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Role of the acetyltransferase AAC(6′)-*Iz* modifying enzyme in aminoglycoside resistance in *Stenotrophomonas maltophilia*

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***Stenotrophomonas maltophilia* is an emerging nosocomial pathogen that displays high-level intrinsic resistance to multiple antibiotics including aminoglycosides. A gene [*aac(6′)-Iz*] encoding an aminoglycoside-modifying enzyme, AAC(6′)-*Iz* acetyltransferase, was recently cloned and sequenced in *S. maltophilia*, but its importance with respect to aminoglycoside resistance in this organism was not determined. Using a homologous gene replacement approach, mutants carrying unmarked chromosomal deletions of the *aac(6′)-Iz* gene were constructed in wild-type and *in vitro*-selected aminoglycoside-resistant *S. maltophilia*. AAC(6′)-*Iz*-deficient mutants derived from both wild-type and aminoglycoside-resistant strains displayed an increase in susceptibility to amikacin, netilmicin, sisomicin and tobramycin (4- to 32-fold decrease in MICs), known substrates for AAC(6′)-I enzymes. The cloned *aac(6′)-Iz* gene restored the aminoglycoside resistance of the *aac(6′)-Iz* mutants, and could also confer aminoglycoside resistance upon *Escherichia coli*. To assess the significance of the *aac(6′)-Iz* gene with respect to the aminoglycoside resistance of clinical strains, its distribution was assessed in 65 clinical isolates from two hospitals. Using PCR, Southern hybridization, RT–PCR and/or nucleotide sequencing, the *aac(6′)-Iz* gene was identified in 57% of the isolates. Susceptibility tests indicated a good correlation between the presence of the *aac(6′)-Iz* gene and the resistance to tobramycin, netilmicin and sisomicin in these strains. These results indicate that the *aac(6′)-Iz* gene is an important contributor to aminoglycoside resistance in clinical strains of *S. maltophilia*, particularly to tobramycin.**

Keywords: aminoglycoside, resistance, *Stenotrophomonas maltophilia*

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Introduction

Previously known as *Pseudomonas maltophilia* and *Xanthomonas maltophilia*, *Stenotrophomonas maltophilia* is an aerobic Gram-negative bacterium ubiquitous in nature.[1] This organism has increasingly emerged as a nosocomial pathogen, particularly as a cause of life-threatening infections in immunocompromised patients.[2–5] An important feature of *S. maltophilia* is that this microorganism displays high-level intrinsic resistance to a variety of classes of antibiotics, including β-lactams, quinolones and aminoglycosides.[6–10] This high-level resistance makes the treatment of *S. maltophilia* infections difficult and also constitutes one of the important dangers of colonization and infection with this bacterium.[3,8,11,12]

S. maltophilia shows unusually high levels of resistance to aminoglycoside antibiotics, despite the fact that most Gram-negative bacilli are generally quite susceptible to this class of antibiotics.[7,13–15] Mechanisms of bacterial resistance to aminoglycosides are many, and include reduced uptake, mutational modification of the 16S rRNA, mutational modification of ribosomal proteins, enzymic modification of the 16S rRNA and enzymic modification of antibiotics.[16,17] More recently, active efflux of aminoglycosides has emerged as an additional mechanism of aminoglycoside resistance in Gram-negative bacteria,[18,19] including *S. maltophilia*.[10,20] Still, aminoglyco-

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side resistance among Gram-negative bacilli is predominantly due to enzymic modification of aminoglycosides by a family of enzymes, including *O*-nucleotidyltransferases, *O*-phosphotransferases and *N*-acetyltransferases (AACs).[16,17,21] The latter, AACs, are acetylCoA-dependent acetyltransferases that primarily modify amino groups and often lead to resistance to the aminoglycosides that possess amino groups.[21,22] The presence of an AAC aminoglycoside-modifying enzyme in *S. maltophilia* was first suggested by King *et al.*,[23] whereas Vanhoof *et al.*[24] postulated an *O*-nucleotidyltransferase in this organism. Still, the genetic identity of the modifying enzymes in *S. maltophilia* remained unknown. A gene encoding the AAC(6′)-*Iz* acetyltransferase of *S. maltophilia* was previously cloned and sequenced.[25] This enzyme was proposed to be the determinant for intrinsic aminoglycoside resistance in *S. maltophilia*,[25] although no direct evidence was provided. Here we report on the influence of chromosomal deletions of the *aac(6′)-Iz* gene on aminoglycoside resistance of *S. maltophilia*, and the distribution of the *aac(6′)-Iz* gene in clinical isolates of *S. maltophilia*.

Materials and methods

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *S. maltophilia* strain K1668 derived from strain K1449 was a multidrug-resistant mutant selected on ciprofloxacin and cefsulodin, and this mutant strain displayed increased resistance to aminoglycosides, β-lactams and fluoroquinolones via an efflux-mediated mechanism.[20] Sixty-five clinical isolates of *S. maltophilia* included strains K1013 to K1029 from Kingston General Hospital (Kingston, Ontario, Canada) and strains K1319 to K1366 from Mount Sinai Hospital (Toronto, Ontario, Canada). Luria–Bertani (LB) broth [1% (w/v) Difco tryptone, 0.5% (w/v) Difco yeast extract and 0.5% (w/v) NaCl] and agar [LB broth containing 1.5% (w/v) agar] were used as the growth media throughout, and bacterial cells were cultivated at 37°C. In some instances, aminoglycoside susceptibilities were carried out using nutrient broth (NB) [5% (w/v) Difco beef extract, 3% (w/v) Difco peptone]. Plasmids were maintained in *E. coli* with appropriate antibiotic selection [pBluescript II SK(+), 100 mg/L ampicillin; pRK415[20] and pEX18Tc,[26] 10 mg/L tetracycline; and pRK2013,[20] 50 mg/L kanamycin].

Antimicrobial susceptibility assay

Drug susceptibility testing was carried out by two-fold serial dilution using LB or NB media (1 mL) with an inoculum of 5×10^5 cells/mL. Data were reported as MICs, which reflected the lowest concentration of antibiotic inhibiting visible cell growth after an overnight incubation at 37°C.

Table 1. *S. maltophilia* strains and plasmids used in this study

Strain or plasmid	Description[a]	Source or reference
Strains		
ATCC13637	wild-type, parent strain	29
ULA-511	wild-type, parent strain	28
K1449	L1 and L2 β-lactamase-deficient mutant of ULA-511	28
K1668	multidrug-resistant derivative of K1449	this study
K1669	K1449 Δ*aac(6′)-Iz*	this study
K1670	K1668 Δ*aac(6′)-Iz*	this study
K1319	clinical isolate	this study
K1671	K1319 Δ*aac(6′)-Iz*	this study
K1324	clinical isolate	this study
K1672	K1324 Δ*aac(6′)-Iz*	this study
Plasmids		
pBluescript II SK(+)	phagemid cloning vector; 2.96 kb; MCS Ap^r	Stratagene
pRK415	broad-host-range cloning vector; 10 kb, p*lac* MCS Tc^r	20
pEX18Tc	broad-host-range gene replacement vector; 6.35 kb; *sacB* Tc^r	26
pRK2013	broad-host-range helper vector; Tra^+ Km^r	20
pLZ650	pBluescript II SK(+)::Δ*aac(6′)-Iz*	this study
pLZ651	pBluescript II SK(+)::*aac(6′)-Iz*	this study
pLZ653	pEX18Tc::Δ*aac(6′)-Iz*	this study
pLZ655	pRK415::*aac(6′)-Iz*	this study

[a] Ap^r, ampicillin resistant; Km^r, kanamycin resistant; Tc^r, tetracycline resistant; MCS, multiple cloning site; p*lac*, *lac* promoter.

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DNA methodology

Basic DNA procedures, including restriction endonuclease digestions, ligations, transformations and agarose gel electrophoresis were carried out as described by Sambrook *et al*.[27] The alkaline lysis method or a plasmid midi kit (Qiagen Inc., Mississauga, Ontario, Canada) was used to isolate plasmids from *E. coli* DH5α and *S. maltophilia*.[27] The genomic DNA of *S. maltophilia* was extracted as described previously.[28] DNA fragments used in cloning were extracted from agarose gels using Prep-A-Gene (Bio-Rad Labs, Richmond, CA, USA) as per the manufacturer's instructions. Nucleotide sequencing of plasmid-borne DNA or PCR products was carried out by Cortec DNA Services Inc. (Kingston, Ontario, Canada) using universal or custom primers. Compilation of DNA sequence data was carried out using DNAMAN (Version 4.11; Lynnon Biosoftware, Vaudreuil, Quebec, Canada).

PCR amplification and cloning of aac(6′)-Iz

The *aac(6′)-Iz* gene (GenBank accession no. AF140221) was amplified from genomic DNA of *S. maltophilia* ULA-511 on a ~1 kb fragment using primers smaac1xz and smaac4xz (Table 2) and conditions described previously,[20] although with a modified annealing temperature of 56°C for 1 min. The *aac(6′)-Iz*-containing PCR product was purified using a Qiaquick PCR purification kit (Qiagen Inc.), and following digestion of the PCR products with *Hin*dIII and *Sst*I it was cloned into *Hin*dIII–*Sst*I-restricted pBluescript II SK(+). The nucleotide sequence of *aac(6′)-Iz* in the resultant plasmid, pLZ651, was confirmed by nucleotide sequencing. The *aac(6′)-Iz* gene of pLZ651 was then released by digestion with *Hin*dIII and *Sst*I, and cloned into *Hin*dIII–*Sst*I-restricted pRK415, yielding plasmid pLZ655. This plasmid was subsequently mobilized from *E. coli* DH5α into *S. maltophilia* via a triparental mating procedure employing the helper strain MM294 carrying vector pRK2013.[20] Transconjugants were selected on LB agar supplemented with 5 mg/L norfloxacin (to counterselect the *E. coli*) and 50 mg/L tetracycline (to select plasmid-bearing transconjugants). PCR as above was also used to assess the presence of the *aac(6′)-Iz* gene in 65 clinical isolates of *S. maltophilia*.

Construction of Δaac(6′)-Iz mutants

To construct Δ*aac(6′)-Iz* mutants, two PCR assays were carried out to amplify two ~0.5 kb DNA fragments [upstream and downstream of the *aac(6′)-Iz* gene sequence to be deleted]. Sequences 5′ to the deletion were amplified from genomic DNA of *S. maltophilia* ULA-511 using primers smaac1xz and smaac2xz (Table 2), whereas sequences 3′ to the deletion were amplified using primers smaac3xz and smaac4xz (Table 2). The two PCR products were digested with *Hin*dIII–*Xba*I and *Xba*I–*Sst*I, respectively, and cloned into *Hin*dIIIa–*Sst*I-restricted pBluescript II SK(+) via three-piece ligation, yielding pLZ650. The intragenic deletion of 107 bp generated in the *aac(6′)-Iz* gene of pLZ650 was confirmed by nucleotide sequencing. Following digestion of pLZ650 with *Hin*dIII and *Sst*I, the Δ*aac(6′)-Iz* gene-containing fragment was cloned into gene replacement vector pEX18Tc previously digested with *Hin*dIII and *Sst*I. The resultant plasmid, pLZ653, was used to transform *E. coli* S17-1, from which it was mobilized into strains K1449 and K1668 and several clinical isolates via conjugation as described.[10,20] Transconjugants carrying pLZ653 in the chromosome were selected on LB agar containing tetracycline (40 mg/L for strain K1449; 60 mg/L for strain K1668; 50 mg/L for strains K1319 and K1324) and norfloxacin (2.5–5 mg/L; for counter-selection). Transconjugants were then streaked on to LB agar containing 10% (w/v) sucrose and sucrose-resistant colonies arising after overnight incubation at 37°C were screened for the presence of the *aac(6′)-Iz* deletion using PCR and/or Southern hybridization (see below).

Southern hybridization

Genomic DNA of *S. maltophilia* was digested with *Pst*I and the resultant DNA fragments were separated on a 0.8% (w/v) agarose gel before being blotted onto positively charged Nylon membranes (Roche, Laval, Quebec, Canada). Mem-

Table 2. Oligonucleotide sequences

Oligonucleotide name	Oligonucleotide sequence
Smaac1xz	5′-ACATAAGCTTGTTCCTGTGGCGCAGCCT-3′
Smaac2xz	5′-ACGTTCTAGACTGCGTCAGCTCCTCCA-3′
Smaac3xz	5′-ACGTTCTAGAGTCTTCGCCGGTGGGGTTCCTG-3′
Smaac4xz	5′-ACTAGAGCTCGCGCCGAGGCAGAATTCCA-3′
Smaac5xz	5′-CAGTTGCGTCTCGGCCTG-3′
Smaac6xz	5′-ATGCGGAAATAGACGACC-3′
Smaac7xz	5′-CTGTGGCCTGATGCCGATGA-3′
Smaac8xz	5′-GCGACTGTCCGAAGCCAGTT-3′

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Table 3. Effect of the *aac(6′)-Iz* gene on aminoglycoside susceptibility of *S. maltophilia* and *E. coli*

Strain	Relevant characteristics	Plasmid[a]	MIC (mg/L) AMK	GEN	KAN	NEO	NET	SIS	STR	TOB
S. maltophilia										
K1449	parent	none	8	8	4	4	8	8	16	8
K1449	parent	pRK415	8	8	4	4	8	8	16	16
K1449	parent	pLZ655	32	8	8	16	64	64	16	128
K1669	K1449 Δ*aac(6′)-Iz*	none	4	2	4	2	2	1	16	0.5
K1669	K1449 Δ*aac(6′)-Iz*	pRK415	4	2	4	2	4	0.5	16	1
K1669	K1449 Δ*aac(6′)-Iz*	pLZ655	32	8	8	16	32	64	16	128
K1668	K1449 MDR	none	64	16	64	64	64	64	64	64
K1668	K1449 MDR	pRK415	64	16	64	64	64	64	64	128
K1668	K1449 MDR	pLZ655	128	16	64	64	128	128	64	512
K1670	K1668 Δ*aac(6′)-Iz*	none	32	8	64	64	8	2	64	2
K1670	K1668 Δ*aac(6′)-Iz*	pRK415	32	8	64	64	4	1	64	4
K1670	K1668 Δ*aac(6′)-Iz*	pLZ655	128	16	64	64	128	64	64	512
E. coli										
DH5α	wild-type	pRK415	0.016	0.004	0.008	0.016	0.004	0.001	0.064	0.008
DH5α	wild-type	pLZ655	0.032	0.008	0.016	0.032	0.032	0.008	0.064	0.032

AMK, amikacin; GEN, gentamicin; KAN, kanamycin; NEO, neomycin; NET, netilmicin; SIS, sisomicin; STR, streptomycin; TOB, tobramycin.
[a]Plasmid pRK415, vector; pLZ655, carrying *aac(6′)-Iz*.

branes were subjected to overnight hybridization with an *aac(6′)-Iz*-specific probe and detected using the DIG High Prime DNA labelling and detection starter kit II (Roche) as per the manufacturer's instructions. The *aac(6′)-Iz* probe was obtained from pLZ651 by PCR amplification of a 370 bp intragenic fragment using primers smaac5xz and smaac6xz as above. The *aac(6′)-Iz* PCR product was purified as above and labelled using the DIG High Prime DNA labelling kit (Roche) according to the manufacturer's instructions.

RT–PCR

RT–PCR was carried out as published previously[28] using the *aac(6′)-Iz*-specific primers smaac5xz and smaac6xz and 40 cycles of amplification.

Results and discussion

Influence of the aac(6′)-Iz gene on aminoglycoside resistance

S. maltophilia generally displays high level resistance to a wide variety of antibiotics, including aminoglycosides,[6,10,28,29] a result confirmed here (Tables 3 and 4). To assess the role of the previously described *aac(6′)-Iz* gene in the aminoglycoside resistance of *S. maltophilia*, chromosomal deletions of the *aac(6′)-Iz* gene were carried out as described in Materials and methods. Elimination of *aac(6′)-Iz* in the wild-type *S. maltophilia* strain K1449 (yielding strain K1669) resulted in an increase in susceptibility to the 2-deoxystreptamine aminoglycoside antibiotics including netilmicin, sisomicin, tobramycin and neomycin. This is not unexpected as these aminoglycosides are known substrates for the AAC(6′)-I-modifying enzymes since their 6′-NH_2 group of the 6-aminohexose is subject to acetylation.[21,22,25,30] Interestingly, gentamicin susceptibility is also increased four-fold upon loss of the *aac(6′)-Iz* gene (Table 3). Gentamicin is a complex of four closely related subspecies C1, C1a, C2 and a minor component C2a, where all differ structurally at the site of acetylation, the 6′-NH_2 group.[22] Gentamicins C1a, C2 and C2a, however, do present a 6′-NH_2 and are likely to be modified at this site.[22] These gentamicin components are similarly modified by other AAC(6′)-I enzymes.[31–33] The observation that susceptibility to kanamycin was not affected by loss of AAC(6′)-Iz is, perhaps, surprising given the presence of the 6′-NH_2 on this aminoglycoside. Still, it has previously been demonstrated that the *in vitro* substrate profiles of aminoglycoside-modifying enzymes do not necessarily completely mirror the aminoglycoside resistance profile of organisms expressing these enzymes.[31,33,34] Also, aminoglycoside resistance is often multifactorial in nature, being affected by, for example, decreased permeability, active efflux and multiple aminoglycoside-modifying enzymes. Thus, the lack of an effect of the *aac(6′)-Iz* deletion on kanamycin resistance in strains K1669 and K1670 might be explained by the presence of an aminoglycoside phosphotransferase [APH(3′)] in these

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Table 4. Status of the *aac(6′)-Iz* gene and aminoglycoside susceptibility of clinical isolates of *S. maltophilia*[a]

Strain[b]	Status of *aac(6′)-Iz*[c]	MIC (mg/L)							
		AMK	GEN	KAN	NEO	NET	SIS	STR	TOB
ULA-511	+	16	16	16	64	8	2	64	32
K1013 (2)	+	16	16	8	16	8	2	32	32
K1016 (8)	–	2	1	1	2	0.5	0.125	8	1
K1018 (12)	–	<1	<0.5	1	<2	0.03	0.125	8	0.5
K1019 (14)	+	8	8	8	16	4	4	32	16
K1023 (22)	–	4	1	4	2	0.5	0.125	32	0.5
K1028 (16098)	+	8	8	8	8	8	2	16	32
K1319 (116)	+	64	64	256	256	>64	128	512	512
K1671 [K1319 Δ*aac(6′)-Iz*]	–	32	16	64	256	16	2	512	4
K1320 (158)	–	16	2	8	32	0.5	0.5	64	2
K1321 (112)	+	128	32	128	>256	2	0.25	128	16
K1323 (33)	+	32	32	32	32	32	8	32	64
K1324 (128)	+	8	4	4	8	16	4	16	2
K1672 [K1324 Δ*aac(6′)-Iz*]	–	8	4	4	8	8	1	16	2
K1328 (72)	–	16	8	32	128	2	2	64	8
K1329 (63)	–	64	8	128	256	4	2	128	8
K1337 (20)	–	4	2	1	8	0.5	0.25	32	1
K1351 (188)	+	32	16	16	256	>64	64	64	256
K1352 (76)	–	16	3	8	16	2	1	64	4
K1353 (13)	+	32	4	128	256	1	0.5	64	16
K1354 (62)	–	32	16	32	32	4	2	128	8
K1355 (75)	–	32	8	16	16	4	1	128	4
K1356 (32)	+	16	32	16	32	32	8	32	64
K1357 (27)	+	32	32	16	128	>64	128	64	256
K1360 (152)	+	128	32	256	1024	64	16	512	512
K1366 (140)	+	64	16	128	1024	8	2	128	32

[a]MIC was determined in nutrient broth at 37°C after 24 h incubation. Abbreviations are defined in the legend of Table 3.
[b]Hospital laboratory designations in parentheses.
[c]The status of the *aac(6′)-Iz* gene was determined by PCR, Southern hybridization and/or nucleotide sequencing. +, present; –, absent.

strains. Although AAC(6′)-Iz is the only aminoglycoside-modifying enzyme to be described to date in *S. maltophilia*, Vanhoof *et al.*[24] have proposed the presence of other aminoglycoside-modifying enzymes in this organism.

Deletion of the *aac(6′)-Iz* gene also compromised the aminoglycoside resistance of the multidrug-resistant mutant K1668 (see strain K1670), although resistance levels remained higher than that observed for the Δ*aac(6′)-Iz* derivative of wild-type strain K1449 (i.e. strain K1669). This makes sense since the elevated aminoglycoside resistance seen in strain K1668 was attributable to active efflux and/or other unidentified aminoglycoside-modifying enzymes, and was not expected to be affected by the *aac(6′)-Iz* deletion.[20] The *aac(6′)-Iz* deletion did not influence the activity of other antibiotics, including fluoroquinolones (norfloxacin and ciprofloxacin), tetracycline and chloramphenicol (data not shown). As expected, the cloned *aac(6′)-Iz* gene (pLZ655) restored the aminoglycoside resistance of the Δ*aac(6′)-Iz* mutants and enhanced the resistance of wild-type and multidrug-resistant strains of *S. maltophilia* (Table 3). These results confirm the role of the *aac(6′)-Iz* gene in aminoglycoside resistance in *S. maltophilia*. Moreover, introduction of the cloned *aac(6)-Iz* gene into *E. coli* also enhanced resistance to tobramycin, netilmicin and sisomicin (four- to eight-fold increase in the MICs; Table 3), indicating that the acetyltransferase is operational in other organisms, where it can contribute to aminoglycoside resistance.

Conservation of the aac(6′)-Iz gene in S. maltophilia

To determine whether the *aac(6′)-Iz* gene was ubiquitous in *S. maltophilia*, amplification of the gene was carried out from a number of clinical strains using primers that annealed upstream and downstream of the *aac(6′)-Iz* gene present in strain ULA-511. Of the two reference strains (i.e. ATCC-13637 and ULA-511) and 65 clinical isolates tested, the resultant PCR products generally displayed three patterns. First, most *S. maltophilia* strains (37/65) yielded a 1 kb frag-

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ment, the size expected from the sequence data (GenBank accession no. AF140221),[25] and DNA sequencing of several of these (e.g. ULA-511) confirmed the presence of the intact *aac(6′)-Iz* gene.[25] Less frequently (21/65), a PCR product of ~0.6 kb was obtained. Nucleotide sequencing of this smaller fragment, obtained from six representative clinical isolates, K1018 and K1025 from Kingston General Hospital, and K1337, K1352, K1354 and K1355 from Mount Sinai Hospital in Toronto, revealed the absence of the *aac(6′)-Iz* gene. Interestingly, however, the nucleotide sequences immediately upstream and downstream of the *aac(6′)-Iz* gene[25] (GenBank accession no. AF140221, derived from ATCC13637) were retained in these apparently *aac(6′)-Iz*$^-$ strains, indicating that there was a specific loss of the *aac(6′)-Iz* gene only. Several base pair differences were, however, noted in these upstream/downstream sequences in the various *aac(6′)-Iz*$^-$ mutants examined, indicating that they were probably not derived from a single clone. Some strains (7/65) produced inconsistent PCR products of variable size, which might indicate the absence of the *aac(6′)-Iz* gene or, possibly, alterations in sequences immediately upstream or downstream of the gene. A second PCR was undertaken using *aac(6′)-Iz*-specific intragenic primers (i.e. smaac5xz and smaac6xz, or smaac7xz and smaac8xz; Table 2). The expected PCR product of ~0.3 kb was amplified from genomic DNA of those strains containing the *aac(6′)-Iz* gene (e.g. strains ATCC13637, ULA-511, K1019, K1327, K1341 and K1366) (data not shown). In contrast, those strains that previously failed to amplify a 1 kb fragment using primers annealing immediately upstream and downstream of the *aac(6′)-Iz* gene also failed to yield a PCR product with the intragenic primers (data not shown). These data are consistent with the absence of the acetyltransferase gene in these strains.

Using a combination of dot blots, Southern hybridization with an intragenic *aac(6′)-Iz*-specific probe and PCR, Lambert *et al.*[25] previously demonstrated that this gene was conserved in all 80 strains examined and at the same location in the genome. To reconcile these data with our own, Southern hybridization was employed to re-examine the distribution of the *aac(6′)-Iz* gene in the aforementioned *S. maltophilia* clinical isolates (Figure 1). Using a 370 bp intragenic *aac(6′)-Iz*-specific probe, strains confirmed as having the *aac(6′)-Iz* gene hybridized with the expected ~1 kb *Pst*I fragment (e.g. Figure 1). Intriguingly, strains confirmed as having a specific deletion of the *aac(6′)-Iz* gene also yielded a hybridization signal, which was consistently ~0.6 kb in size (e.g. Figure 1). This result was consistent with the probe hybridizing to the same *Pst*I fragment that was, however, smaller due to loss of the *aac(6′)-Iz* sequences. Possibly, the probe is able to cross-hybridize with sequences upstream or downstream of the *aac(6′)-Iz* gene, although no obvious *aac(6′)-Iz*-like sequences were identified. Alternatively, a related gene conserved elsewhere in the chromosome may be cross-hybridizing in these instances.[21] Interestingly, those strains

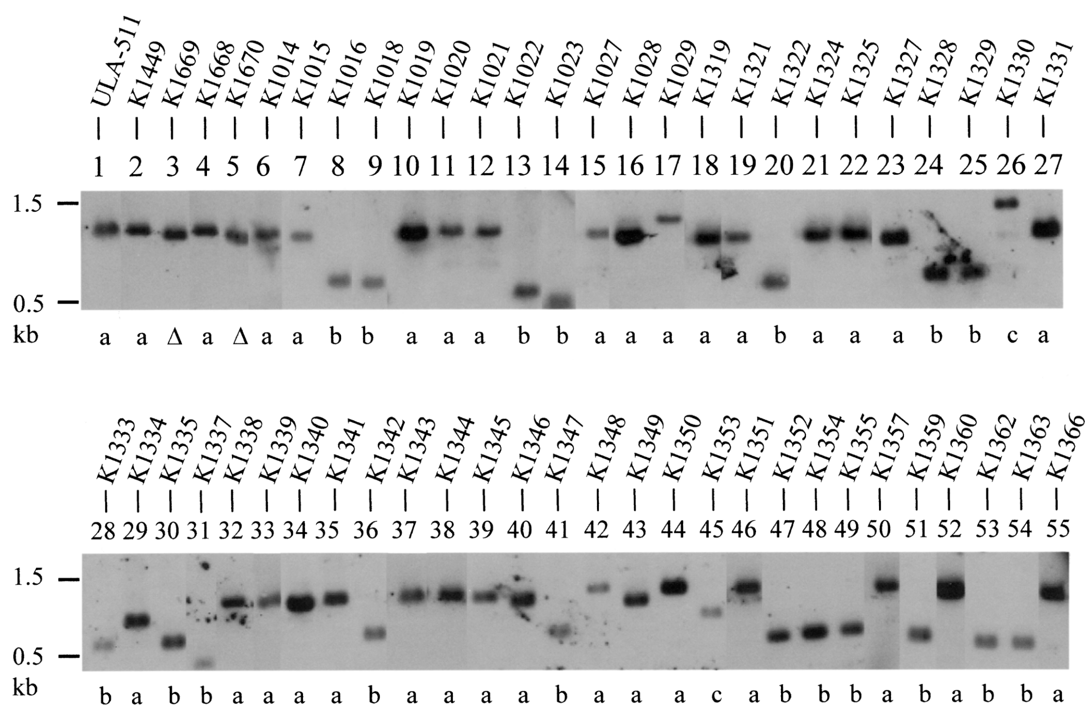

Figure 1. Southern hybridization of *Pst*I-digested genomic DNA of *S. maltophilia* strains using a DIG-labelled intragenic *aac(6′)-Iz* gene probe. Strain designations are indicated above the lanes. Three patterns of hybridization are seen and are indicated under the lanes by 'a' [~1.1 kb band that contains the *aac(6′)-Iz* gene], 'b' [~0.6 kb product that lacks the *aac(6′)-Iz* gene] or 'c' [inconclusive hybridizing signal(s)]. Hybridization patterns for strains carrying a 107 bp deletion in the *aac(6′)-Iz* gene are indicated by 'Δ'. DNA size markers are shown on the left.

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that failed to yield specific PCR products with primers internal or external to the *aac(6′)-Iz* gene also yielded modified hybridization signals, although these varied from strain to strain (e.g. Figure 1). Whether this means that an *aac(6′)-Iz* gene is present but in a variety of locations in the genomes of these strains, in contrast to earlier reports where its position appeared to be conserved,[25] or that this gene is absent and Southern hybridization is identifying related sequences is unclear. The fact that strains with precise deletions of the *aac(6′)-Iz* gene nonetheless elicit a hybridization signal certainly suggests that some cross-reactivity is possible with this probe. Moreover, the Lambert *et al.*[25] dot blot and PCR data were in complete agreement as regards the presence of the *aac(6′)-Iz* gene in all strains of *S. maltophilia* examined, again suggesting that the less stringent Southern hybridization might here be detecting related as opposed to *aac(6′)-Iz*-specific sequences. In any case, the *aac(6′)-Iz* gene does not appear to be completely conserved in *S. maltophilia*, and so is unlikely to be a housekeeping resistance gene. The differences between the results presented here and those reported by Lambert *et al.*[25] may reflect geographical issues (isolates from Canada versus isolates from France).

To assess whether there was a correlation between the presence of the *aac(6′)-Iz* gene and resistance to aminoglycosides in clinical strains of *S. maltophilia*, the susceptibility of 23 representative strains to several aminoglycosides was tested. As shown in Table 4, and with the exception of strain K1324, isolates possessing the *aac(6′)-Iz* gene were more resistant to tobramycin (MIC > 16 mg/L) than strains lacking this gene (MIC < 8 mg/L). The low tobramycin MIC for the *aac(6′)-Iz*-containing strain K1324 was explained, however, by the observation that deletion of the *aac(6′)-Iz* gene in this strain failed to impact tobramycin susceptibility, in contrast to deletions in other *aac(6′)-Iz*-containing strains (e.g. K1671), which substantially increased tobramycin susceptibility (see Table 4 and strain K1671). Apparently, the *aac(6′)-Iz* gene of strain K1324 is non-functional or its activity is occluded by other resistance mechanism(s). A similar correlation was observed when gentamicin susceptibility was examined, with MICs > 8 mg/L for most strains possessing the acetyltransferase gene (Table 4). Still, there was substantial variability in terms of the tobramycin resistance levels seen in the various *aac(6′)-Iz*-containing strains, with some strains, e.g. K1319 and K1360, highly resistant (MIC of 512 mg/L) with others, e.g. K1321 and K1353, much less so (MIC 16 mg/L). MICs of tobramycin will, to some extent at least, reflect expression of the *aac(6′)-Iz* gene and, indeed, RT–PCR seemed to support this (Figure 2). Expression of the *aac(6′)-Iz* gene was, for example, markedly higher in the former strains compared with the latter (Figure 2, compare lanes 4 and 8 with lanes 5 and 7). As expected, too, strains lacking *aac(6′)-Iz* (e.g. K1018 and K1023) failed to yield an RT–PCR product (Figure 2, lanes 2 and 3). The presence of the acetyltransferase

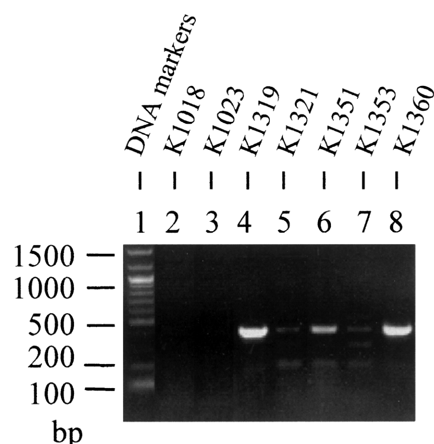

Figure 2. Expression of the *aac(6′)-Iz* gene in clinical isolates of *S. maltophilia* as assessed using RT–PCR. Strain designations are indicated above the lanes. DNA size markers are shown in lane 1.

also correlated with resistance to netilmicin (and to a lesser extent sisomicin), with *aac(6′)-Iz*-containing strains demonstrating netilmicin MICs of >4 mg/L, whereas those without this gene had MICs of <4 mg/L (Table 4). From a clinical standpoint, then, whereas the occurrence of the *aac(6′)-Iz* gene is not universal in *S. maltophilia*, its presence (and level of expression) correlates with resistance to tobramycin in particular and to some extent to gentamicin. As such, it is likely to be an important determinant of resistance to these agents in this organism.

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