglycosides tested by us) amikacin, sisomicin, and tobramycin (data not shown). Total RNA purification and reverse transcription-PCR, both performed using the protocol described previously (8), but with the reverse transcription-PCR primers *aph-RTF* (5′-AGGAACAGGGACAACCCG-3′) and *aph-RTR* (5′-CCAGGTCTTCATGATCGG-3′), confirmed that isolate K279a expresses *aph* under normal laboratory growth conditions (data not shown). Accordingly, to test whether *aph* is a true determinant of intrinsic aminoglycoside resistance in K279a, we disrupted the gene in an otherwise wild-type background. We did this by first PCR amplifying *aph* in two nonoverlapping fragments, the 5′ proximal of which had a HindIII site inserted at its 3′ end and the 3′ proximal of which had a HindIII site inserted at its 5′ end. The primer pairs used (with the HindIII sites underlined) were *aph-delF* (5′-CAGTAATGGACCTGTGGGC-3′) and *aph-HindR* (5′-AAGCTTTGAAAGTCGGAA GTGA-3′) and *aph-HindF* (5′-AAGCTTGTAGCGGCATGC CATC-3′) and *aph-delR* (5′-CACCATCAGGTTCCGGCAA). The two resultant amplicons were digested with HindIII, mixed, and ligated together to produce a mutant *aph* allele with a 111-bp internal deletion (i.e., a frameshift). This so-called *aph*^{FS} allele was cloned into the suicide gene replace-

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TABLE 1. MICs of various aminoglycosides against recombinant *E. coli* DH5 α producing or not producing *S. maltophilia* APH(3')-IIc and against *S. maltophilia* K279a and an *aph*^{FS} derivative

Strain or recombinant and condition	MIC (mg/liter) of ^a :								
	AMI	BUT	GENT	KAN	LIV	NEO	PARO	SIS	TOB
DH5 α ::pBAD									
No arabinose	0.5	0.125	1	0.25	4	0.5	0.25	0.125	0.5
With arabinose	0.5	0.125	1	0.25	4	0.5	0.25	0.125	0.5
DH5 α ::pBAD[<i>aph</i> (3')-II]									
No arabinose	0.5	0.125	1	0.25	4	0.5	0.25	0.125	0.5
With arabinose	4	4	1	>256	8	64	128	0.125	8
K279a	64	8	16	256	128	512	256	8	32
K279a (<i>aph</i> ^{FS})	64	1	16	8	128	64	64	8	32

^a MICs were determined using agar dilution on Muller-Hinton agar. Abbreviations: AMI, amikacin; BUT, butirosin; GENT, gentamicin; KAN, kanamycin; LIV, lividomycin; NEO, neomycin; PARO, paromomycin; SIS, sisomicin; TOB, tobramycin.

ment vector pEXTc (10) and the recombinant plasmid mobilized into *S. maltophilia* K279a through conjugation with *E. coli* SM10. Selection for gene replacement was performed as described previously (8, 10), and mutants were confirmed by PCR and sequencing. MICs of a variety of aminoglycosides against K279a and the *aph*^{FS} mutant are presented in Table 1. These data show that *aph* contributes to intrinsic kanamycin, neomycin, butirosin, and paromomycin resistance in *S. maltophilia*, confirming the result of the *E. coli* heterologous host experiment. We can therefore conclude with confidence that the newly identified *aph*(3')-II gene from *S. maltophilia* encodes a protein that modifies kanamycin, neomycin, butirosin, and paromomycin, thus contributing to the resistance of *S. maltophilia* isolates to these drugs and, together with the previously described AAC(6')-Iz, resistance to all aminoglycosides tested except gentamicin. Because this represents the third characterized example of an APH(3')-II enzyme, we have named it APH(3')-IIc.

In *S. maltophilia* K279a, *aph*(3')-IIc appears to be a chromosomal gene. There is no evidence of linked mobile genetic elements or of a difference in guanine-cytosine content indicative of the gene being acquired recently by *S. maltophilia* (data not shown). To confirm whether this is the case, we used PCR to test for the presence of homologues of the *aph*(3')-IIc gene in a collection of previously characterized clinical *S. maltophilia* isolates collected from Europe and North, South, and Central America (7). PCR using the *aph*-F and *aph*-R primer set (above) gave strong PCR products of the correct size when genomic DNA from 50% of the isolates was used as template; some other isolates gave very weak bands (data not shown). We therefore made another primer set to amplify a 344-bp region of the core of K279a *aph*(3')-IIc beginning 218 bp downstream of its start. The primers were *aph*int-F (5'-AGG AACAGGGACAAACCG-3') and *aph*int-R (5'-CCAGGTCTTCATGATCGG-3'). Intriguingly, the use of this second primer set gave appropriately sized PCR products only when the template used was genomic DNA from those isolates that did not give a strong positive PCR product with the full-length primer set (data not shown). The exception to this rule is K279a itself, which gave a product with both primer sets. Therefore, these data suggest that while a homologue of the *aph*(3')-IIc gene is present in all the isolates tested, supporting

our conclusion that this is an intrinsic resistance gene in *S. maltophilia*, there is some sequence heterogeneity. This was not unexpected given the degree of genetic variability known to occur among different *S. maltophilia* isolates (3, 7, 8).

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