Characterization of the Chromosomal aac(6\')-Iz Gene of Stenotrophomonas maltophilia

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Received 7 April 1999/Returned for modification 30 June 1999/Accepted 27 July 1999

The aac(6\')-Iz gene of Stenotrophomonas maltophilia BM2690 encoding an aminoglycoside 6\'-N-acetyltransferase was characterized. The gene was identified as a coding sequence of 462 bp corresponding to a protein with a calculated mass of 16,506 Da, a value in good agreement with that of ca. 16,000 found in vitro coupled transcription-translation. Analysis of the deduced amino acid sequence indicated that the protein was a member of the major subfamily of aminoglycoside 6\'-N-acetyltransferases. The enzyme conferred resistance to amikacin but not to gentamicin, indicating that it was an AAC(6\') of type I. The open reading frame upstream from the aac(6\')-Iz gene was homologous to the fprA gene of Myxococcus xanthus (61% identity), which encodes a putative pyridoxine (pyridoxamine) 5'-phosphate oxidase. Pulsed-field gel electrophoresis of total DNA from BM2690 and S. maltophilia ATCC 13637 digested with XbaI, DraI, and SpeI followed by hybridization with rRNA and aac(6\')-Iz-specific probes indicated that the gene was located in the chromosome. The aac(6\')-Iz gene was detected by DNA-DNA hybridization in all 80 strains of S. maltophilia tested. The MICs of gentamicin against these strains of S. maltophilia were lower than those of amikacin, netilmicin, and tobramycin, indicating that production of AAC(6\')-Iz contributes to aminoglycoside resistance in S. maltophilia.

Stenotrophomonas maltophilia (formerly Xanthomonas mal- tophila) is a free-living, ubiquitous, nonfermentative gram-negative bacillus. It is an important opportunistic nosocomial pathogen in neutropenic, immunocompromised, and debilitated patients. S. maltophilia is associated with a wide variety of clinical infections, including bacteremia, endocarditis, meningitis, respiratory and urinary tract infections, and wound sepsis (13). More recently, increasing chronic colonization by this bacterial species of the lower respiratory tract in patients with cystic fibrosis has been reported (2, 12).

S. maltophilia is characterized resistant to most broad-spectrum antimicrobial agents, including aminoglycosides, carbapenems, cephalosporins, penicillins, and quinolones. These antibiotics are seldom used alone (13), and synergism in vitro has been observed for certain combinations (5, 29, 49). The resistance of S. maltophilia is mainly attributed to permeability barriers (8, 25, 47). For instance, growth temperature-dependent variation in susceptibility to aminoglycosides, between 30 and 37°C, could be due to changes in the conformation of the outer membrane (30, 31, 46). Efflux pumps could also play a role in multiple resistance, but this mechanism has not yet been documented in this species. In addition to intrinsic resistance by impermeability, S. maltophilia can inactivate antibiotics by synthesis of detoxifying enzymes. Metallo-\beta-lactamases and cephalosporinases have been extensively investigated (9, 26, 36), and modification of aminoglycosides by O-nucleotidyl-ation and N-acetylation has been reported, but the responsible genes have not been identified (20, 45). Among the various aminoglycoside-modifying enzymes, the AAC(6\') family is of particular interest, since it modifies antibiotics of therapeutic importance, such as amikacin, isepamicin, gentamicin, netilmicin, and tobramycin. The sequences of 22 aac(6\') genes encoding type I enzymes which modify amikacin but not gentamicin have been determined. Certain genes are associated with mobile elements, including plasmids, transposons, and integron cassettes (4, 40), whereas others are species specific, such as aac(6\')-Ic of Serratia marcescens (41); aac(6\')-Ii of Enterococcus faecium (6); and aac(6\')-Ig, -Ir, -Ik, and -Ir to -Iw of proteolytic genomospecies of Actinobacter (22, 23, 34, 35). In this work, we describe the aac(6\')-Iz gene indigenous to S. maltophilia which contributes to aminoglycoside resistance in the species.

MATERIALS AND METHODS

Bacterial strains and plasmids. Epidemiologically unrelated clinical isolates of S. maltophilia from different sources, including strain BM2690 isolated from the sputum of a patient with cystic fibrosis, were used. The strains were isolated between 1992 and 1998 at the Hopital Saint Michel in Paris, France. Eight additional strains isolated from cystic fibrosis patients were from Hopital Robert Debré in Paris. S. maltophilia ATCC 13637 was also included in this study. Certain strains and plasmids used in this study are listed in Table 1.

Strain identification and growth conditions. Identification was performed with API 20 NE strips (bioMérieux, La-Balme-les-Grottes, France). All the isolates were resistant to imipenem. The strains were grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) or on Mueller-Hinton (MH) agar (Sanofi Diagnostics Pasteur, Marne-la-Couquette, France). MH medium supplemented with 0.005% 5-bromo-4-chloro-3-indolyl-\beta-D-galactopyranoside (IPTG) was used to detect production of \(\beta\)-galactosidase. MICs on MH agar containing serially twofold-diluted aminoglycosides were determined by the method of Steers et al. (42) with ca. 10\(^6\) CFU per spot. The plates were incubated at 37°C for 18 h. The activity of 2\(^\prime\)- and 6\(^\prime\)-N-ethyllactimicin was studied by diffusion on MH agar at 37°C with disks containing 100 \(\mu\)g of antibiotic.

Assay for aminoglycoside-modifying enzymes. The activity of aminoglycoside-modifying enzymes was detected in bacterial extracts by the phosphocellulose paper-binding technique (17). The reaction was allowed to proceed for 30 min at 37°C.

Genetic techniques. Transformation of Escherichia coli IM83 was performed as described previously (38). The antibiotics for selection were ampicillin (100 \(\mu\)g/ml) and tobramycin (5 \(\mu\)g/ml).

Preparation and analysis of DNA. Isolation of total DNA and large-scale preparation of plasmid DNA were done as described previously (38). Electrophoresis was performed in 0.8% agarose gels (Sigma Chemical Co., St. Louis, Mo.) with a Tris-borate buffer system.

For the analysis of total DNA by pulsed-field gel electrophoresis (PFGE), strains were grown in 2-ml volumes of brain heart infusion broth. After centrifugation at 32 g for 10 min, the cell pellet was suspended in 750 \(\mu\)l of 10 mM Tris base, 10 mM EDTA, 10 mM EGTA, 1 M NaCl (pH 7.5). Agarose plugs were prepared as described previously (17).

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made from a 1:1 mixture of 1.8% insert agarose (FMC BioProducts, Rockland, Maine) and the cell suspension. Each plug was placed in 1 ml of lysis buffer (6 mM Tris-HCl, 1 M NaCl, 100 mM EDTA, 0.5% Brij 58 [Sigma], 0.2% deoxycholic acid [Sigma], 0.5% N-lauroyl sarcosine [Sigma], 1 mg of lysosome [Sigma]/ml) for 1 h at 37°C. Genomic DNA in agarose plugs was then treated for 16 h at 55°C with 1 ml of TE buffer (10 mM Tris base, 1 mM EDTA) containing 100 μg of proteinase K (Sigma). After three 1-h washes with TE buffer, the plugs were digested with 20 U of restriction enzyme DnlI, XbaI, or SpeI (Boehringer, Mannheim, Germany) for 20 h at 37°C. They were then loaded into the wells of a 0.8% agarose gel in 45 mM Tris-borate, 1 mM EDTA (pH 8.0), and genomic DNA was separated by PFGE at 4.5 V/cm for 21 h at 14°C with a Chef DRIII apparatus (Bio-Rad, Ivory-sur-Seine, France). The pulse time was 5 to 50 s with linear ramping. Lambda ladder PFGE I (Boehringer) was incorporated as a size standard. The electrophoresis products were visualized by ethidium bromide staining for 30 min.

DNA techniques. For dot blotting and Southern hybridization, DNA was immobilized on Nytran membranes (Schleicher & Schuell, Dassel, Germany). Prehybridization and hybridization were carried out as described previously (38).

The aac(6′)-Iz-specific probe was obtained by amplification of a 367-bp fragment of plasmid DNA obtained from E. coli JM83/pAT680 with primers 5′-TGTACCCGTGATCGCCA and 5′-ACTGTCCGAAGCCAGTT deduced from E. coli JM83/pAT681, as previously (28), and radiolabeled with [32P]dCTP according to the recommendations of the manufacturer. The physical link between aac(6′)-Iz and the pdrH-like gene was studied with primers C (5′-AGCCAGATTGGCGCGTGG) and D (5′-TAGACGACCCGTTCGGTCTC) located in these genes, respectively (Fig. 1), under PCR conditions similar to those indicated above.

DNA sequencing. The 1.1-kb PstI fragment was cloned into bacteriophage M13 derivatives (Boehringer) and sequenced by the dideoxynucleotide chain terminating technique with synthetic oligonucleotides and the Sequenase version 2.0 DNA-sequencing kit (United States Biochemical Corp., Cleveland, Ohio) according to the recommendations of the manufacturer. DNA fragments were resolved by electrophoresis on 8% polyacrylamide gels containing 8 M urea. Nucleotide and amino acid sequences were analyzed and compared by using the GenBank, EMBL, and Swiss-Prot databases with the FASTA program (Genetics Computer Group, Madison, Wis.).

Analysis of plasmid-encoded proteins. The proteins specified by the recombinant plasmids were synthesized in a coupled in vitro transcription-translation system (50). The proteins were labeled with [35S]methionine and processed for electrophoresis in a sodium dodecyl sulfate-polyacrylamide gel as described previously (38).

Enzymes and chemicals. T4 DNA ligase was from Amersham (Little Chalfont, Buckinghamshire, England), lysosome was from Sigma Chemical Co., and RNase A (bovine pancreas) was from Calbiochem-Behring (La Jolla, Calif.). [32P]dCTP, [1-14C]acetyl coenzyme A, and [35S]methionine were from Boehringer. DNA fragments were labeled with [32P]dCTP according to the recommendations of the manufacturer. The physical link between aac(6′)-Iz and the pdrH-like gene was studied with primers C (5′-AGCCAGATTGGCGCGTGG) and D (5′-TAGACGACCCGTTCGGTCTC) located in these genes, respectively (Fig. 1), under PCR conditions similar to those indicated above.

TABLE 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli JM83</td>
<td>araD(lac mutant proAB)</td>
<td>[8040(lacZ)M15]</td>
</tr>
<tr>
<td>S. maltophilia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BM2690</td>
<td>Ak Gm Is Km Tm</td>
<td>Clinical isolate</td>
</tr>
<tr>
<td>ATCC 13637</td>
<td>Ak Gm Is Km Tm</td>
<td>Type strain</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUC18</td>
<td>Tra Mob Ap</td>
<td>48</td>
</tr>
<tr>
<td>pAT680</td>
<td>Tra Mob Ak Is Km Tm</td>
<td>1.1-kb PstI fragment from BM2690 into pUC18</td>
</tr>
<tr>
<td>pAT681</td>
<td>Tra Mob Ak Is Km Tm</td>
<td>903-bp EcoRI-PstI fragment from pAT680 into pUC18</td>
</tr>
</tbody>
</table>

"Tra", non-self-transferable; Mob, non mobilizable; Ak, amikacin resistance; Ap, ampicillin resistance; Is, isepamicin resistance; Gm, gentamicin resistance; Km, kanamycin resistance; Nt, netilmicin resistance; Tm, tobramycin resistance.

FIG. 1. Sequence of S. maltophilia aac(6′)-Iz and part of pdrH-like gene and predicted amino acid sequences. The putative ribosome binding site (RBS) and potential -35 and -10 promoter sequences are underlined. The start and stop codons are depicted in boldface, with the stop codons indicated by asterisks.
micin, and 6'-N-ethylnetilmicin, Schering-Plough Research Institute (Ken-
ilworth, N.J.); and nalidixic acid, Sterling Winthrop (New York, N.Y.).

Nucleotide sequence accession number. The nucleotide sequence of aac(6\(^{-}\)\(^{9}\))-Iz has been deposited in the GenBank data library under accession no. AF140221.

RESULTS AND DISCUSSION

Aminoglycoside resistance of \(S.\) \textit{maltophilia} BM2690. The MICs of aminoglycosides against \(S.\) \textit{maltophilia} BM2690 indicated that amikacin, isepamicin, netilmicin, and tobramycin were slightly less active against this strain than gentamicin (Table 2). Similar results were observed for most \(S.\) \textit{maltophilia} strains (Table 3). The variability in aminoglycoside MICs could be due to alterations in penetration of the antibiotics in the cells, as has been observed in \textit{Pseudomonas} spp. Disk-agar diffusion tests indicated that the activity of 2'-N-ethylnetilmicin against \(S.\) \textit{maltophilia} BM2690 was diminished compared with that of 6'-N-ethylnetilmicin. These two compounds have similar intrinsic activities against aminoglycoside-susceptible strains, and the difference observed can be taken as evidence for production of a 6'-N-acetyltransferase.

Aminoglycoside-modifying enzyme in \(S.\) \textit{maltophilia} BM2690. Extracts of \(S.\) \textit{maltophilia} BM2690 were shown to contain aminoglycoside acetyltransferase activity. The substrate profile of the enzyme was consistent with an AAC(6\(^{-}\)\(^{9}\))-I, since amikacin, gentamicins C1a and C2, isepamicin, kanamycin B, netilmicin, tobramycin, and 2'-N-ethylnetilmicin were modified whereas gentamicin C1 and 6'-N-ethylnetilmicin were not (data not shown).

Cloning and sequencing of the aac(6\(^{-}\)\(^{9}\))-I gene. Total DNA from BM2690 and pUC18 DNA were digested with PstI, mixed, ligated, and introduced by transformation into \(E.\) \textit{coli} JM83. Transformants selected on medium containing ampicillin plus tobramycin were screened for their plasmid content by agarose gel electrophoresis of crude bacterial lysates.

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC ((\mu g/ml))*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amikacin</td>
</tr>
<tr>
<td>(S.) \textit{maltophilia} BM2690</td>
<td>256</td>
</tr>
<tr>
<td>(E.) \textit{coli} JM83</td>
<td>0.5</td>
</tr>
<tr>
<td>(E.) \textit{coli} JM83/pAT680</td>
<td>4</td>
</tr>
</tbody>
</table>

* MICs determined on MH agar at 37°C.

FIG. 2. Alignment of the deduced amino acid sequences of AAC(6\(^{-}\)\(^{9}\))-I. In addition to \(S.\) \textit{maltophilia} AAC(6\(^{-}\)\(^{9}\))-Iz, the sequences are from \(S.\) \textit{marcescens} \[AAC(6\(^{-}\)\(^{9}\))-Ic\] (41), \(K.\) \textit{pneumoniae} \[AAC(6\(^{-}\)\(^{9}\))-I\] (43), \(Acinetobacter\) \textit{baumannii} [AAC(6\(^{-}\)\(^{9}\)))-I] (22), \(A.\) \textit{baumannii} [AAC(6\(^{-}\)\(^{9}\)))-I] (23), \(Acinetobacter\) sp. 6 [AAC(6\(^{-}\)\(^{9}\)))-I] (34), the PBW301 plasmid of \(E.\) \textit{aerogenes} [AAC(6\(^{-}\)\(^{9}\)))-I] (4), \(Acinetobacter\) sp. 14 [AAC(6\(^{-}\)\(^{9}\)))-I] (35), \(Acinetobacter\) sp. 15 [AAC(6\(^{-}\)\(^{9}\)))-I] (35), \(Acinetobacter\) sp. 16 [AAC(6\(^{-}\)\(^{9}\)))-I] (35), \(Acinetobacter\) sp. 17 [AAC(6\(^{-}\)\(^{9}\)))-I] (35), \(Acinetobacter\) ungrouped 640 [AAC(6\(^{-}\)\(^{9}\)))-I] (35), and \(Acinetobacter\) ungrouped BM2722 [AAC(6\(^{-}\)\(^{9}\)))-I] (35). The dashes indicate gaps introduced to optimize sequence similarity. The alignments were derived with the Clustal V program (19). Identical amino acids are indicated by asterisks. The dots indicate conservative amino acid substitutions corresponding to the following exchange groups: A, G, P, S, H, K, R, F, W, Y, D, E, N, Q, I, L, M, and V; and C (11).
smallest recombinant plasmid, pAT680, contained a 1.1-kb PstI fragment that conferred aminoglycoside resistance by synthesis of an AAC(6')-I. Analysis of the insert in pAT680 revealed two open reading frames (ORFs) homologous to sequences in the GenBank sequence library. Nucleotides 1 to 393 were 69% identical to the 3' terminus of the fprA gene, which encodes a putative pyridoxaminephosphate oxidase in Myxococcus xanthus (18). Nucleotides 411 to 849 were 59% identical to the aac(6')-Ic gene of S. marcescens. The search for stop codons indicated that the aac(6')-Iz gene was included in an ORF located between the TGA codons at coordinates 335 and 849 (Fig. 1). Two potential translation initiation codons, CTG and GTG, were located at positions 366 and 390, respectively, but neither was preceded by a typical ribosome binding site. This may account for the low-level aminoglycoside resistance observed in E. coli JM83/pAT680 (Table 1). Promoter sequences were not readily apparent by analysis of the region upstream from aac(6')-Iz; a putative promoter may consist of 235 (TTGGCG) (position 149 to 154) and 210 (TCGAAG) motifs separated by 17 nucleotides (the underlined nucleotides indicate identity with the consensus 235 and 210 promoter elements recognized by the E. coli σ70 factor). It is also possible that aac(6')-Iz and a pdxH-like gene form a single transcription unit, similar to aacC3 and cysC in Pseudomonas aeruginosa (44).

Analysis of the AAC(6')-Iz protein. To confirm that the ORF between nucleotides 335 and 849 encodes the AAC(6')-Iz protein,
tein, a 903-bp EcoRI-PstI fragment was subcloned into pUC18, generating pAT681. The proteins specified by pUC18 and pAT681 were characterized in an E. coli in vitro coupled transcription-translation system. A band of approximately 16,000 Da, which should correspond to the resistance protein, was encoded by pAT681 but not by the pUC18 control (data not shown). This apparent molecular mass was in good agreement with the 16,506 Da calculated for the predicted amino acid sequence. On the basis of sequence relationships, three subfamilies of AAC(6') have been distinguished. AAC(6')-I is a member of the major subfamily which contains AAC(6')-Ic specific for S. marcescens (41) (46% identity); AAC(6')-Id found in Klebsiella pneumoniae (39) (42% identity); AAC(6')-Ii from Enterobacter cloacae (43) (45% identity); AAC(6')-II from Enterobacter aerogenes (4) (44% identity); AAC(6')-Ih from Acinetobacter baumannii (23) (42% identity); and AAC(6')-Ig, -Ij, -Ik, -Ir, -Is, -It, -Iu, -Iv, and -Ix specific for the various species of proteolytic Acinetobacter (22, 23, 34, 35) (42 to 46% identity) (Fig. 2). The relatedness of the major subfamily AAC(6')-I is shown in Fig. 3.

Analysis of the protein deduced from the second ORF. The deduced C-terminal portion of the putative protein encoded by the truncated DNA sequence upstream from the aac(6')-Iz gene had homology with pyridoxine (pyridoxamine) 5'-phosphate oxidase proteins found in M. xanthus (18) (61% identity), Saccharomyces cerevisiae (24) (45% identity), and E. coli (21) (39% identity). It is likely that this DNA corresponds to the analogous gene in S. malophilia. Pyridoxine (pyridoxamine) 5'-phosphate oxidase is a key enzyme in biosynthesis of the essential coenzyme pyridoxal 5'-phosphate, which participates in many aspects of amino acid metabolism. The fact that, in S. malophilia BM2690, aac(6')-Iz was adjacent to a gene involved in essential host metabolism strongly suggests a chromosomal location for that gene. Furthermore, the G+C content of the aac(6')-Iz was 69% similar to that of S. malophilia, in agreement with an indigenous origin of this gene.

Distribution and location of the aac(6')-Iz gene. Total DNA from 80 S. malophilia strains was spotted on a Nytran membrane and hybridized with an intragenic aac(6')-Iz probe. The gene was found to be present in all the strains (data not shown). To determine the location of the aac(6')-Iz gene, PFGE was performed with total DNA of S. malophilia BM2690 and ATCC 13637 digested with XbaI, DraI, and SpeI. The fragments were transferred to Nytran membranes and hybridized successively with an rRNA and an aac(6')-Iz probe (Fig. 4). The aac(6')-Iz gene was located in a ca. 510-kb XbaI fragment in S. malophilia BM2690 and in a ca. 680-kb SpeI fragment in S. malophilia ATCC 13637 which cohybridized with the rRNA probe. These results confirmed that the aac(6')-Iz gene was located in the chromosome of S. malophilia. In addition, the adjacent location of aac(6')-Iz and pdhH-like genes in the remaining strains of S. malophilia was demonstrated by amplification of a 676-bp fragment with primers C, specific for pdhH, and D, specific for aac(6')-Iz (data not shown).

Conclusions. Like S. marcescens (41), E. faecium (6), and the various species of proteolytic Acinetobacter (35), S. malophilia harbors a “housekeeping” aac(6')-I. This gene, probably weakly expressed, contributes to aminoglycoside resistance in this species as indicated by the slight difference in susceptibility to gentamicin and to the other aminoglycosides (Table 3). The origin of aminoglycoside resistance genes is still a matter of controversy (33). A possibility is that they derive from antibiotic-producing microorganisms in which they are required for self-protection against the toxic aminoglycosides (3, 7, 10). Alternatively, they could derive from housekeeping genes involved either in cellular metabolism or in peptidoglycan or lipopolysaccharide metabolism (27). These two hypotheses are not mutually exclusive, and there are strong arguments in favor of each proposal, depending on the bacterial species considered. It has been established that the resident AAC(2')-Ia in Providencia stuartii can O-acetylate peptidoglycan (27, 32), and a similar function for the AAC(2')-Id of Mycobacterium seg- matis is possible (1). The functional role, if any, of AAC(6')-Iz in S. malophilia remains unknown.

ACKNOWLEDGMENTS

This work was supported by a grant from the Association Francaise de Lutte contre la Mucoviscidose and by a Bristol-Myers Squibb Unrestricted Biomedical Grant in Infectious Diseases. We thank E. Bingen for the gift of strains.

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