Identification and functional dissection of AAC(6')-Iaf, a novel isozyme of aminoglycoside 6'-*N*-acetyltransferase from multidrug-resistant *Pseudomonas aeruginosa*

（多剤耐性緑膿菌由来アミノグリコシド剤耐性因子 AAC(6')–Iaf の同定と機能解析）

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Abbreviations

AAC   aminoglycoside acetyltransferase

AG   aminoglycoside

AME   aminoglycoside modifying enzyme

BLAST   basic local alignment search tool

BSA   bovine serum albumin

CoA   coenzyme A

DTNB   5, 5'-Dithiobis(2-nitrobenzoic acid)

GCN5   general control non-repressed protein 5

GNAT   GCN5-related *N*-acetyltransferase

kb   kirobase (1000 base pairs)

MDR   multidrug-resistant, or multidrug-resistance

MIC   minimum inhibitory concentration

ORF   open reading frame

PAGE   polyacrylamide gel electrophoresis

PBS   phosphate buffer saline

PFGE   pulsed-field gel electrophoresis

PVDF   polyvinylidene fluoride

RDK   rapid diagnostic kit

SDS   sodium dodecyl sulfate

TLC   thin-layer chromatography

Tris   tris (hydroxymethyl) aminomethane

Chapter 1. Introduction

1-1. Clinical significance of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative bacterium that is widely distributed throughout nature, in, for example, soils, water, plants, and animals including humans. *P. aeruginosa* is a rod-shaped bacterium with a monotrichate flagellum and motility (Yoshida, 2007). Due to its simple nutritional requirements, *P. aeruginosa* can tolerate many physical conditions, allowing it to persist in various environments, including both community and hospital settings. Community reservoirs of this organism include swimming pools, whirlpools, hot tubs, contact lens solution, home humidifiers, soils and rhizospheres, and vegetables (Harris *et al*., 1984). In hospital, *P. aeruginosa* can be isolated from a variety of sources, including respiratory therapy equipment, antiseptics, soaps, sinks, mops, medicines, and hydrotherapy pools (Yoshida, 2007, Sekiguchi *et al*., 2007b).

Although *P. aeruginosa* is a ubiquitous organism, most serious infections due to it are acquired in hospitals, with compromised patients, including with cancer, cystic fibrosis, and severe burns, being at high risk for these infections (Morrison & Wenzel, 1984, Erol *et al*., 2004, Ohara & Itoh, 2003, Thuong *et al*., 2003). Rates of colonization in these patients may be over 50%, whereas representative rates of colonization in healthy humans are normally 0 to 2% for skin, 0 to 3.3% for the nasal mucosa, and 0 to 6.6% for the throat (Morrison & Wenzel, 1984).

1-2. Multidrug-resistant (MDR) *P. aeruginosa*

Since *P. aeruginosa* is intrinsically resistant to many antibiotics (Li *et al*., 1994, Lister *et al*., 2009), a limited number of antibiotics have been used to treat patients infected with this organism. These antibiotics include β-lactams such as ceftazidime and imipenem, aminoglycosides such as amikacin and tobramycin, and fluoroquinolones such as ofloxacin and ciprofloxacin. Recently, however, *P. aeruginosa* strains resistant to these antibiotics have emerged, and are becoming more widespread. The most serious problem is the emergence of multidrug-resistant (MDR) *P. aeruginosa* strains, which are resistant to all β-lactams, aminoglycosides and quinolones. In Japan, the criteria of the Ministry of Health, Labor, and Welfare define an MDR *P. aeruginosa* strain as one resistant to carbapenem (MIC $\geq$16 μg/ml), amikacin (MIC $\geq$32 μg/ml) and fluoroquinolone (MIC $\geq$4 μg/ml) (Kirikae *et al*., 2008). Infections caused by MDR *P. aeruginosa* are difficult to treat because of the limited susceptibility of these bacteria to antimicrobial agents, and many such infections are fatal. Hence, the rapid detection of such bacteria is crucial for early infection control measures, thus preventing nosocomial infections.

1-3. Aminoglycosides

Aminoglycosides are a vital component for antipseudomonal chemotherapy in patients with infectious diseases (Magnet & Blanchard, 2005). The aminoglycosides, which may be natural or semisynthetic soluble compounds, include amikacin, arbekacin, gentamicin, kanamycin, neomycin, netilmicin, paromomycin, tobramycin, and lividomycin. Due to the large numbers of amino and hydroxyl groups in each molecule, they are positively charged at biological pH. Aminoglycosides have been categorized into two classes, the 4,6-substituted and 4,5-substituted deoxystreptamines (Fig. 1-1) (Mingeot-Leclercq & Tulkens, 1999, Mingeot-Leclercq *et al*., 1999). The former class consists of 2-deoxystreptamine, with two sugar groups, a 6-aminohexose and a 3-aminohexose, attached to positions 4 and 6, respectively. Based on differences in their side chains, these compounds can be categorized into several derivatives. The 4,5-substituted deoxystreptamines consist of a common neamine, with a glycopyranosyl ring attached to position 4 of a 2-deoxystreptamine moiety. The core may be further substituted by one (ribostamycin), two (neomycin and paromomycin), or three (lividomycin A) additional sugars attached to position 5 of the 2-deoxystreptamine.

The primary target of aminoglycosides is the bacterial small ribosomal subunit. Aminoglycoside binding to the 16S rRNA, at the tRNA acceptor A site (aminoacyl site), inhibits the translation process by causing misreading and/or hindering the translocation step (Fig. 1-2) (Fourmy *et al*., 1996, Carter *et al*., 2000). This action stabilizes the interaction between transfer RNA and messenger RNA in the A site by decreasing transfer RNA dissociation rates, and interferes with proofreading steps that ensure translational fidelity.

A. 4,6-disubstituted
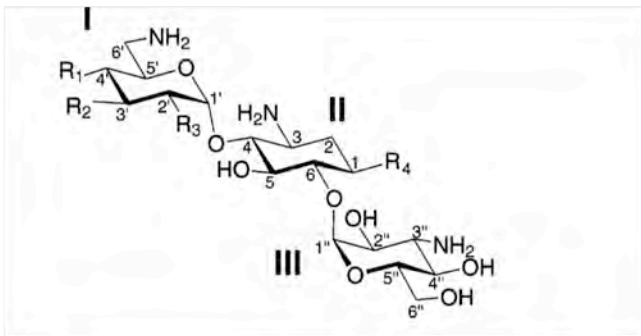
Aminoglycoside	R_1	R_2	R_3	R_4
Kanamycin A	OH	OH	OH	NH_2
Kanamycin B	OH	OH	NH_2	NH_2
Tobramycin	OH	H	NH_2	NH_2
Dibekacin	H	H	NH_2	NH_2
Arbekacin	H	H	NH_2	HN–C(OH)–...–NH_2
Amikacin	OH	OH	OH	HN–C(OH)–...–NH_2

B. 4,5-disubstituted
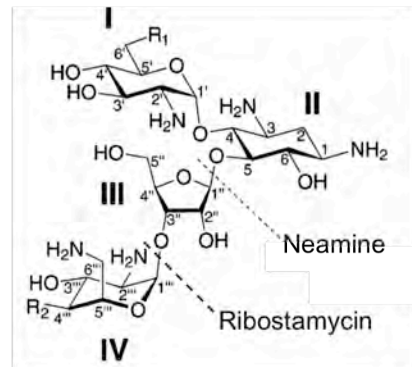
Aminoglycoside	R_1	R_2
Neamine	NH_2	OH
Ribostamycin	NH_2	OH
Neomycin B	NH_2	OH
Paromomycin	OH	OH
Lividomycin A	OH	O–

Fig. 1-1. Chemical structures of aminoglycosides.
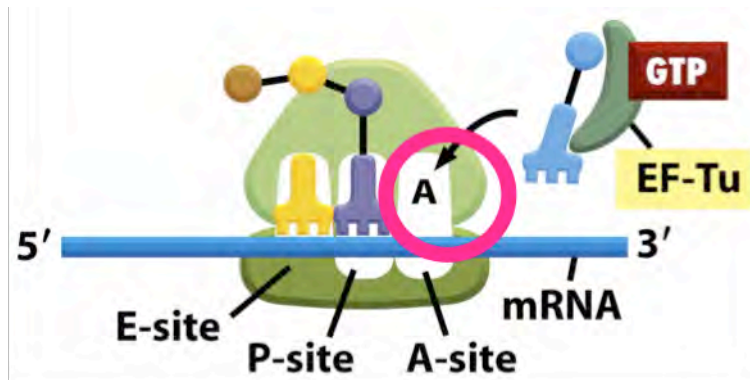
Molecular biology of the cell 4th.

Figure 1-2. Target site of aminoglycosides. The target site of aminoglycoside is enclosed in a red circle. The primed ring of aminoglycosides (ring I in Fig. 1-1) binds to the major groove at the A site of 16S ribosomal RNA.

1-4. Mechanisms of aminoglycoside resistance

Bacterial resistance to aminoglycosides can result from several causes, including decreased membrane permeability, the modification of 16S rRNA or ribosomal proteins, and the enzymatic modification of the aminoglycosides (Magnet & Blanchard, 2005). The first two causes are endogenous and are caused by the accumulation of mutations within the genome, whereas the third is exogenous. In *P. aeruginosa* isolates, resistance to aminoglycosides is due primarily to exogenous factor, the production of aminoglycoside-modifying enzymes (AMEs). To date, three types of AMEs have been identified in clinical isolates resistant to antibiotics:, aminoglycoside phosphoryltransferases (APHs), aminoglycoside acetyltransferases (AACs), and aminoglycoside adenylyltransferases (AADs) (Wright, 1999). Aminoglycoside modification catalyzed by

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these enzymes result in a marked reduction of aminoglycoside affinity for target ribosomal RNA, resulting in a reduced ability to inhibit bacterial translational.

AACs are one of AMEs that transfer acetyl groups of acetyl coenzyme A (acetyl-CoA) to the amino groups of aminoglycosides. The AACs can be grouped into four classes, AAC(1), AAC(2'), AAC(3'), and AAC(6'), based on the acetylation sites of the aminoglycosides side chains (Table 1-1) (Shaw *et al.*, 1993).

Table 1-1. Classification of aminoglycoside acetyltransferase.

Acetyltransferase	Type	Resistance to
AAC(6')	I *	AMK, DBK, KAN, SIS, TOB
	II	DBK, GEN, AKN, SIS, TOB
AAC(3)	Ia, Ib	GEN, SIS
	IIa, Iib, IIc	DBK, GEN, SIS, TOB
	IIIa, IIIb, IIIc	DBK, GEN, KAN, LIV, NEO, SIS, TOB
	IV	DBK, GEN, SIS, TOB
	VII	GEN
AAC(1)	-	LIV
AAC(2')	Ia	DBK, GEN, NEO, TOB

N-acetylation at the 6' position, catalyzed by AAC(6'), is one of the most prevalent forms of aminoglycoside modification observed in clinical isolates of *P. aeruginosa* clinical isolates resistant to aminoglycosides. Two types of AAC(6')s have been described: AAC(6')-I, which confers resistance to amikacin (AMK) but not to gentamicin (GEM), and AAC(6')-II, which confers resistance to GEM but not to AMK. To date, at least 30

AAC(6')-I enzymes, designated AAC(6')-Ia to AAC(6')-Iae, have been identified and characterized (Fig. 1-3). In Japan, 4 kinds of AAC(6')-I proteins, AAC(6')-Ib, -Iad, -Iae, and -Isa were identified in clinical isolates and laboratory strain (Hamano *et al*., 2004, Sekiguchi *et al*., 2005, Vakulenko & Mobashery, 2003, Doi *et al*., 2004). In contrast, only two AAC(6')-II enzymes have been identified to date.

The recent availability of representative crystal structures for AACs has greatly expanded our knowledge of enzyme function, revealing unexpected and exciting associations with other families of enzymes, such as eukaryotic histone acetyltransferase (Dyda *et al*., 2000). Furthermore, the complete genome sequences of several bacteria have revealed many potential aminoglycoside resistance elements.

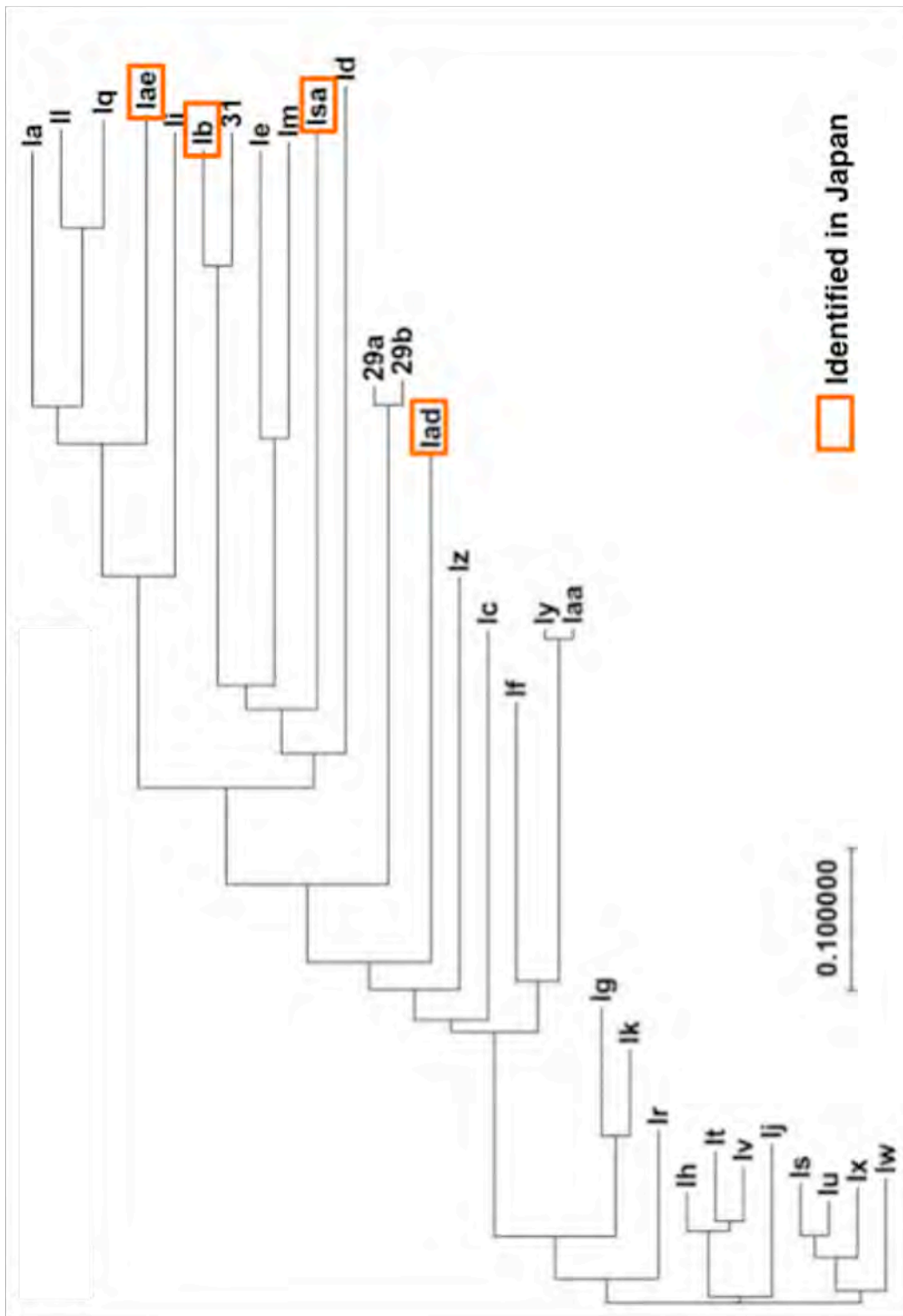**Figure 1-3.** Dendrogram for AAC(6')-I family proteins. The dendrogram was constructed using the ClustalW computer program, available on the National Institute of Genetics website (http://www.ddbj.nig.ac.jp/E-mail/clustalw-e.html). The figure was illustrated with the TreeViewPPC program. Branch lengths correspond to the numbers of amino acid exchanges.AAC(6')-I enzymes enclosed with orange square was identified in Japan.

1-5. Class 1 integron – Import system of antibiotic resistance genes

Most exogenous genes that encode antibiotic-inactivating enzymes are found in class 1 integrons (Levesque *et al.*, 1995, Rowe-Magnus & Mazel, 2002), posing a major problem in the treatment of infectious diseases. Integrons are genetic elements that contain the components of the genetic determinants of a site-specific recombination system that recognizes and captures gene cassettes (Stokes & Hall, 1989). Bacteria harboring integrons can incorporate and express genes with potentially adaptive functions, including antibiotic resistance genes, as described above, as well as several genes that encode proteins involved in other adaptive functions, including virulence factors, metabolic enzymes, and restriction enzymes (Ogawa & Takeda, 1993, Vaisvila *et al.*, 2001).

These integrons possess have been found to possess conserved segments at each end, separated by a variable region that could include antibiotic resistance gene cassettes (Fig. 1-4) (Hall & Collis, 1995, Hall *et al.*, 1994). The 5' conserved segment (5'CS) contains the *intI* gene, which encodes the enzyme integrase, as well as an adjacent recombination site (*attI*). The 3' conserved segment (3'CS) contains the *qacED1* and *sulI* genes, which are associated with resistance to disinfectants (Hall *et al.*, 1994).

Gene cassettes are integrated into integrons by site-specific recombination catalyzed by the integrase IntI (Mazel, 2006). This enzyme binds to both the *attI* site in the 5'CS of the integron and a short sequence, called the 59-base element (59-be), located downstream of the gene cassette (Stokes *et al.*, 1997). Although the 59-be itself is not highly conserved and its length varies from 57 to 141 bases, it has a two-fold axis of symmetry for site-specific recombination. There are two highly conserved core sites in the 59-be, a 7-bp core site with the consensus sequence GTTAGGC or GTTRRRY located at the right-hand (RH) end of the

element furthest from the 3' end of the cassette-encoded gene, and an inverse core site with the consensus sequence GCCTAAC or RYYYAAC at the left-hand (LH) end.
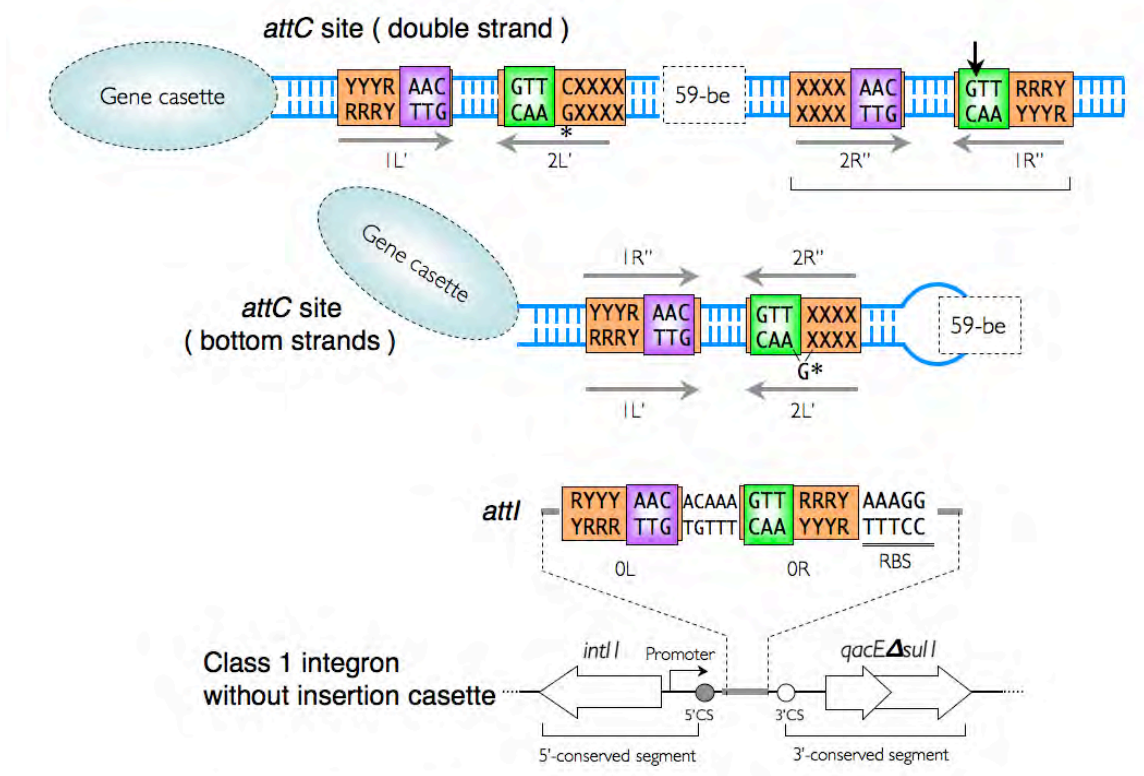
Figure 1-4. Class 1 integron structure and nucleotide sequences required for the integration of antibiotic resistance gene cassettes.

Genes imported into integrons are expressed primarily from a common promoter P1 located in the 5' CS, because gene cassettes generally do not include a promoter. Three versions of P_1 have been identified, containing different combinations of -35 and -10 bp sequences compared with the consensus sequences (Levesque *et al.*, 1994). These sequences include $TGGACAN_{17}$ (a strong promoter), $TGGACAN_{17}TAAGCT$ (a weak promoter), and $TGGACAN_{17}TAAACT$ (a hybrid promoter). When multiple cassettes are imported into an

integron, the genes adjacent to the common promoter P_1 are expressed with the highest efficiency.

In addition to importing gene cassettes, integrons have been found to possess the ability to exchange gene cassettes (Collis & Hall, 1992), suggesting that the order of the gene cassettes could change under the selective pressure of antibiotics. Furthermore, integrons can transfer among bacteria by phages, plasmids, and transposons (Rowe-Magnus *et al*., 2002). Since these horizontal transfers often lead to the rapid dissemination of multiple antibiotic resistance genes within a bacterial population (Fig.1-5), they are often associated with nosocomial outbreaks.
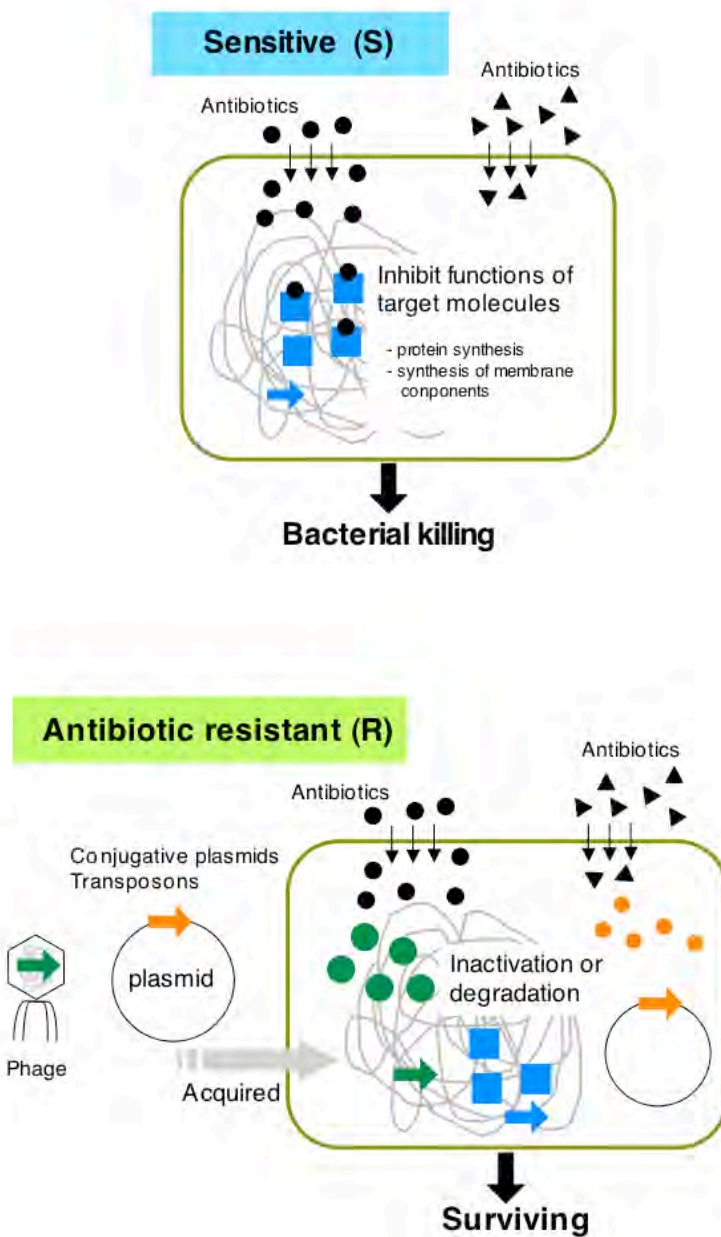
Figure 1-5. Mechanisms of horizontal transfer and acquisition of exogenous antibiotic resistance genes in bacteria.

1-6. Infection controls of drug resistant bacteria

The emergence and spread of antibiotic resistant, especially MDR, bacteria remains a global public health concern. In the fields of clinical microbiology and preventive medicine, nosocomial infections caused by antibiotic resistant bacteria have been monitored by pulsed-field gel electrophoresis (PFGE) and by the determination of antibiotic resistance profiles based on susceptibility testing (Jenney *et al*., 2003). Although these techniques are effective for understanding of nosocomial outbreak, it has never been determined exactly why bacteria become antibiotic resistant bacteria.

Many mechanisms responsible for antibiotic resistance have been identified, and hundreds of resistance genes have been characterized in both Gram-negative and Gram-positive species. Recently emerging antibiotic resistance factors, including metallo-β-lactamase NDM-1 (Kumarasamy *et al*., 2010, Yong *et al*., 2009) and KPC-1 (Pournaras *et al*., 2009, Kitchel *et al*., 2009, Baraniak *et al*., 2009), have been carefully monitored worldwide as causative factors of nosocomial infections, suggesting that molecularly-based rapid detection system may be effective in monitoring prevalent antibiotic resistant bacteria in clinical settings. Among the molecular-based methods of detecting antibiotic resistance factors are multiplex PCR, real-time PCR, DNA sequencing, and a plethora of hybridization-based techniques. These methods, however, are considered unsuitable for practical applications, because they are time consuming and require special devices for sample preparation and detection of suspected bacteria. For laboratory medical technologists, the usability of the technology is the most important factor.

1-7. Outline of thesis

This study primarily aimed to clarify the molecular mechanisms of antibiotic resistances in nosocomial pathogens, especially MDR *P. aeruginosa*. Elucidation of these molecular pathways has the potential to promote the development of a molecularly-based rapid method of detecting antibiotic resistant bacteria. In Chapter 2, I describe screening for antibiotic resistance genes, to understand the factors responsible for the as yet unknown mechanisms of antibiotic resistance of MDR *P. aeruginosa*. In Chapter 2, the AAC(6')-Iaf, which was newly identified during screening, was dissected to characterize its role in antibiotic resistances in *P. aeruginosa*. Finally, in Chapter 4, I attempted to develop a novel rapid detection system using monoclonal antibodies that specifically recognize AAC(6')-Iae, the most prevalent aminoglycoside resistance factor in Japan.

Chapter 2. Identification of the *aac(6')-Iaf* gene from MDR *P. aeruginosa* clinical isolates

We previously described a nosocomial outbreak of catheter-associated urinary tract infection with an MDR *P. aeruginosa* strain, IMCJ2.S1, in a hospital in Miyagi prefecture in Japan (Sekiguchi *et al*., 2007a). IMCJ2.S1 was found to harbor an aminoglycoside 6'-*N*-acetyltransferase gene, *aac(6')-Iae*, in a chromosomal integron. Subsequently, MDR *P. aeruginosa* strains carrying *aac(6')-Iae* were isolated in Miyagi, Tokyo, and Hiroshima. Since then, the *aac(6')-Iae* gene in *P. aeruginosa* clinical isolates has been monitored during the surveillance of MDR *P. aeruginosa* in hospitals throughout Japan (Kirikae *et al*., 2008, Sekiguchi *et al*., 2007a). During surveillance in the western part of Japan, two MDR *P. aeruginosa* clinical isolates without the *aac(6')-Iae* gene were newly identified. Each of these isolates contained a novel aminoglycoside 6'-*N*-acetyltransferase gene, *aac(6')-Iaf*. In this chapter, I describe the genetic background of this gene.

2-1. Results and discussion

2-1-1. Characterization of the parental strain of AAC(6')-Iaf

During surveillance, 355 clinical isolates of *P. aeruginosa* were analyzed. The results of antibiotic resistance gene screening indicated that 208 isolates (59%) carry the *aac(6')-Iae* gene, whereas the mechanism of aminoglycoside resistance 147 isolates (41%) were unclear.

Of 147 *aac(6')-Iae*-negative strains, two *P. aeruginosa* clinical strains, IMCJ798 and IMCJ799, were focused, because both IMCJ798 and IMCJ799 had high-level multidrug-resistant phenotypes, showing resistance to all antibiotics except for gentamicin. In particular, they were highly resistant to β-lactams, amikacin, and ofloxacin. These results were similar to those of IMCJ2.S1, except for resistances to arbekacin and gentamicin (Table 2-1).

Table 2-1. Antimicrobial susceptibility of *P. aeruginosa* IMCJ798 and 799.

Isolate name	MIC (μg/ml) of:										
	PIP	TZP	CAZ	IPM	MEM	ATM	AMK	ABK	GEM	OFX	PMB
IMCJ 798	256	256	512	128	>512	64	128	8	4	>128	4
IMCJ 799	256	256	512	128	>512	64	128	16	2	>128	4
IMCJ2.S1	256	256	512	128	512	128	128	2	16	128	2
ATCC27853	<4	4	<1	4	1	2	2	<0.5	<1	<0.5	2

[a] PIP, piperacillin; TZP, piperacillin-tazobactam ; CAZ, cetazidime ; IPM, imipenem ; MEM, meropenem ; ATM, aztreonam ;
AMK, amikacin ; ABK, arbekacin ; GEM, gentamicin ; OFX, ofloxacin ; PMB, polymyxin B.

The PFGE patterns of *Spe*I- and *Xba*I-digested fragments of IMCJ798 and IMCJ799 DNA were identical, but differed from those of IMCJ2.S1 (Fig. 2-1). The PFGE patterns of IMCJ798 and IMCJ799 showed similarities to IMCJ2.S1 of 56.4% (*Spe*I) and 70.5% (*Xba*I), respectively. Thus, the genotypic properties of IMCJ798 and IMCJ799 differed from those of IMCJ2.S1, although they had similar phenotypes.
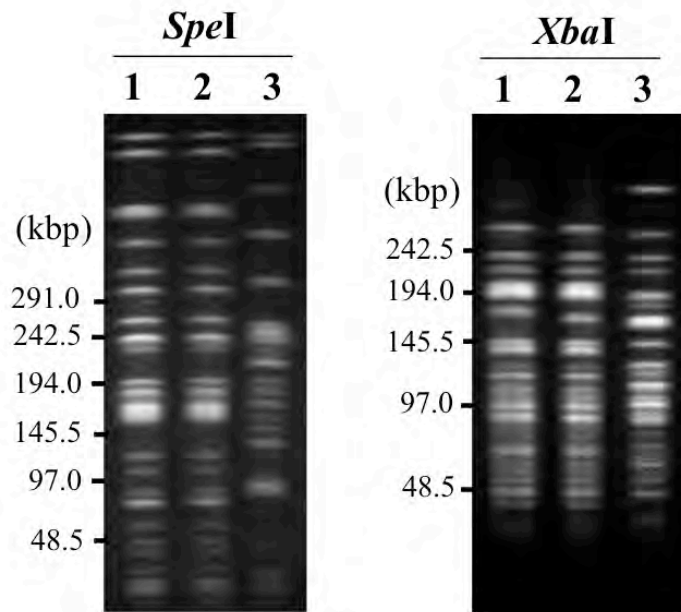
Figure 2-1. PFGE patterns of *Spe*I- and *Xba*I-digested genomic DNA from multidrug-resistant *P. aeruginosa* strains IMCJ798 and IMCJ799. The DNA fragments were detected by EtBr staining. Lane 1, IMCJ798; lane 2, IMCJ799; lane 3, IMCJ2.S1.

2-1-2. Genetic environment of the *aac(6')-Iaf* gene.

To identify the drug resistance genes of IMCJ798 and IMCJ799, the variable region of class 1 integrons was amplified with primers 5'CS and 3'CS (Table 2-4, below). An amplicon of 1.7-kbp generated from both strains was found to be identical by DNA sequencing. Sequence analysis revealed a variable region containing two cassettes and carrying a novel *aac(6')* gene and the bla_{IMP-1} metallo-β-lactamase gene, respectively (Fig. 2-2A). The novel *aac(6')* gene comprised an ORF of 552-bp, starting with a TTG codon, and its sequence showed 94% identity to *aac(6')-Iq* from *Klebsiella pneumoniae* (Centron & Roy, 1998) and 91% identity to *aac(6')-Im* from *Citrobacter freundii* (Hannecart-Pokorni *et al.*, 1997).

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Based on the standard nomenclature of AAC enzymes (Vanhoof *et al*., 1998), this ORF was named *aac(6')-Iaf*. The nucleotide sequence of In123 reported here has been deposited in the EMBL/GenBank/DDBJ databases and assigned accession number AB462903.

The 5'-CS and 3'-CS of the integron were further mapped by PCR cartography using external primers (Table 2-3, and Fig. 2-2B). Typical 59-base elements (Stokes *et al*., 1997) were observed in both cassettes (Fig. 2-2C), indicating that the *aac(6')-Iaf* genes in *P. aeruginosa* IMCJ798 and IMCJ799 are localized within the class 1 integron. Since this integron was not found in any database, we named it In123. The full length sequence of In123 is shown in Fig.2-3.

In addition, the *aac(6')-Iaf* gene was found to have a 34.4% G+C content, whereas the average G+C content of the *P. aeruginosa* PAO1 and *Klebsiella pneumoniae* MGH78578 genomes are 66.6% and 57.1%, respectively. (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi?view=1)

These findings suggested that the *aac(6')-Iaf* gene may have been derived from species with intrinsically low G+C content, not from *Pseudomonas* or *Klebsiella* species.

Figure 2-2. Genetic structure of In123. (A) Schematic map of In123. Primers A, B, C, D, E, F, G and H are described in Table 2. Arrows indicate primer location and direction. (B) PCR amplification with primer sets described above. PCR products were separated in 1% agarose gel electrophoresis. Lanes: 1, B+F; 2, A+D; 3, B+D; 4, C+D; 5, C+E; 6, C+F; 7, C+G; 8, C+H. (C) The 59-be in integron cassettes. Putative integrase binding domains, 1L and 2L at the left-hand (LH) and 2R and 1R at the right-hand (RH) are boxed. Recombination cross-over occurred at a unique point between adjacent G and C residues, indicated by vertical arrows. The extra base in 2L is marked with an asterisk. Inverted repeats are underscored with arrows.

A

intI1 5'CS *aac(6')-Iaf* *bla*IMP-1 3'CS *qacEΔsul1*

A B C D E F G H

1744-bp

1678-bp

698-bp

552-bp

1419-bp

1598-bp

1937-bp

2778-bp

B

M 1 2 3 4 5 6 7 8 M

3.0
2.5
2.0
1.5
1.0
0.75
0.50
0.25
(kbp)

C

LH consensus   RH consensus

aac(6')-Iaf ACA GCCCAAC AACCG CTTCAACC -69bp- GGTT-AAG CGAAT GTTAGAA AAG
                                    *

*bla*IMP-1 AAT TTCTAAC AAGTC GTTGCAGC -88bp- GCTG-AAC TTAGC GTTAGAT GCA
                 1L   2L   2R   1R
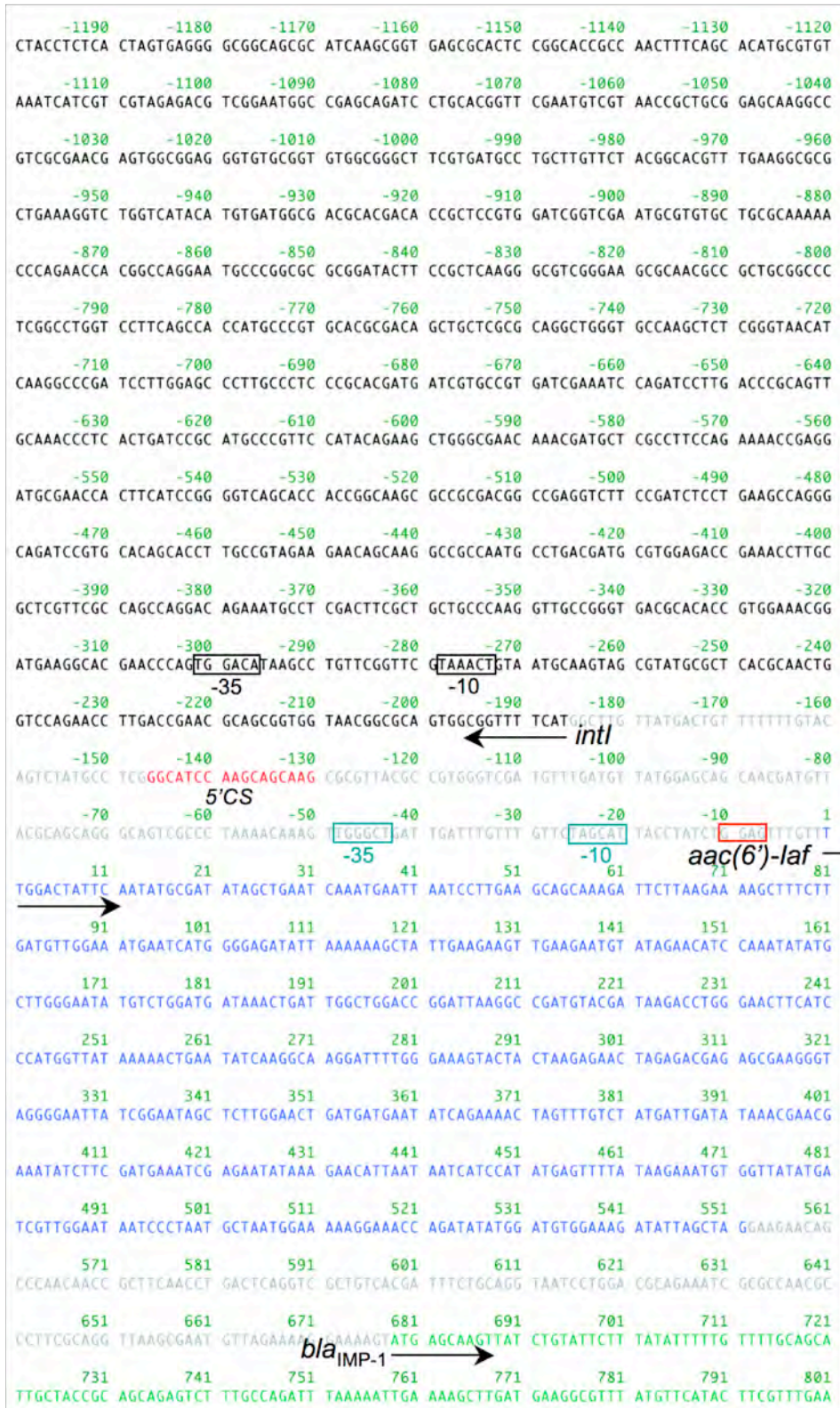   *

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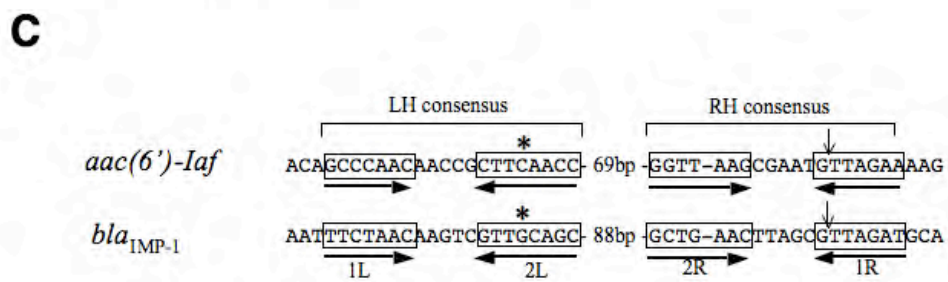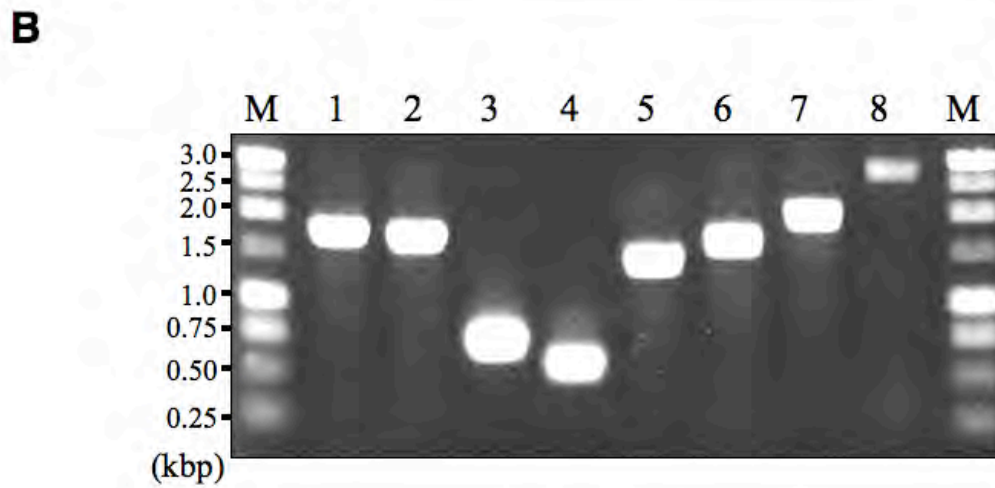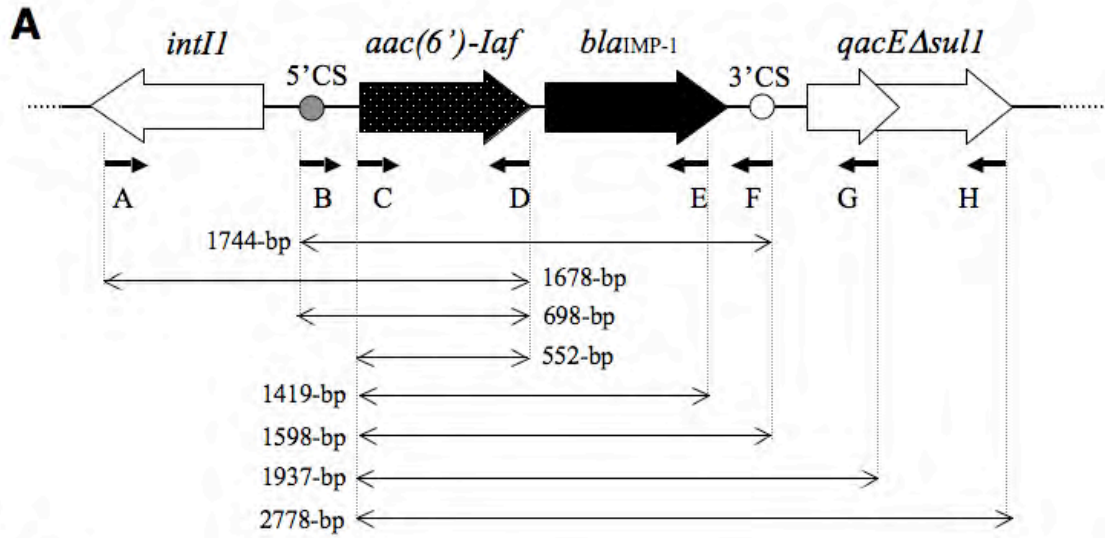
Figure 2-3. Full DNA sequences of In123 encoded by *aac(6')-lae*. The black square denoted the first promoter and the green square denotes the second promoter of *aac(6')-laf*. The red square shows the putative SD sequence.

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      811         821         831         841         851         861         871         881
GAAGTTAACG GGTGGGGCGT TGTTCCTAAA CATGGTTTGG TGGTTCTTGT AAATGCTGAG GCTTACCTAA TTGACACTCC

      891         901         911         921         931         941         951         961
ATTTACGGCT AAAGATACTG AAAAGTTAGT CACTTGGTTT GTGGAGCGTG GCTATAAAAT AAAAGGCAGC ATTTCCTCTC

      971         981         991        1001        1011        1021        1031        1041
ATTTTCATAG CGACAGCACG GGCGGAATAG AGTGGCTTAA TTCTCGATCT ATCCCCACGT ATGCATCTGA ATTAACAAAT

     1051        1061        1071        1081        1091        1101        1111        1121
GAACTGCTTA AAAAAGACGG TAAGGTTCAA GCCACAAATT CATTTAGCGG AGTTAACTAT TGGCTAGTTA AAAATAAAAT

     1131        1141        1151        1161        1171        1181        1191        1201
TGAAGTTTTT TATCCAGGCC CGGGACACAC TCCAGATAAC GTAGTGGTTT GGTTGCCTGA AAGGAAAATA TTATTCGGTG

     1211        1221        1231        1241        1251        1261        1271        1281
GTTGTTTTAT TAAACCGTAC GGTTTAGGCA ATTTGGGTGA CGCAAATATA GAAGCTTGGC CAAAGTCCGC CAAATTATTA

     1291        1301        1311        1321        1331        1341        1351        1361
AAGTCCAAAT ATGGTAAGGC AAAACTGGTT GTTCCAAGTC ACAGTGAAGT TGGAGACGCA TCACTCTTGA AACTTACATT

     1371        1381        1391        1401        1411        1421        1431        1441
AGAGCAGGCG GTTAAAGGGT TAAACGAAAG TAAAAAACCA TCAAAACCAA GCAACTAAAT TTCTAACAAG TCGTTGCAGC

     1451        1461        1471        1481        1491        1501        1511        1521
ACGCCACTAC GTGGCTGGAC AGTTTGTAAG TTGCGCTTTT GTGGTTTGCT TCGCAAAGTA TTCCACAACG CGCAACTTAC

     1531        1541        1551        1561        1571        1581        1591        1601
AAACTGCCGC TGAACTTAGC GTTAGATGCA CTAAGCACAT AATTGCTCAC AGCCAAACTA TCAGGTCAAG TCTGCTTTTA
                                                                          3'CS

     1611        1621        1631        1641        1651        1661        1671        1681
TTATTTTTAA GCGTGCATAA TAAGCCCTAC ACAAATTGGG AGATAGTCA TGAAAGGCTG GCTTTTTCTT GTTATCGCAA
                                              qacEΔ ———————————→

     1691        1701        1711        1721        1731        1741        1751        1761
TAGTTGGCGA AGTAATCGCA ACATCCGCAT TAAAATCTAG CGAGGGCTTT ACTAAGCTTG CCCCTTCCGC CGTTGTCATA

     1771        1781        1791        1801        1811        1821        1831        1841
ATCGGTTATG GCATCGCATT TTATTTTCTT TCTCTGGTTC TGAAATCCAT CCCTGTCGGT GTTGCTTATG CAGTCTGGTC

     1851        1861        1871        1881        1891        1901        1911        1921
GGGACTCGGC GTCGTCATAA TTACAGCCAT TGCCTGGTTG CTTCATGGGC AAAAGCTTGA TGCGTGGGGC TTTGTAGGTA

     1931        1941        1951        1961        1971        1981        1991        2001
TGGGGCTCAT AATTGCTGCC TTTTTGCTCG CCCGATCCCC ATCGTGGAAG TCGCTGCGGA GGCCGACGCC ATGGTGACGG
                                                                     sul1 ———————————→

     2011        2021        2031        2041        2051        2061        2071        2081
TGTTCGGCAT TCTGAATCTC ACCGAGGACT CCTTCTTCGA TGAGAGCCGG CGGCTAGACC CCGCCGGCGC TGTCACCGCG

     2091        2101        2111        2121        2131        2141        2151        2161
GCGATCGAAA TGCTGCGAGT CGGATCAGAC GTCGTGGATG TCGGACCGGC CGCCAGCCAT CCGGACGCGA GGCCTGTATC

     2171        2181        2191        2201        2211        2221        2231        2241
GCCGGCCGAT GAGATCAGAC GTATTGCGCC GCTCTTAGAC GCCCTGTCCG ATCAGATGCA CCGTGTTTCA ATCGACAGCT

     2251        2261        2271        2281        2291        2301        2311        2321
TCCAACCGGA AACCCAGCGC TATGCGCTCA AGCGCGGCGT GGGCTACCTG AACGATATCC AAGGATTTCC TGACCCTGCG

     2331        2341        2351        2361        2371        2381        2391        2401
CTCTATCCCG ATATTGCTGA GGCGGACTGC AGGCTGGTGG TTATGCACTC AGCGCAGCGG GATGGCATCG CCACCCGCAC

     2411        2421        2431        2441        2451        2461        2471        2481
CGGTCACCTT CGACCCGAAG ACGCGCTCGA CGAGATTGTG CGGTTCTTCG AGGCGCGGGT TTCCGCCTTG CGACGGAGCG

     2491        2501        2511        2521        2531        2541        2551        2561
GGGTCGCTGC CGACCGGCTC ATCCTCGATC CGGGGATGGG ATTTTTCTTG AGCCCCGCAC CGGAAACATC GCTGCACGTG

     2571        2581        2591        2601        2611        2621        2631        2641
CTGTCGAACC TTCAAAAGCT GAAGTCGGCG TTGGGGCTTC CGCTATTGGT CTCGGTGTCG CGGAAATCCT TCTTGGGCGC

     2651        2661        2671        2681        2691        2701        2711        2721
CACCGTTGGC CTTCCTGTAA AGGATCTGGG TCCAGCGAGC CTTGCGGCGG AACTTCACGC GATCGGCAAT GGCGCTGACT

     2731        2741        2751        2761        2771        2781        2791        2801
ACGTCCGCAC CCACGCGCCT GGAGATCTGC GAAGCGCAAT CACCTTCTCG GAAACCCTCG CGAAATTTCG CAGTCGCGAC

     2811        2821        2831        2841        2851        2861        2871        2881
GCCAGAGACC GAGGGTTAGA TCATGCCTAG
```

2-1-3. Location of In123.

Class 1 integrons are frequently located on plasmids and they can be transferred across bacteria (Bissonnette & Roy, 1992). Using genomic DNA digested with I-*Ceu*I, plasmid preparation, transformation, conjugation, and Southern hybridization were performed to determine the location and transmission ability of In123 in IMCJ798 and IMCJ799. *P. aeruginosa* GN17203, which harbors pMS350 containing bla_{IMP-1}, was used as the positive control (Watanabe *et al*., 1991). The plasmid carriage and antibiotic resistance genes, and the role in the conjugation test of the strains used in this experiment were summarized in Table 2-2.

Table 2-2. Strains used to determine the integron location.

Strain	Plasmid	Antibiotic resistance genes		The role in the conjugation test
		aac(6')-laf	bla_{IMP-1}	
GN17203	+	–	+	donor
PAO1	–	–	–	recipient
IMCJ798	–	+	+	donor
IMCJ799	–	+	+	donor

Initially, the plasmid DNA were prepared as described in Materials and Methods, but no plasmid was detected in IMCJ798 and IMCJ799 by electrophoresis; in contrast, pMS350 was detected in *P. aeruginosa* GN17203 (data not shown). The plasmid DNA preparations were used to transform *E. coli* DH5α and *P. aeruginosa* PAO1 by electroporation. No transformants were obtained on LB agar plates containing amikacin. In conjugation tests using *P. aeruginosa* ATCC 27853 RFP[r] as a donor strain, amikacin resistance was not transferred from IMCJ798 or IMCJ799 to *P. aeruginosa* ATCC 27853 RFP[r], whereas carbapenem resistance was transferred from *P. aeruginosa* GN17203 to ATCC 27853 RFP[r].

To confirm that In123 is located on the chromosome, PFGE analysis and Southern hybridization using *P. aeruginosa* genomic DNA digested with I-*Ceu*I were performed. In all strains, four chromosomal fragments of various sizes and encoding ribosomal RNA (*rRNA*) were detected by the gene probes (PAO1: 4,063, 950, 775 and 475 kb; GN17203: ca. 3,600, 1,500, 945 and 900 kb; IMCJ798 and IMCJ799: ca. 4,500, 950, 900 and 480 kb) (Fig. 2-4A and 2-4C, and left panels in Fig. 2-4B and 2-4D). The *aac(6')-Iaf* probe bound to a 4,500-kb fragment of IMCJ798 and IMCJ799. The band bound by the *aac(6')-Iaf* probe was also bound by the *rRNA* gene probe. In IMCJ clinical isolates, the bla_{IMP-1} probe detected the same fragment as the *aac(6')-Iaf* and *rRNA* gene probes. In addition, the bla_{IMP-1} probe bound to a 700-kbp extrachromosomal fragment that was not bound by the *rRNA* gene probe, suggesting that this extrachromosomal fragment may correspond to pMS350 in GN17203 (Watanabe *et al*., 1991). Another smaller bla_{IMP-1} probe specific band detected in IMCJ clinical isolates in Fig. 2-4B was not observed in Fig. 2-4D, probably due to differences in electrophoretic conditions (see figure legend). It is likely that this band was not resolved under the conditions used in Fig. 2-4C and 2-4D.

Collectively, these results strongly suggest that In123, which encodes *aac(6')-Iaf*, is located on the chromosome, not on a plasmid, in *P. aeruginosa* IMCJ798 and IMCJ799. Although other class 1 integrons, including *aac(3)-Ib*, *aac(3)-Ic*, and *aac(6')-Iae* in *P. aeruginosa* (Riccio *et al.*, 2003, Schwocho *et al.*, 1995) and *aac(3)-Id* in *Vibrio fluvialis* (Ahmed *et al.*, 2004), were also reported located on the chromosome, our results are the first to show that an integron integrated into the pseudomonal chromosome could be detected by both I-*Ceu*I genome typing and Southern blotting with an rRNA probe.
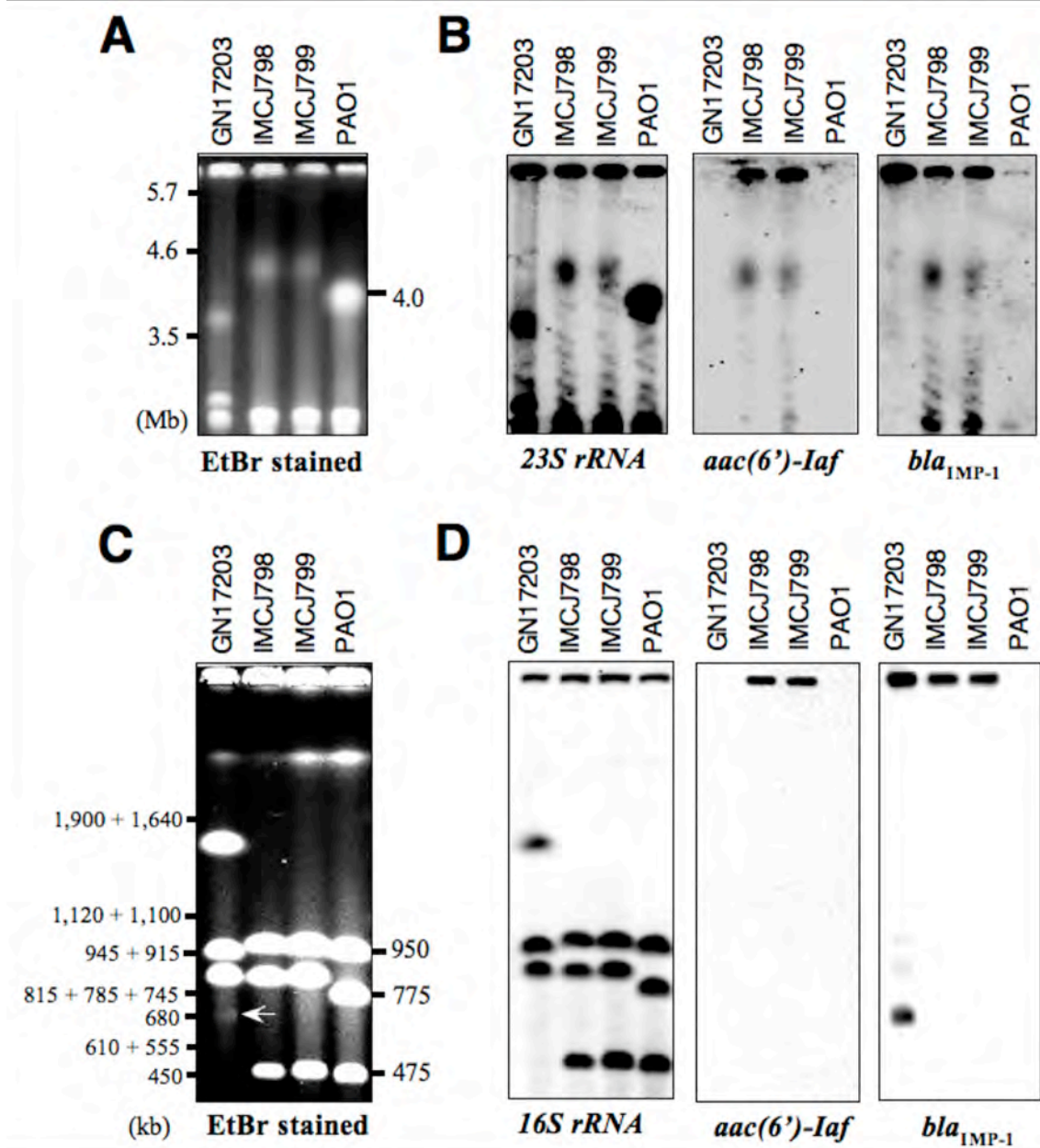
Figure 2-4. PFGE patterns (A and C) and southern bybridization (B and D) of *P. aeruginosa* genomic DNA digested with I-CeuI. PFGE of *P. aeruginosa* genomic DNA was digested with I-*Ceu*I and electrophoresed under two conditions: (panel A) 106° angle, 0.8% agarose and linear switching times of 20 to 30 min for 48 h with a voltage gradient of 2 V/cm for separation of the largest 4.0-Mb fragment of PAO1; and (panel C) 120° angle, 1% agarose, and nonlinear switching times of 5.3 to 120 sec for 19.5 h with a voltage gradient of 6 V/cm for separation of the 950-, 775- and 475-kb fragments of PAO1 in. The molecular standards were *Schizosaccharomyces pombe* (A) and *Saccharomyces cerevisiae* YPH80 (C) DNAs. The arrowhead in panel C indicates the location of an extrachromosomal band that may correspond to pMS350. DNA fragments shown in panels A and C were transferred to membranes, and hybridized with probes for rRNA genes*, aac(6')-Iaf*, and *bla*IMP-1, as shown in panel B and D.

2-1-4. Comparison of AAC(6')-Iaf with other AAC(6')-I enzymes

The AAC(6')-Iaf aminoglycoside acetyltransferase encoded by the first cassette gene in In123 consisted of 183 amino acids. The amino acid sequence of AAC(6')-Iaf was compared with the sequence of other AAC(6')-I enzymes. As shown in Fig. 2-5, the deduced molecular phylogeny of these sequences suggests that all the AAC(6')-I enzymes can be classified into three subfamilies (Vakulenko & Mobashery, 2003); the first containing AAC(6')-Ib and -Ie; the second containing AAC(6')-Ic, -Id, and -Ih; and the third containing AAC(6')-Ia, -Iae, and –Iq. AAC(6')-Iaf was found to belong to the third subfamily, whose members show considerable phylogenetic distance from the other two subfamilies (Sekiguchi *et al*., 2005, Doi *et al*., 2004, Vakulenko & Mobashery, 2003). Using multiple-sequence alignments, AAC(6')-Iaf showed 91%, 87%, 63%, 57%, and 35% identity to AAC(6')-Iq from *Klebsiella pneumoniae* (Centron & Roy, 1998), AAC(6')-Im from *Citrobacter freundii* (Hannecart-Pokorni *et al*., 1997), AAC(6')-Ia from *Shigella sonnei* (Tenover *et al*., 1988), AAC(6')-Iae from *Pseudomonas aeruginosa* (Sekiguchi *et al*., 2005), and AAC(6')-Ii from *Enterococcus faecium* (Costa *et al*., 1993), respectively (Fig. 2-6A).
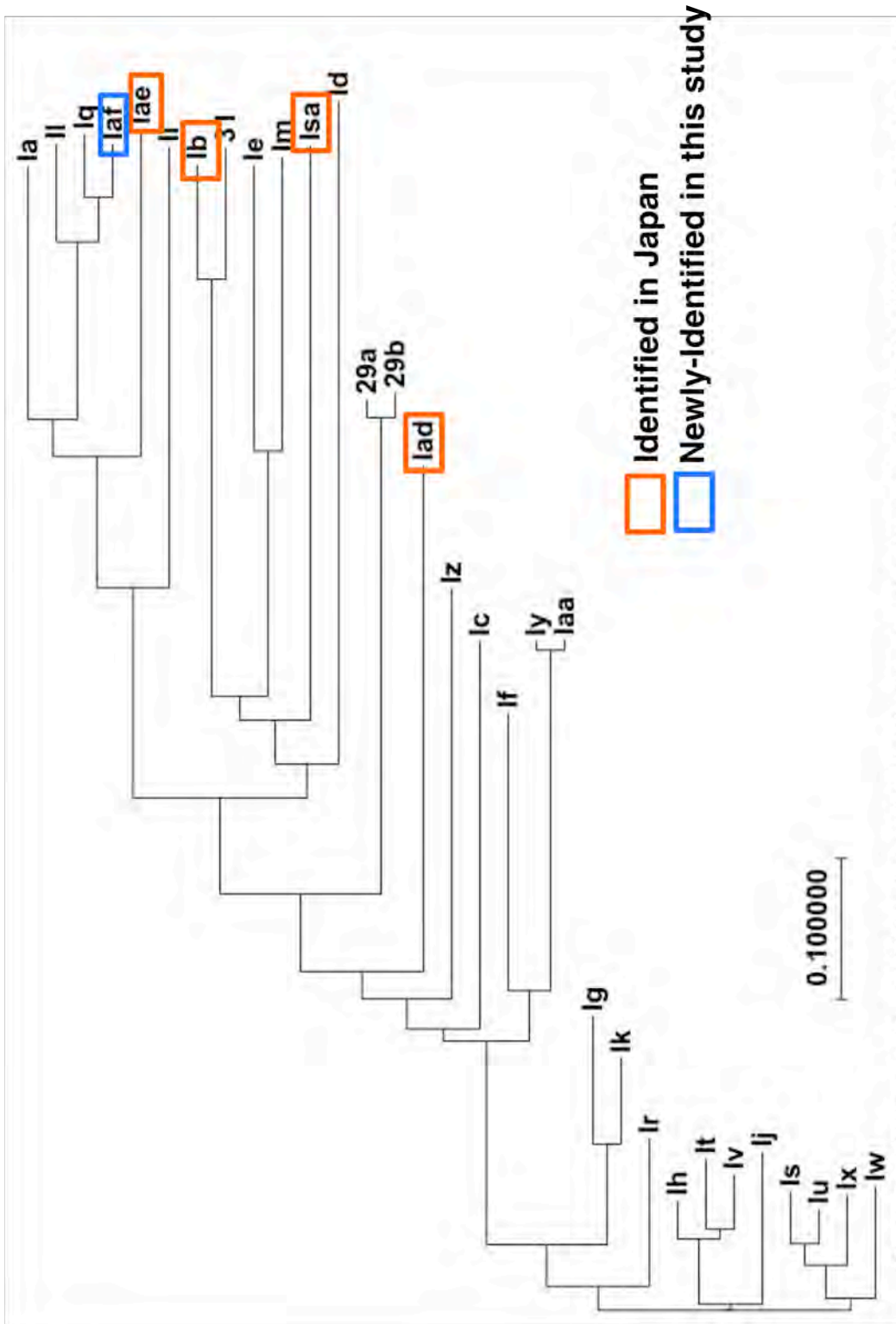
Figure 2-5. Dendrogram for AAC(6')-I family proteins. The dendrogram was constructed using the ClustalW computer program, available on the National Institute of Genetics website (http://www.ddbj.nig.ac.jp/E-mail/clustalw-e.html). The figure was illustrated with the TreeViewPPC program. Branch lengths correspond to the numbers of amino acid exchanges. AAC(6')-I enzymes enclosed with orange square was identified in Japan. AAC(6')-Iaf that was newly identified in this study is enclosed with blue square.
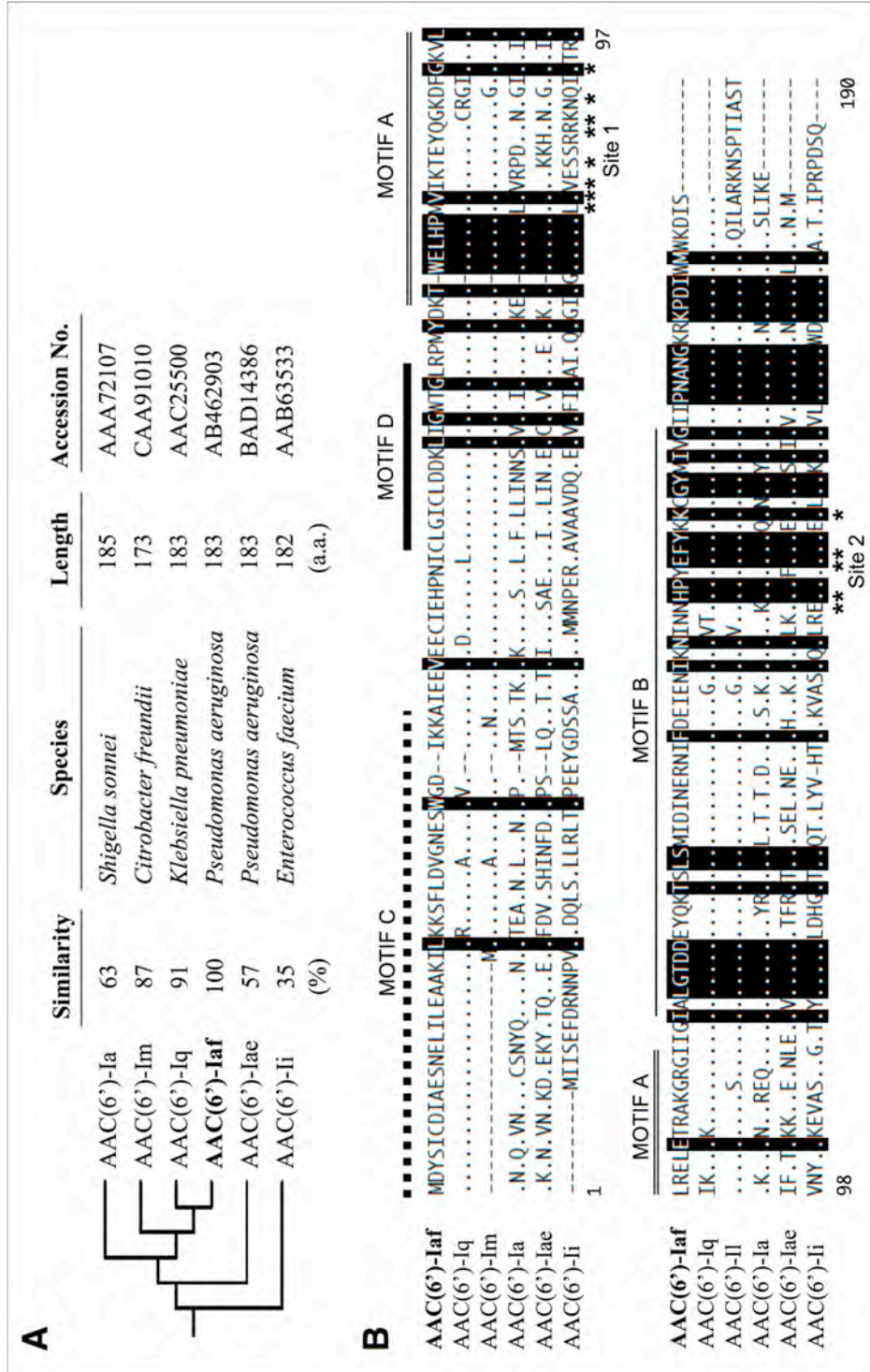
Figure 2-6. Phylogenic tree and amino acid sequence alignments of the AAC(6')-I subfamily. (A) Calculation of phylogenic relationships using the CLUSTAL W2 program. (B) Dots indicate amino acids identical to those of AAC(6')-Iaf, with black-highlighted amino acids being conserved residues among this AAC(6')-I subfamily. Dashes represent gaps introduced to optimize alignment.

2-1-5. Structural alignment of AAC(6')-Iaf

AAC(6')-I family proteins are members of the GCN5-related *N*-acetyltransferase (GNAT) superfamily (Dyda *et al*., 2000, Vetting *et al*., 2005). The GNATs constitute the catalytic core of this large superfamily of biologically important enzymes, which catalyze the transfer of acetyl groups from acetyl coenzyme A (AcCoA, the "donor") to a primary amine (the "acceptor") (Fig.2-7). Members of the GNAT superfamily must accommodate a wide range of primary amine-carrying acceptor substrates, varying from small compounds such as serotonin to large protein molecules such as histones. Despite functional variations across the GNAT superfamily, with different substrate, the topology of these proteins is nearly identical.
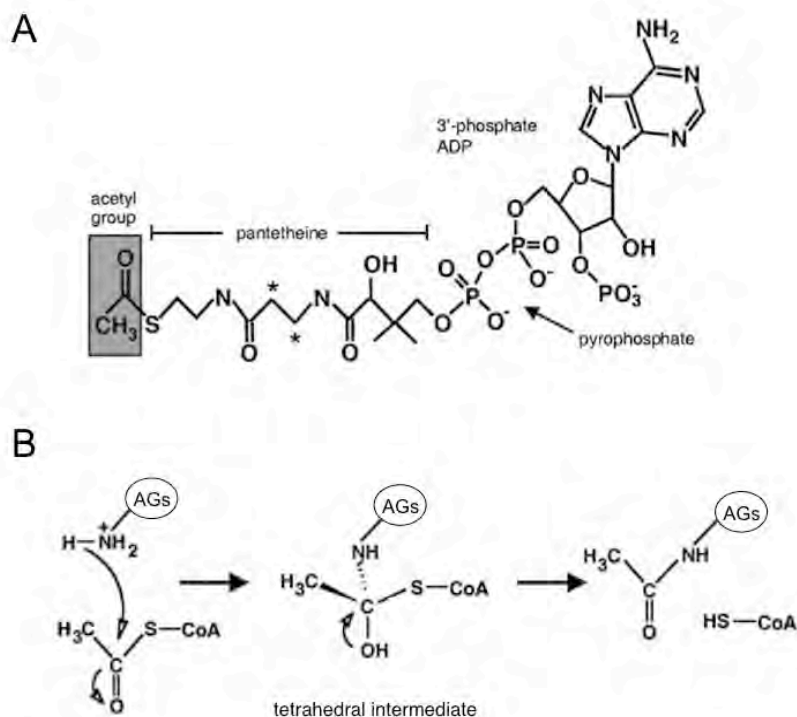

Figure 2-7. (A) Chemical structure of acetyl coenzyme A (Acetyl-CoA). (B) The reaction catalyzed by *N*-acetyltransferases, in which the nucleophilic attack of a primary amine on the acyl carbon of the acetyl group occurs via a tetrahedral intermediate. AGs indicate aminoglycosides.

As shown in Fig. 2-8, the structure of GNAT superfamily proteins has isoform including four conserved four motifs (C, D, A, and B) (Neuwald & Landsman, 1997). To perform structure-based alignments, the 2D alignment of AAC(6')-Iaf and monomeric AAC(6')-Ii protein (PDB ID: 1N71), which also belongs to the third subfamily, were assessed using ESPript software (Gouet *et al*., 2003) (Fig. 2-9). The SWISS-MODEL program was also used to construct the model structure of AAC(6')-Iaf, based on the structure of the monomeric AAc(6')-Ii protein (PDB ID: 1N71) (Kiefer *et al*., 2009). The computationally-predicted three-dimensional structure of AAC(6')-Iaf indicated that this protein possessed a single domain consisting of 5 alpha helices and 7 beta sheets, similar to the structure of AAC(6')-Ii. The analysis using GASH program in PDBj revealed that the RMSD (Root Mean Square Deviation) value for structural simirality of AAC(6')-Ii and AAC(6')-Iaf model was 0.66. The four motifs (C, D, A, and B) of most AAC(6')-I enzymes were also observed in AAC(6')-Iaf (Fig. 2-9A). The crystal structure of AAC(6')-Ii shows two acetyl-CoA binding sites positioned between the two arms, with one binding site located on loop beta 4-3 and helix alpha 3, and the other on loop alpha4-alpha5 and helix alpha5 (Burk *et al*., 2003, Burk *et al*., 2005). These 2 putative sites required for acetyl-CoA binding were also found in AAC(6')-Iaf (Fig. 2-6, asterisks), suggesting that AAC(6')-Iaf may act as an aminoglycoside 6'-*N*-acetyltransferase.
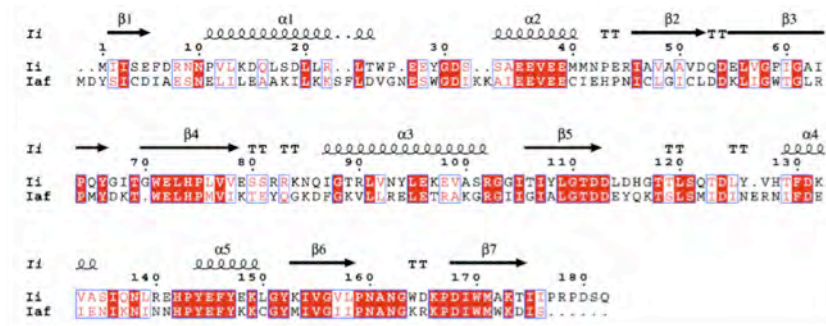
AAC(6')-Ii
from _Enterococcus faecium_

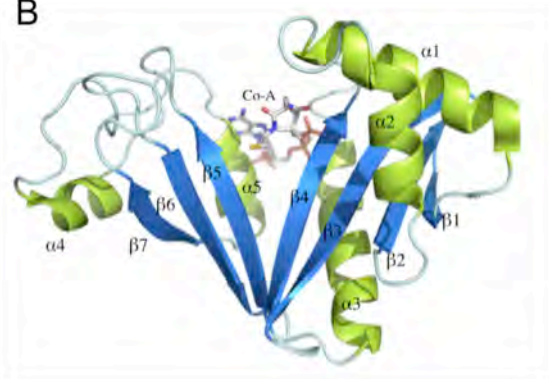**AAC(6')-Ib**
from _Escherichia coli_

AAC(6')-Iy
from _Salmonella enteritidis_

Figure 2-8. The crystal structures of known AAC(6')-I proteins. The gray circle indicates the substrate pocket.

Figure 2-9. (A) Sequence alignment of AAC(6')-Iaf from *P. aeruginosa* (Iaf) with aminoglycoside 6'-N-acetyltransferase AAC(6')-Ii from *E. faecium* (Ii). Secondary-structure elements from the respective proteins were obtained using DSSP (Kabsch & Sander, 1983). The figure was produced using ESPript (Gouet et al.,1999). Alignment was performed using CLUSTALW2. (B) Crystal structure of AAC(6')-Ii monomer containing Coenzyme A (PDB ID: 1n71). Alpha helixes are shown in blue and beta sheets in lime green. (C) Model structure of AAC(6')-Iaf. The model was created using the alignment mode in the SWISS-MODEL Workspace (Arnold et al., 2006), with the structure of AAC(6')-Ii (1n71) used as the template.

2-2. Materials and Methods

2-2-1. Bacterial strains

Two *P. aeruginosa* clinical isolates, IMCJ798 and IMCJ799, were obtained individually from two patients, one with a urinary tract infection and the other with a decubitus ulcer. *P. aeruginosa* ATCC 27853 was obtained from the American Type Culture Collection (Manassas, VA) and used as a reference strain for antibiotic susceptibility testing. *Escherichia coli* strains DH5α (Takara Bio, Shiga, Japan) and JM109 (Stratagene, La Jolla, Calif) were used as hosts for recombinant plasmids. *E. coli* BL21(DE3) (Invitrogen, Carlsbad, CA) was used for expression of recombinant *aac(6')-Iaf*. The rifampicin-resistant *P. aeruginosa* mutant ATCC 27853 RFP^r was used for conjugation. *P. aeruginosa* GN17203, carrying a plasmid pMS350 containing *bla*$_{IMP-1}$ (Watanabe *et al.*, 1991), was kindly provided by S. Iyobe (Kitasato University, Sagamihara, Japan).

Table 2-3. Strains used in this study.

Strain name	Genotype or description	Reference or source
P. aeruginosa IMCJ 798	Clinical isolate	Patient
P. aeruginosa IMCJ 798	Clinical isolate	Patient
P. aeruginosa GN17203	Clinical isolate carrying blaIMP-encoded plasmid pMS350	Inoue et al.
E. coli JM109	F$^-$ [*traD36 proAB+ lac Iq lacZ*ΔM15] *recA1 endA1 gyrA96 thi hsdR17*(r$_K^-$ m$_K^+$) e14$^-$ (*mcrA$^-$*) *supE44 relA1* Δ (*lac-proAB*)	stratagene
E. coli BL21(DE3)	F$^-$ *ompT hsdSB*(r$_n$- m$_n$-) gal(*λcI 857 ind1 Sam7 nin5 lacUV5-T7gene1*) *dcm(DE3)*	invitrogen

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2-2-2. Antimicrobial agents

Amikacin (AMK) and imipenem (IPM) were obtained from Banyu Pharmaceutical Co. (Tokyo, Japan); arbekacin (ABK) and dibekacin (DIB) from Meiji Seika Kaisha, Ltd. (Tokyo, Japan); aztreonam (ATM) from Eisai (Tokyo, Japan); ceftazidime (CAZ) from GlaxoSmithKline K.K. (Tokyo, Japan); gentamicin (GEN) and a neomycin B and C mixture (NEO) from Nacalai Tesque, Inc. (Kyoto, Japan); isepamycin (ISP), netilmicin (NET) and sisomicin (SIS) from Schering-Plough K.K. (Osaka, Japan); kanamycin A (KAN) and polymyxin B (PMB) from Sigma-Aldrich (St. Louis, MO); meropenem (MEM) from Sumitomo Pharmaceutical Co., Ltd. (Osaka, Japan); ofloxacin (OFX) from Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan); piperacillin (PIP) and piperacillin-tazobactam (TZP) from Tomiyama Pure Chemical Industries, Ltd. (Tokyo, Japan); and tobramycin (TOB) from Towa Pharmaceutical Co., Ltd. (Osaka, Japan).

2-2-3. Determination of antibiotic susceptibility

Minimal inhibitory concentrations (MICs) were determined using a micro dilution method according to the protocols recommended by the Clinical and Laboratory Standards Institute (CLSI, 2006).

2-2-4. PCR amplification of class 1 integrons

Genomic DNAs were extracted as described (Sambrook, 2001) and used as PCR templates. Class 1 integrons were detected by PCR using 5'CS and 3'CS primers as described (Levesque *et al*., 1995, Collis & Hall, 1992) and genetically mapped using the primers listed in Table 2-4. An Expand High Fidelity PCR system (Roche Diagnostics

GmbH, Penzberg, Germany) was used in all PCR amplifications. All PCR products were

sequenced to identify genes and their order in the integron.

2-2-5. DNA sequencing

DNA sequences were determined using an ABI PRISM 3100 sequencer (Applied

Biosystems). Homology searches of nucleotide and translated protein sequences were

performed using BLAST (Altschul *et al*., 1997, Altschul *et al*., 2005). Multiple-sequence

alignments and searches for ORFs were performed using the Clustal W2 program (Larkin *et

al*., 2007) and GENETYX software (Genetyx, Tokyo, Japan). The dendrogram for AACs

was calculated with the CLUSTAL W2 program (Larkin *et al*., 2007).

Table 2- 4. Primers used in this study (1)

Primer	Sequence ^{a} (5' to 3')	Location in Fig. 2	Description	Reference
5'CS	GGCATCCAAGCAGCAAG	B	5'-end common segment of class 1 integrons	29
3'CS	AAGCAGACTTGACCTGA	F	3'-end common segment of class 1 integrons	29
intI-R	TGCGTGTAAATCATCGTCGT	A	Positions 196-177 in *intI1*	38
qacEdelta-R	GCAATTATGAGCCCCATACC	G	Positions 287-268 in *qacEΔ1*	38
sul1-R	GGGTTTCCGAGAAGGTGATT	H	Positions 787-768 in *sul1*	38
aac(6')Iaf-F	TTGGACTATTCAATATGCGA	C	Positions 1-20 in *aac(6')-Iaf*	This study
aac(6')Iaf-R	CTAGCTAATATCTTTCCACA	D	Positions 552 end-533 in aac(6')-Iaf	This study
blaIMP1-F	GAAGTTAACGGGTGGGGCG		Positions 124-142 in *bla* $_{IMP-1}$	This study
blaIMP1-R	CTTTAACCGCCTGCTCTAAT	E	Positions 700-681 in *bla* $_{IMP-1}$	This study
16S-rRNA_F	ATGCAAGTCGAGCGGATGAAGGGAG		Positions 55-79 in 16S rRNA gene	This study
16S-rRNA_R	TAGTCGACATCGTTTACGGCGTGGA		Positions 822-798 in 16S rRNA gene	This study
23S-rRNA_F	CGAGGACAGTGTATGGTGGGCAGT		Positions 2207-2231 in 23S rRNA gene	This study
23S-rRNA_R	CTCAACGCCTCACAACGCTTACACA		Positions 2856-2832 in 23S rRNA gene	This study
PstI-aac-F	aactgcagGGCTTGTTATGACTGTTTTT		Sequence from 185-bp upstream region of *aac(6')-Iaf* with PstI site	This study
EcoRI-aac-R	ggaattcCTAGCTAATATCTTTCCACA		Positions 552-533 in *aac(6')-Iaf* with EcoRI site	This study
SphI-aac-F	aaagcatgcgATGGACTATTCAATATGCGA		Positions 1-20 in *aac(6')-Iaf* with *S*phI ^{b}	This study
PstI-aac-R	aactgcagCTAGCTAATATCTTTCCACA		Positions 552-533 in *aac(6')-Iaf* with PstI site	This study

^{a} Lowercase letters represent restriction enzyme recognition sites attached on the 5' end of primers.

^{b} The initiation codon TTG in *aac(6')-Iaf* was replaced with ATG.

2-2-6. Pulsed-field gel electrophoresis (PFGE)

DNA plugs were prepared as described (Grundmann *et al*., 1995) and digested overnight at 37°C with *Spe*I and *Xba*I (Takara Bio.). PFGE analysis was performed as described (Sekiguchi *et al*., 2005).

2-2-7. Large plasmid extraction and transformation

Plasmid DNA was extracted from *P. aeruginosa* as described (Kado & Liu, 1981; Casse, 1979), with modifications. Briefly, bacterial pellets were lysed in 2 ml lysis buffer (50mM Tris-Cl, 20mM EDTA, 4% SDS, pH 12.6), followed by gentle shaking for 30 min at 37°C. The lysate was neutralized by adding 400 μl 1M Tris-Cl (pH7.5), and the proteins were precipitated by adding 250 μl 5M NaCl. Each solution was extracted with an equal volume of phenol-chloroform (1:1, vol/vol). The plasmid DNA in the aqueous phase was precipitated by adding a two-fold volume of 100% ethanol, and the DNA pellet was collected. Plasmid DNA preparations were analyzed by electrophoresis on 0.7 % agarose gels in 0.5X Tris-borate-EDTA buffer at 4°C.

Plasmids isolated from *P. aeruginosa* strains were used to transform *E. coli* DH5α and *P. aeruginosa* PAO1 by electroporation using Gene Pulser Xcell (Bio-Rad Laboratories, Hercules, CA). The transformants were cultured on LB agar plates containing 20 μg/ml amikacin for 24 h at 37ºC.

2-2-8. Transfer of aminoglycoside resistance

Drug resistance was transferred from *P. aeruginosa* clinical isolates to a rifampicin-resistant mutant of *P. aeruginosa*, ATCC 27853 RFP[r], using the broth mating

method (Kato *et al*., 1982). The transconjugants were selected on Mueller-Hinton agar plates containing rifampicin (200 μg/ml) and imipenem (16 μg/ml) or amikacin (20 μg/ml).

2-2-9. Genome typing with I-*Ceu*I and Southern blot analysis

DNA plugs containing total genomic DNA of isolates were digested overnight with I-*Ceu*I. The DNA fragments were separated by PFGE, and Southern hybridization was performed using the ECL Direct Nucleic Acid Labeling and Detection system (GE Healthcare, Tokyo, Japan), according to the manufacturer's instructions, to determine whether In123 was located chromosomally, as described (Liu *et al*., 1993, Head & Yu, 2004, Poirel *et al*., 2003). Probes for *aac(6')-Iaf*, *bla*$_{\text{IMP-1}}$, *16S rRNA* and *23S rRNA* were amplified by PCR from IMCJ798 using the primer sets, aac(6')Iaf-F/aac(6')Iaf-R, blaIMP-1-F/blaIMP-1-R, 16S-rRNA-F/16S-rRNA-R and 23S-rRNA-F/23S-rRNA-R, respectively (Table 2-3).

Chapter 3. Functional dissection of AAC(6')-Iaf

Using integron sequencing, MDR *P. aeruginosa* strains IMCJ798 and IMCJ799 were found to contain a novel chromosomal class 1 integron, In123, which included *aac(6')-Iaf* as the first cassette gene. The encoded protein AAC(6')-Iaf was found to consist of 183 amino acids, with 91% and 87% identity to AAC(6')-Iq and AAC(6')-Im, respectively. In this chapter, the distribution of the *aac(6')-Iaf* gene to aminoglycoside resistances in MDR *P. aeruginosa*, and the biochemical properties of this gene product were assessed.

3-1. Results and discussion

3-1-1. Effects of *aac(6')-Iaf* on aminoglycoside resistance

To determine whether *aac(6')-Iaf* mediates aminoglycoside resistance, the MIC values for various aminoglycosides were assessed using *E. coli* JM109 transformants carrying pSTV-*aac*WT or pSTV-*aac*(TTG→ATG). The plasmid pSTV-*aac*WT contains both *aac(6')-Iaf* and its 185-bp upstream region from IMCJ798. In *E. coli* JM109, the *aac(6')-Iaf* gene was expressed from its native promoter and initiation codon. In pSTV-*aac*(TTG→ATG), the putative initiation codon TTG was replaced with ATG (Fig. 3-1), to overcome its low-level expression in *E. coli* caused by a rare initiation codon, as described for *aac(6')-Iq* (Mauch *et al.*, 1990, Centron & Roy, 1998).
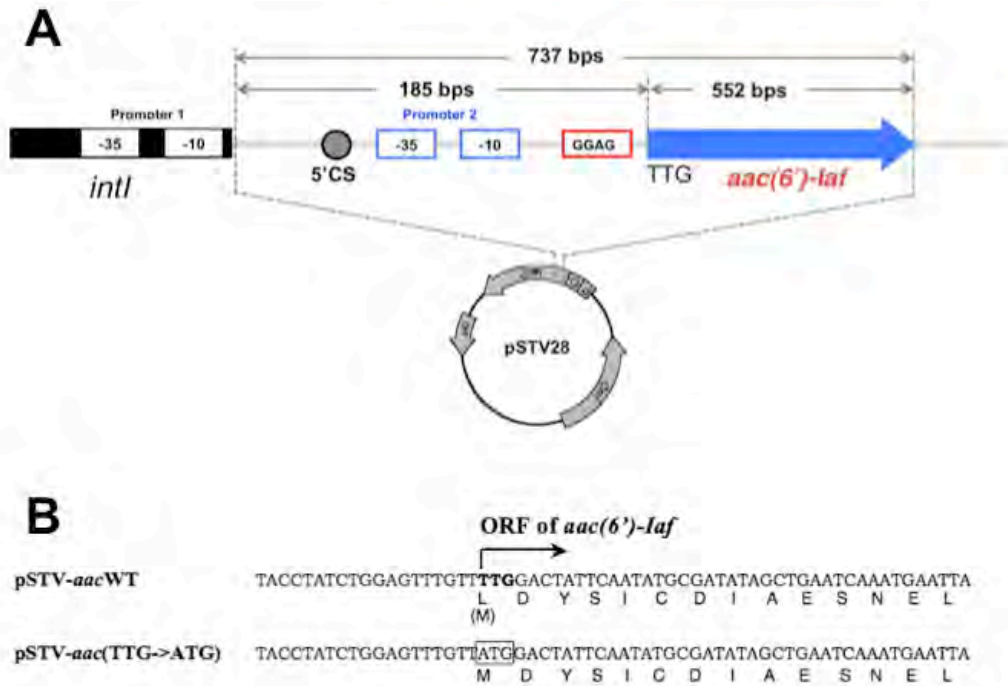
Figure 3-1. (A) Construction of the expression vector for AAC(6')-Iaf. (B) Partial sequences of the *aac(6')-Iaf* gene and its promoter region in pSTV-*aac*WT and pSTV-*aac*(TTG→ATG). Putative initiation codons are shown in bold type, and initiation codons altered to ATG are boxed. The N-terminal sequence of native AAC(6')-Iaf, determined by the Edman degradation method, is underlined.

In the aminoglycoside susceptibility test using parental strains of the *aac(6')-Iaf* gene, both *P. aeruginosa* IMCJ798 and IMCJ799 were resistant to amikacin, dibekacin, isepamicin, kanamycin, netilmicin, and tobramycin, but were sensitive to gentamicin (Table 3-1). The profile of aminoglycoside resistance in *E. coli* JM109 carrying pSTV-*aac*WT or pSTV-*aac*(TTG→ATG) was also tested. These results showed good correlations with those of both IMCJ798 and IMCJ799. The effective increase of MIC values for the same aminoglycosides, amikacin, dibekacin, isepamicin, kanamycin, netilmicin, and tobramycin,

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were observed in these transformants. Furthermore, MIC values for five of these six aminoglycosides were two-fold higher in JM109 carrying pSTV-*aac*(TTG→ATG) than in JM109 carrying pSTV-*aac*WT.

Table 3-1. Aminoglycoside susceptibility of *P. aeruginosa* parental strains and *E. coli* transformants.

Strain	MIC (mg/ml) of:									
	AMK	ABK	DIB	GEM	ISP	KAN	NET	SIS	TOB	NEO
P. aeruginosa IMCJ798	128	8	>128	4	>128	>128	>128	32	32	8
P. aeruginosa IMCJ799	128	16	>128	2	>128	>128	>128	32	32	8
E.coli JM109 pSTV28 [b]	1	2	1	0.5	1	2	0.5	0.5	0.125	2
E.coli JM109 pSTV-*aac* WT [b]	16	4	16	1	4	64	8	1	4	4
E.coli JM109 pSTV-*aac* (TTG->ATG) [b]	32	4	32	1	16	128	8	2	4	4

[a] AMK, amikacin; ; ABK, arbekacin; DIB, dibekacin; GEM, Gentamicin; ISP, isepamicin; KAN, kanamycin A; NET, netilmicin; SIS, sisomicin; TOB, tobramycin; NEO, neomycin.

[b] The MIC values were determined with MH broth containing chroramphenicol (30μg/ml) and each aminoglycosides.

3-1-2. Expression AAC(6')-Iaf in *P. aeruginosa* and *E. coli* transformants

AAC(6')-Iaf production in IMCJ798, IMCJ799, and *E. coli* JM109 transformants was also confirmed by Western blot analysis using polyclonal anti-AAC(6')-Iaf IgG (Fig. 3-2). A band corresponding to AAC(6')-Iaf was present in both IMCJ798 and IMCJ799, but not in the more sensitive strain *P. aeruginosa* ATCC27853. This AAC(6')-Iaf band was also detected in JM109 harboring pSTV-*aac*WT and pSTV-aac(TTG→ATG), but not in JM109 harboring the empty vector pSTV. The level of AAC(6')-Iaf in JM109 carrying pSTV-aac(TTG→ATG) was 2.4-fold higher than in JM109 carrying pSTV-*aac*WT, whereas the intensity of cross-reacting protein bands were unchanged (Fig. 3-2). This increase was

similar to the behavior of the 6-HDNO gene carrying TTG as an initiation codon (Mauch *et al*., 1990). These results demonstrate that the ORF of *aac(6')-Iaf* initiates from TTG and that *aac(6')-Iaf* plays a crucial role in aminoglycoside resistance of *P. aeruginosa* IMCJ798 and IMCJ799.
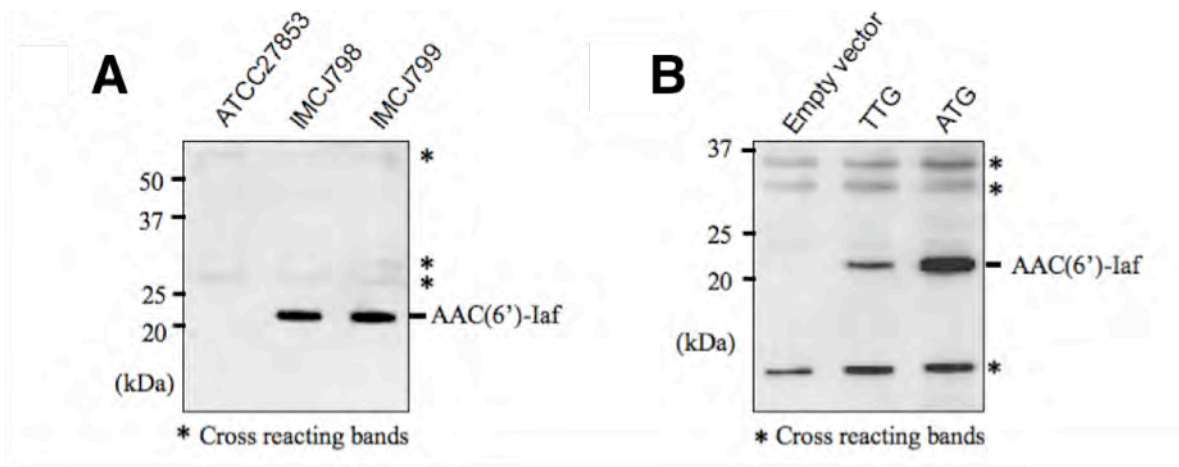

Figure 3-2. Production of AAC(6')-Iaf in *P. aeruginosa* clinical isolates and *E. coli* transformants used in aminoglycoside susceptibility tests. Each lysate was separated by SDS-PAGE (15%) and analyzed by western blotting using polyclonal anti-AAC(6')-Iaf antibodies. The positions of nonspecifically reacting protein bands serving as loading controls are indicated with asterisks. (A) Expression of AAC(6')-Iaf in *P. aeruginosa* clinical isolates of IMCJ798 and IMCJ799. (B) Expression of AAC(6')-Iaf in *E. coli* JM109 carrying pSTV28 (lane 1, empty vector), pSTV-aacWT (lane 2, TTG) and pSTV-aac(TTG→ATG) (lane 3, ATG).

3-1-3. Protein purification and analytical gel filtration chromatography

A recombinant plasmid pQE2-*aac(6')-Iaf* was constructed to express the His-tagged AAC(6')-Iaf protein. A soluble protein extract from *E. coli* harboring pQE2-*aac(6')-Iaf* was analyzed by SDS-PAGE (Fig. 3-3). After induction with IPTG, the His-tagged AAC(6')-Iaf (about 22 kDa, His-tag-included molecular weight), which was in good agreement with the values calculated from the deduced amino acid sequence of the enzyme, was expressed at a high level.

The molecular weight of AAC(6')-Iaf in solution was also estimated during size exclusion chromatography under non-denaturing conditions (Fig. 3-4). During chromatography, a single peak was observed in location indicating that the apparent molecular weight is 45,769 Da. Given that monomeric molecular weight of AAC(6')-Iaf is about 22 kDa, AAC(6')-Iaf was thought be form dimmer in solution (Fig. 3-4). The AAC(6')-Iaf that His-tag was eliminated with TAGzyme (QIAGEN) was also analyzed. Tag-free AAC(6')-Iaf also showed an apparent dimer. In contrast, monomeric isozyme of AAC(6')-Ib was analyzed using same procedure. In the same condition, AAC(6')-Ib showed monomer form in solution as described previously (Burk *et al*., 2003).
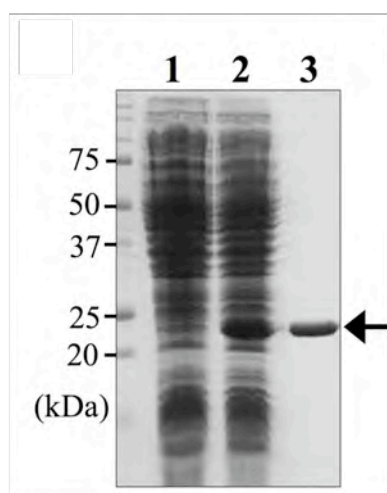

Figure 3-3. SDS-PAGE of overproduced and purified AAC(6')-Iaf. IPTG-induced *E.coli* BL21(DE3)pLysS carrying pQE2 (lane 1), pQE2-aac (lane 2) and purified His-tagged AAC(6')-Iaf (lane 3) were analyzed on 15% SDS-PAGE gels. Proteins were stained with Coomassie Brilliant Blue R-250. The arrow indicates the His-AAC(6')-Iaf band.
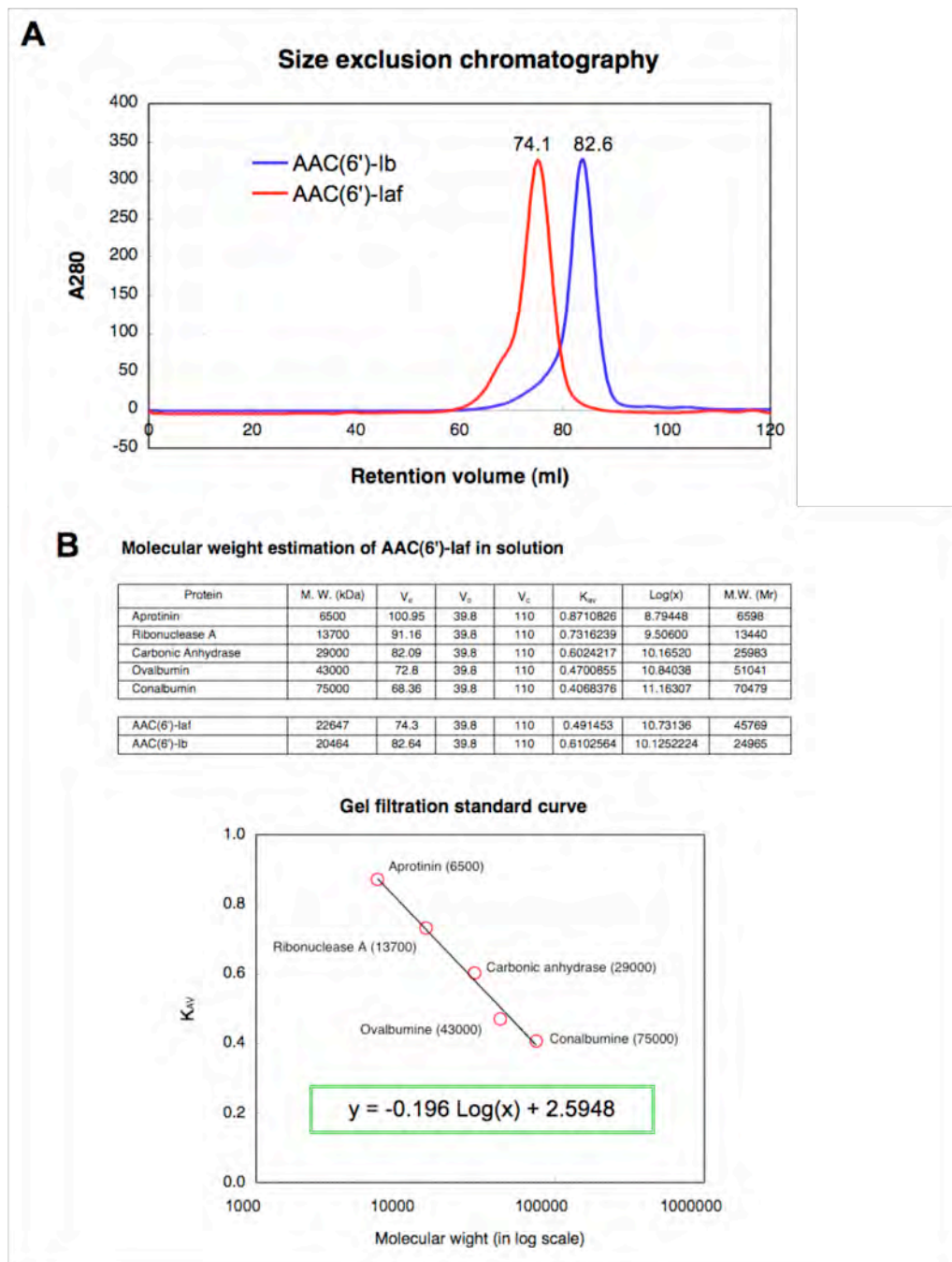
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A

Size exclusion chromatography

AAC(6')-Ib
AAC(6')-Iaf

74.1 82.6

B

Molecular weight estimation of AAC(6')-Iaf in solution

Protein	M. W. (kDa)	V_e	V_o	V_c	K_{av}	Log(x)	M.W. (Mr)
Aprotinin	6500	100.95	39.8	110	0.8710826	8.79448	6598
Ribonuclease A	13700	91.16	39.8	110	0.7316239	9.50600	13440
Carbonic Anhydrase	29000	82.09	39.8	110	0.6024217	10.16520	25983
Ovalbumin	43000	72.8	39.8	110	0.4700855	10.84038	51041
Conalbumin	75000	68.36	39.8	110	0.4068376	11.16307	70479
AAC(6')-Iaf	22647	74.3	39.8	110	0.491453	10.73136	45769
AAC(6')-Ib	20464	82.64	39.8	110	0.6102564	10.1252224	24965

Gel filtration standard curve

Aprotinin (6500)
Ribonuclease A (13700)
Carbonic anhydrase (29000)
Ovalbumine (43000)
Conalbumine (75000)

$$y = -0.196 \, Log(x) + 2.5948$$

Molecular wight (in log scale)

Figure 3-4. (A) Size exclusion chromatography of AAC(6')-Iaf (red) and AAC(6')-Ib (blue). (B) Determination of molecular weights in solution. The molecular weights of AAC(6')-Iaf and -Ib in solution were estimated using the equation shown in the graph.

3-1-4. Determination of N-terminal amino acid sequence

To confirm whether the initiation codon of *aac(6')-Iaf* is TTG, N-terminal sequencing of the native AAC(6')-Iaf protein was performed. Native protein was purified from IMCJ798 using affinity column coupling polyclonal anti-AAC(6')-Iaf rabbit IgG. The molecular mass of purified native AAC(6')-Iaf, as determined by 15% polyacrylamide gel electrophoresis, showed a protein corresponding to the estimated size of approximately 21 kDa (Fig. 3-5). Purified native AAC(6')-Iaf was detected in Western blotting analysis using anti-AAC(6')-Iaf rabbit IgG. The N-terminal sequence of AAC(6')-Iaf was found to be MDYSI (Fig. 3-6). This result demonstrated that the initiation codon of *aac(6')-Iaf* gene is TTG.
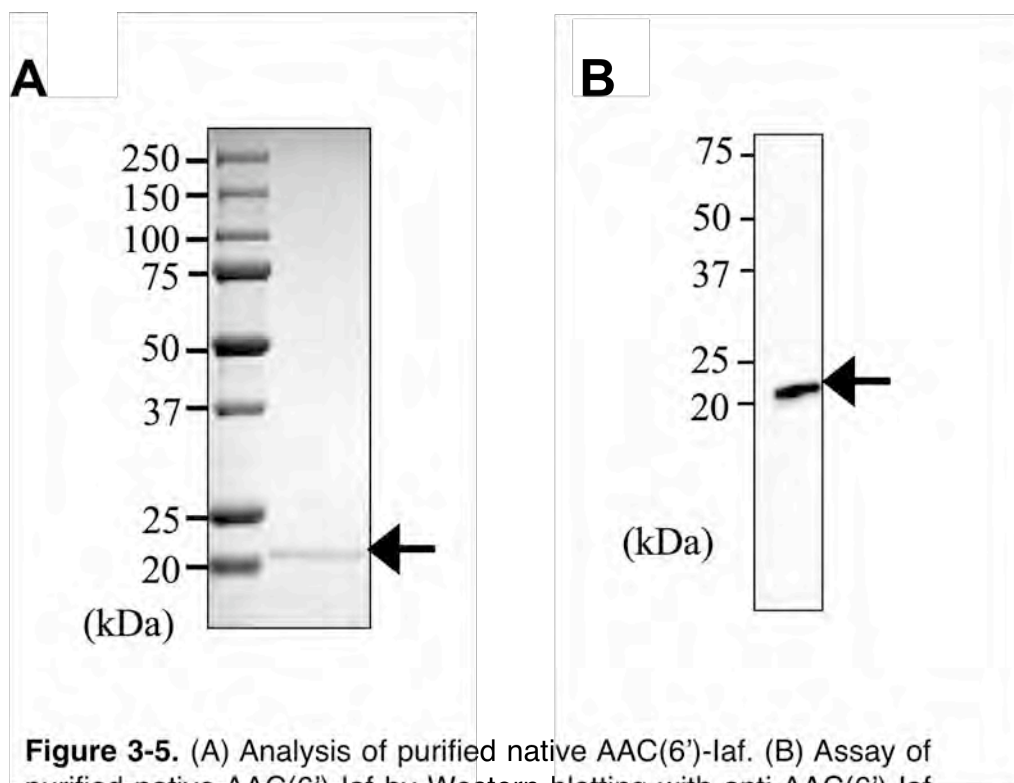
Figure 3-5. (A) Analysis of purified native AAC(6')-Iaf. (B) Assay of purified native AAC(6')-Iaf by Western blotting with anti-AAC(6')-Iaf polyclonal antibody. The arrows indicate native AAC(6')-Iaf band.
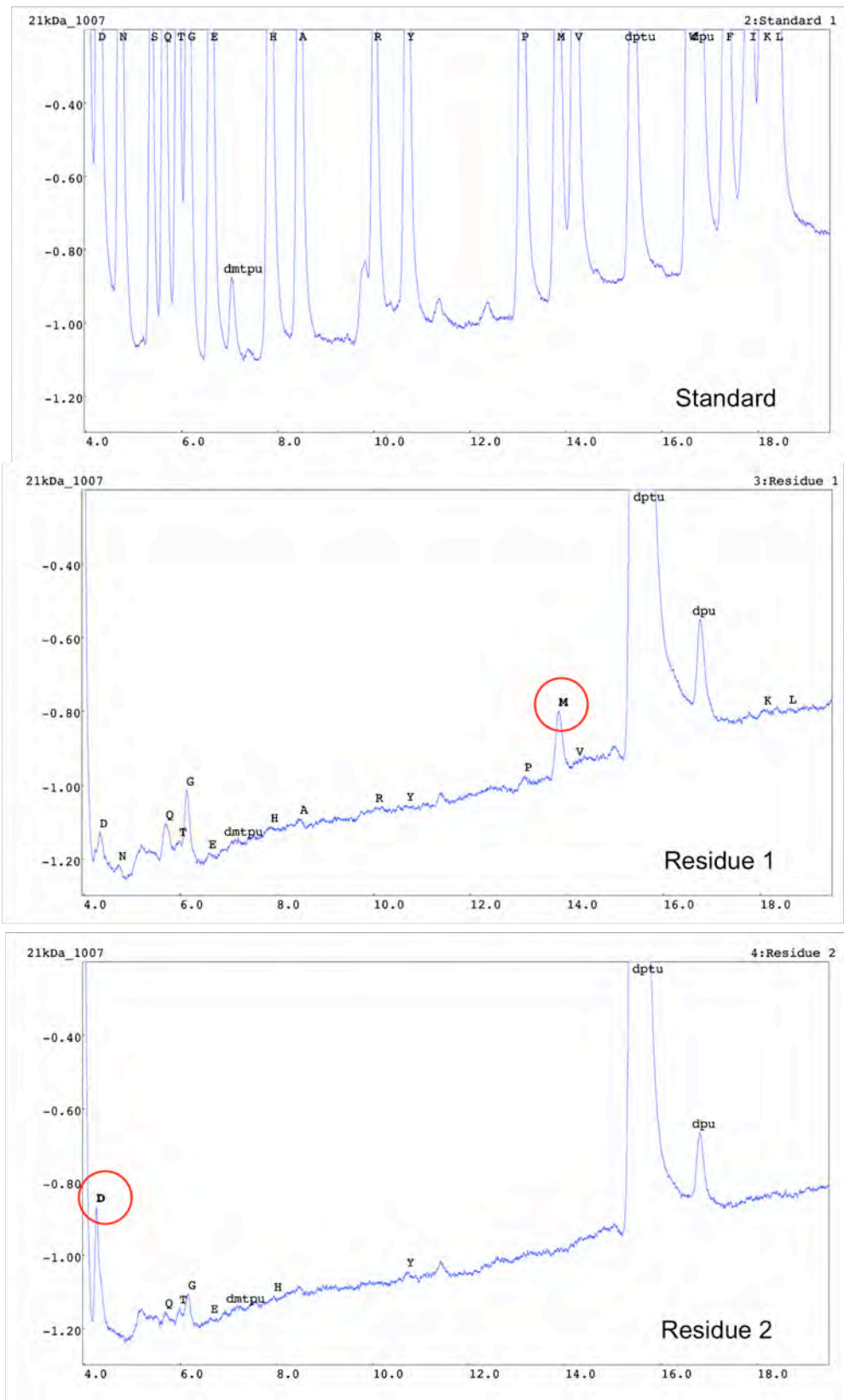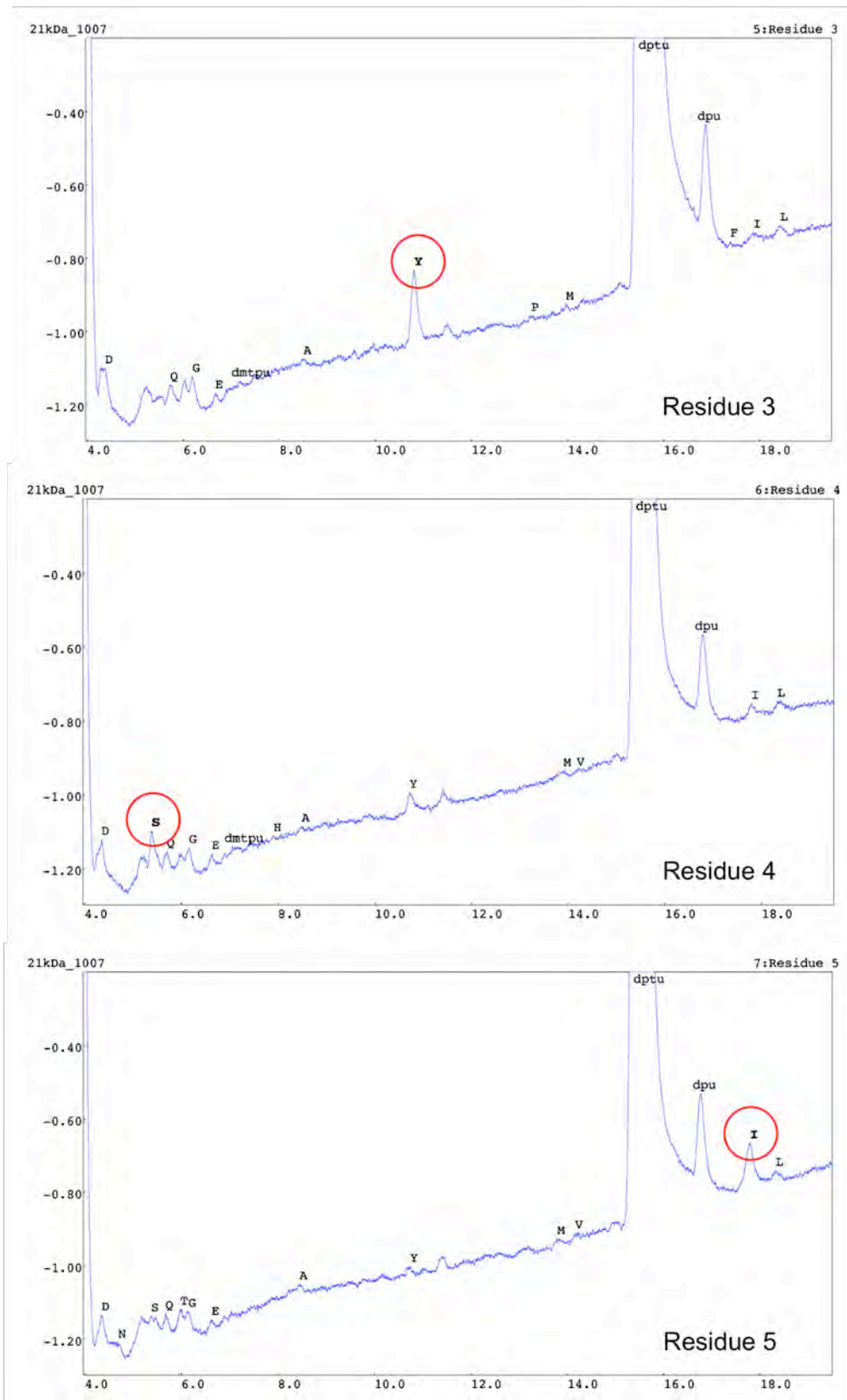
Figure 3-6. Chromatogram of the N-terminal sequence of native AAC(6')-Iaf

3-1-5. Acetylation activity of AAC(6')-Iaf

To examine the biochemical properties of AAC(6')-Iaf in aminoglycoside resistance, the ability of native AAC(6')-Iaf to acetylate was tested by TLC using various aminoglycosides (Fig. 3-7).
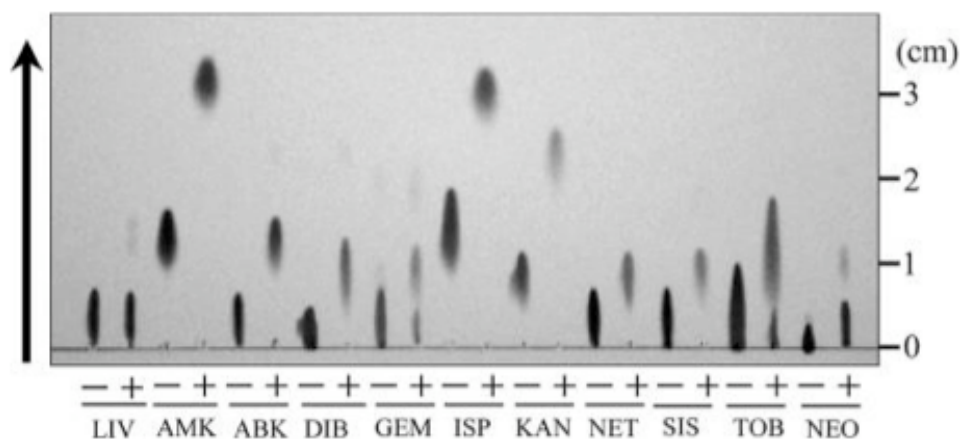

Figure 3-7. Analysis of acetylated aminoglycosides by TLC. Native AAC(6'-Iaf and various aminoglycosides were incubated in the absence (–) or presence (+) of acetyl-CoA. LIV, lividomycin A. The arrow indicates the direction of development.

It was found that, in the presence of acetyl coenzyme A, native AAC(6')-Iaf acetylatd all aminoglycosides with an amino group at the 6' position, showing AAC(6')-Iaf has broad spectrum of aminoglycoside. Commercially available gentamicin is a mixture of derivatives of gentamicin, including gentamicins C1, C1a, C2, and C2b (Vakulenko & Mobashery, 2003, Shaw *et al.*, 1993). Neomycin also consists of derivatives of neomycin B and C. It was found that AAC(6')-Iaf partially acetylated both of these reagents. Surprisingly, lividomycin A, which has a hydroxyl group at the 6' position, was also a substrate for AAC(6')-Iaf, although only an extremely low amount of AAC activity was detected. This partial acetylation of

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lividomycin A suggests that AAC(6')-Iaf may be able to acetylate an alternate amino group at 2' or 3 positions in the aminoglycoside molecule. Additionally, AAC(6')-Iaf was thought to be bifunctional enzyme possessing both *N*-acetyltransfer and *O*-acetyltransfer activity. These observation has shown in AAC(6')-APH(2'') from enterococci and AAC(6')-Isa (Daigle *et al.*, 1999, Hamano *et al.*, 2004). However, the acetylation assay using spectrophotometer indicated that *O*-acetylation of lividomycin A was not observed in the presence of excess amount of AAC(6')-Iaf, whereas *N*-acetylation of aminoglycoside that possess aminogroup at 6' position was observed in the same condition. Collectivelly, These data indicate that *aac(6')-Iaf* encodes a functional aminoglycoside 6'-*N*-acetyltransferase that effectively modifies the amino group at the 6' position of aminoglycosides in vitro.

E. coli JM109 carrying pSTV-*aac*WT expressing exogenous AAC(6')-Iaf did not show reduced susceptibility to arbekacin, gentamicin, or neomycin (Table 3). In comparison, *Enterococcus faecium* producing AAC(6')-Ii was susceptible to neomycin, although AAC(6')-Ii was able to acetylate neomycin (Wright & Ladak, 1997). In the arbekacin-resistant actinomycete strain, arbekacin and neomycin were shown to retain their antibiotic effects even after they were acetylated at their 6' positions by AAC(6')s (Zhu *et al.*, 1999). These results suggest that acetylation of arbekacin and neomycin at their 6' positions does not affect the antimicrobial activities of these drugs. Alternatively, the antimicrobial activity of these antibiotics retained after treatment with AAC(6')-Iaf may be due to residual unacetylated arbekacin or neomycin. In addition, the gentamicin derivatives C1 and C2b carry a methyl group at the 6' position, which may make them refractory to AAC(6')-I enzymes.

3-1-6. Kinetic study of AAC(6')-Iaf

The biochemical properties of AAC(6')-Iaf were also tested by steady state kinetics. Kinetic study was carried out using amikacin, kanamycin-A, and Tobramycin. The kinetic patterns and parameters were described in Fig. 3-8 and Table 3-2, respectively.
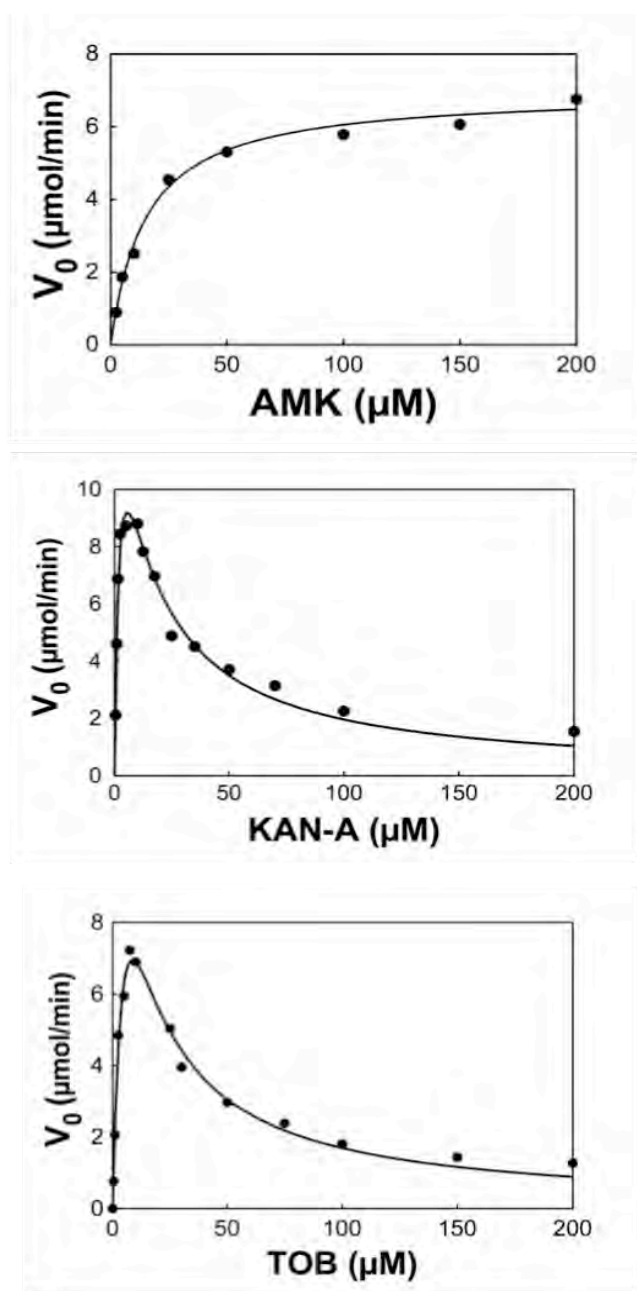

Figure 3-8. Kinetic patterns of AAC(6')-Iaf.

As for amikacin, the plots of initial velocity fitted to Michaelis-Menten model. In contrast, as for kanamycin-A and tobramycin, the plots of initial velocity fitted to substrate inhibition model. Steady-state affinity of amikacin, kanamycin-A, and tobramycin with AAC(6')-Iaf were 15.2 μM, 4.4 μM, and 5.3 μM, respectively. Although these K_m values indicated that interaction of aminoglycosides with AAC(6')-Iaf has high affinity, catalytic turnover rates (k_{cat}) revealed slow reaction (0.11-0.48 per second). The inhibition constants (K_i) of kanamycin-A and tobramycin were 13.2 μM and 9.6 μM, respectively. Specificity constants (k_{cat}/K_m) of AAC(6')-Iaf is all on the order of 10^4 M^{-1} s^{-1}, indicating lower than the other aminoglycoside-inactivating enzymes with high values between 10^8 M^{-1} s^{-1} to 10^9 M^{-1} s^{-1} (McKay, *et al*., 1994, Siregar, *et al*., 1995). These low turnover rates and specificity constants of AAC(6')-Iaf were in good agreement with those of AAC(6')-Ii and -Iy ((Draker & Wright, 2004, Magalhaes, *et al*., 2008).

Table 3-2. Kinetic parameters of AAC(6')-Iaf.

Aminoglycoside	K_m (μM)	k_{cat} (s^{-1})	Ki (μM)	k_{cat}/K_m (M^{-1}s^{-1})
Amikacin	15.2 ± 0.21	0.48	none	6.8×10^4
Kanamycin-A	4.4 ± 0.58	0.11	13.2 ± 2.94	2.6×10^4
Tobramycin	5.3 ± 0.23	0.21	9.6 ± 2.59	3.9×10^4

3-1-7. Catalytic residue of AAC(6')-Iaf

Protonation of the thiol anion of AcCoA by the general base is required to acetylate aminoglycoside (see 2-1-5, and Fig. 2-7, above). The hydroxyl group in the side chain of Tyr147 has been reported to act as a general base in AAC(6')-Ii activity, although this residue did not affect catalysis (Draker & Wright, 2004). Structural alignment of AAC(6')-Ii and AAC(6')-Iaf model, showing that Tyr154 in AAC(6')-Iaf corresponded to Tyr147 in AAC(6')-Ii, suggested that Tyr154 may act as the catalytic residue in AAC(6')-Iaf (Fig. 3-9).
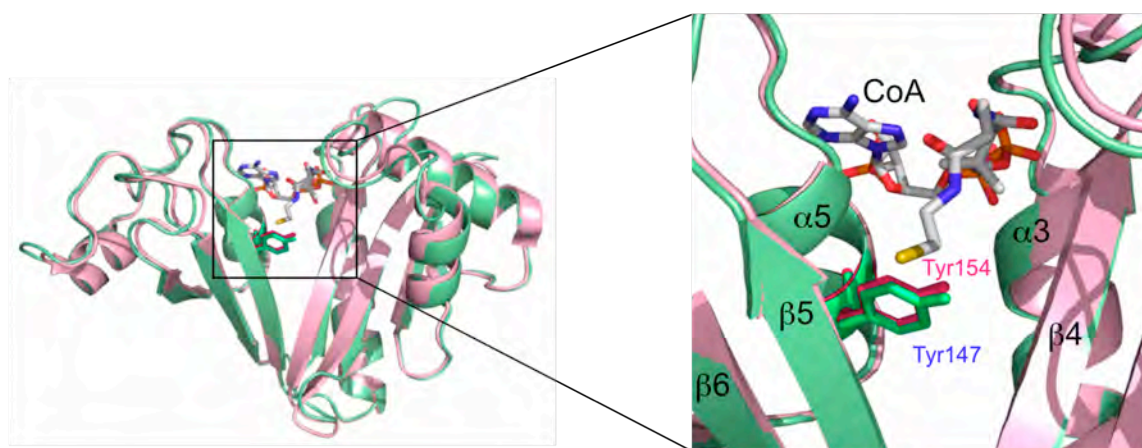

Figure 3-9. Identification of the general base in aminoglycoside acetylation by AAC(6')-Ii. The AAC(6')-Ii-CoA complex structure (PDB:1n71, gray) and the AAC(6')-Iaf model (light pink) were superimposed using MacPymol software. (Right panel) Tyr147 in AAC(6')-Ii is indicated in green, and Tyr154 in AAC(6')-Iaf is indicated in pink.

To examine the role of this residue in aminoglycoside acetylation, Tyr154 was substituted with an Ala residue, and the resulting AAC(6')-Iaf mutant protein, Y154A, was assessed for its susceptibility to aminoglycoside and its ability to acetylate aminoglycoside (Fig. 3-10). In aminoglycoside susceptibility testing, four aminoglycosides that showed high-level resistance when tested with pSTV-*aac*WT (see section 3-1-1 above), were used. MIC values for all aminoglycosides was more than three-fold lower in JM109 carrying pSTV-*aac*Y154 than in JM109 carrying pSTV-*aac*WT (Fig. 3-10).

A

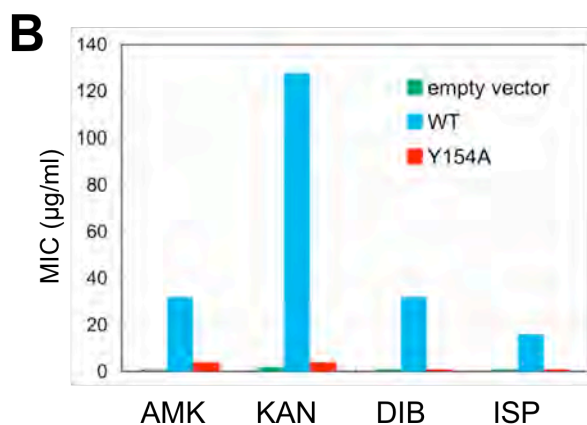| Strain	MIC (μg/ml)			
	AMK	KAN-A	DIB	ISP
JM109 pSTV28	1	2	1	1
JM109 pSTV-aacWT	32	128	32	16
JM109 pSTV-aacY154A	4	4	1	1

B

Figure 3-10. Effect of the Y154A substitution in AAC(6')-Iaf on aminoglycoside resistances. (A) Aminoglycoside susceptibility testing. (B) Plot of the MIC data in A.

In addition, the ability of purified AAC(6')-Iaf Y154A to acetylate these aminoglycosides was more than 10-fold lower than using wild-type protein (Fig. 3-11). These findings suggested that the Tyr154 residue in AAC(6')-Iaf may serve as the general base that protonates the thiolate anion of AcCoA, similar to Y147 in AAC(6')-Ii. However, further experiments including replacement of Tyr154 with Phenylalanine, determinations of steady-state kinetics and analysis of crystal structure, are needed to determine the detailed catalytic mechanism by which of aminoglycoside acetylation by AAC(6')-Iaf.
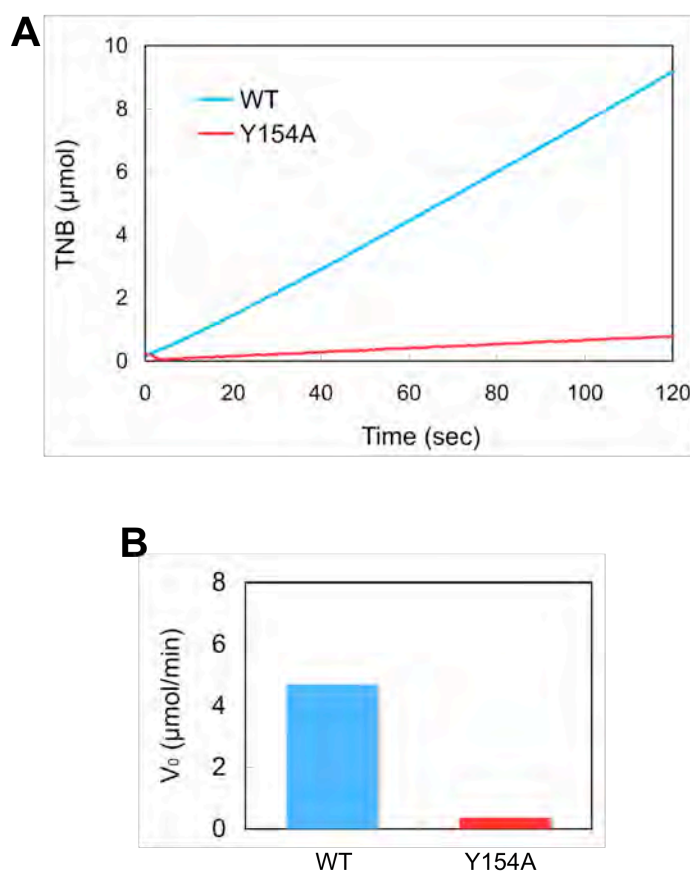

Figure 3-11. Effect of the Y154A substitution in AAC(6')-Iaf on aminoglycoside acetylation activity. (A) The amikacin acetylation was spectrophotometrically monitored at 37°C. (B) Plot of acetylation activity in A.

3-1-8. Amino acid residues essential for aminoglycoside resistance.

As described in the Introduction (Chapter 1, 1-4, above), at least 30 kinds of AAC(6')s have been identified to date. Of these, limited AAC(6')s including the elucidation of the three-dimensional structure of AAC(6')-Ib, -Iy, and -Ii have been characterized biochemically. Although details of the interaction between acetyl-CoA or aminoglycoside have been clearly shown in AAC(6')-Ib and –Iy. As for AAC(6')-Ii, whereas the interaction with AcCoA is analyzed, the interaction with aminoglycoside is still unknown.

To determine the amino acid residues essential for aminoglycoside inactivation by AAC(6')Iaf that belongs to the same subfamily as AAC(6')-Ii, mutational analyses in the putative substrate pocket of AAC(6')-Iaf were performed. The amino acid residues were selected based on the model structure of AAC(6')-Iaf. The replacement of Trp34, Glu78, Asp118, and Asp175 by Ala led to decrease of the MIC values for aminoglycsides (Fig. 3-12A).

The configuration of these residues was analyzed on model structure of AAC(6')-Iaf, and compared with the substrate pocket of AAC(6')-Iy and –Ib (Fig. 3-12B). In both AAC(6')-Iy and –Ib, it is reported that acidic and aromatic amino acids were associated with aminoglycoside binding. The configration of Trp34, Glu78, Asp118, and Asp175 in putative substrate pocket of AAC(6')-Iaf model structure was similar to that of AAC(6')-Iy and AAC(6')-Ib. Additionally, these amino acid residues were conserved in AAC (6') subfamily including AAC(6')-Iaf and AAC(6')-Ii.

Western blotting analysis with anti-AAC(6')-Iaf polyclonal antibodies was indicated that the AAC(6')-Iaf mutants were produced in *E. coli* JM109 as well as AAC(6')-Iaf WT (Fig. 3-12C).

Collectively, Trp34, Glu78, Asp118, and Asp175 in AAC(6')-Iaf were essential amino acid residues for aminoglycoside resistance. However, further analysis such as kinetic study using AAC(6')-Iaf mutant proteins and the structural analysis of AAC(6')-Iaf-CoA-aminoglycoside complex will be require to clarify the detail of the interaction between this enzyme and substrates.
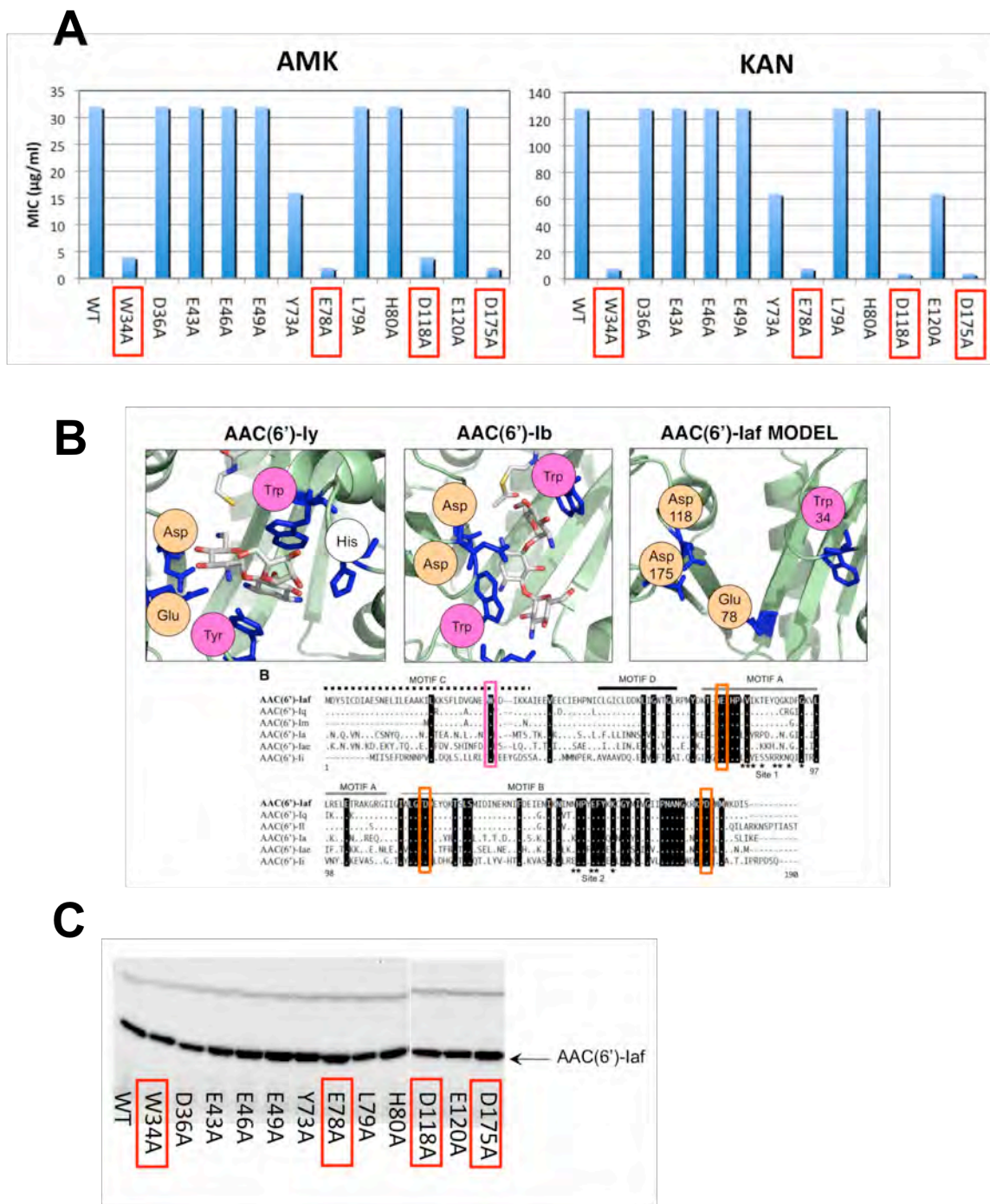
Figure 3-12. (A) The results of aminoglycoside susceptibility testing for amikacin (AMK) and kanamycin-A (KAN). (B) The comparison of the substrate pocket in AAC(6')-I proteins, and the location of amino acid residues essential for aminoglycoside resistance catalyzed by AAC(6')-Iaf, in the sequence alignment. (C) The expression of AAC(6')-Iaf mutants in *E. coli* JM109. Red squares indicate amino acid residues essential for AAC(6')-Iaf activity.

3-1-9. Interaction of substrates with AAC(6')-Iaf

The data from acetylation assay revealed that AAC(6')-Iaf could catalyze aminoglycoside acetylation. These results mean that AAC(6')-Iaf recognizes two substrates, aminoglycoside and acetyl-CoA. The interaction of these substrates with AAC(6')-Iaf was analyzed using isotermal titration calorimetric approach.

The ITC profiles are shown in Fig. 3-13. Interactions of both CoA and aminoglycoside with AAC(6')-Iaf were exothermic reaction. In the interaction between Coenzyme A (CoA) and AAC(6')-Iaf, the binding isotherms revealed that the affinity of CoA in the absence of amikacin was 3-fold lower than that in the presence of CoA, suggesting that the CoA binding may depend on aminoglycoside. In addition, a very small heat release was observed in the binding of CoA-AAC(6')-Iaf without aminoglycoside, showing the weak binding to AAC(6')-Iaf. In contrast, the affinity of amikacin was high with or without CoA (0.1μM or 0.18μM). These results could suggest that aminoglycoside is leading substrate of AAC(6')-Iaf. However, given that CoA used in the ITC experiments is product analogue of AAC(6')-Iaf, further analyses such as dead-end inhibition kinetics is required to clarify the detailed mechanism of AAC(6')-Iaf acetylation.
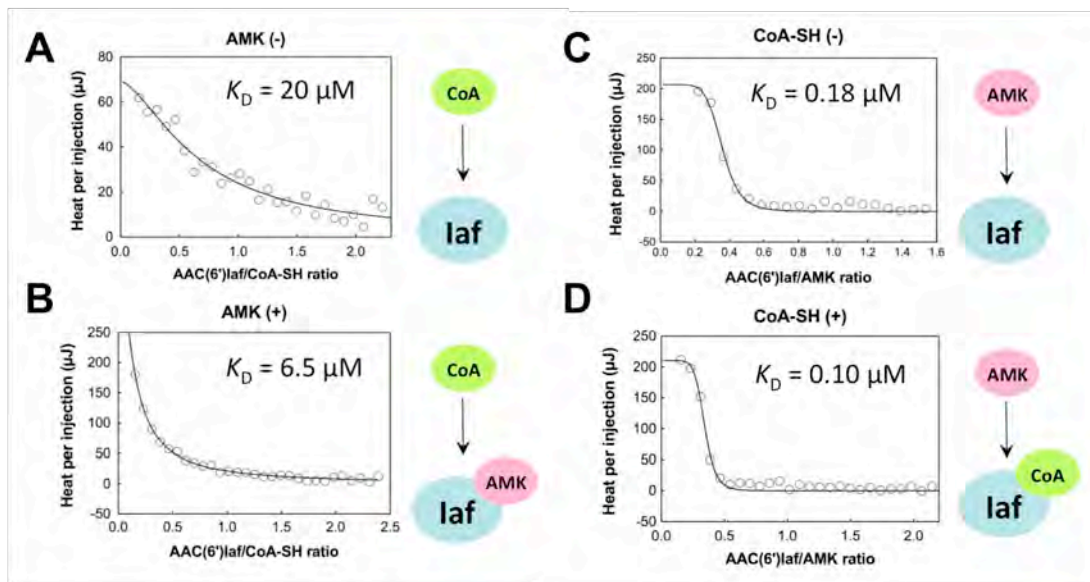
Figure 3-13. Plot of heat per injection (μJ) versus the titrated stoichiometry between enzyme and ligend. The calcuration was performed with NanoAnalyze Software v2.1.9 from TA instruments. (A) CoA-SH was titrated into AAC(6')-Iaf. (B) CoA-SH was titrated into AAC(6')-Iaf-amikacin(AMK) complex. (C) AMK was titrated into AAC(6')-Iaf. (D) AMK was titrated into AAC(6')-Iaf-CoASH complex.

3-2. Materials and Methods

3-2-1. Cloning of the *aac(6')-Iaf* gene

The open reading frame (ORF) of *aac(6')-Iaf* and its 185 bp upstream region, which includes its promoter, were PCR amplified from *P. aeruginosa* IMCJ798 DNA using the primer set PstI-aac_F and EcoRI-aac_R (Table 2-3, above). The PCR products were digested with *Eco*RI and *Pst*I and ligated into the *Pst*I and *Eco*RI sites of pSTV28, at a polarity opposite to the transcriptional direction of the promoter on the vector. The plasmids were used to transform DH5α, and transformants were selected on LB agar containing 30 µg/ml chloramphenicol. The resulting plasmid was designated pSTV-*aac*WT. To determine its MIC values, pSTV-*aac*WT was used to transform *E.coli* JM109, which represses transcription driven by the promoter on the pSTV28 vector.

3-2-2. Site directed mutagenesis

The putative TTG initiation codon on pSTV-*aac*WT was replaced by ATG using primers, 5'-TACCTATCTGGAGTTTGTTATGGACTATTC-3' and 5'-GAATAGTCCATAACAAACTCCAGATAGGTA-3', according to the protocol of QuickChange Site-Directed Mutagenesis kit (Stratagene). The resulting plasmid was designated pSTV-*aac*(TTG→ATG). To determine MIC values for aminoglycosides, these plasmids were used to transform *E. coli* JM109.

The putative catalytic residue, Tyr 154 in the AAC(6')-Iaf was also changed to Alanine residue by site directed mutagenesis using primer described in Table 3-3. The pSTV-*aac*(TTG→ATG) was used as the template plasmid. To determine the function of tyr

154 in AAC(6')-Iaf, the resulting plasmid pSTV-aac Y154A was used to transform *E. coli*
JM109.

The alanine scanning in putative substrate pocket of AAC(6')-Iaf were also performed
QuickChange system using primers listed in Table 3-3. The resulting plasmids were
summarized in Table 3-4. These plasmids were also used to transforme *E. coli* JM109.

Table 3-3. Primers used in the mutational analysis.

Mutation	Primer name	sequence (5' to 3')
W34A	Iaf-W34A_F	TGGAAATGAATCAGCGGGAGATATTAAAAA
	Iaf-W34A_R	TTTTTAATATCTCCCGCTGATTCATTTCCA
D36A	Iaf_D36A_F	TGAATCATGGGGAGCTATTAAAAAAGCTAT
	Iaf_D36A_R	ATAGCTTTTTTAATAGCTCCCCATGATTCA
E43A	Iaf_E43A_F	AAAAGCTATTGAAGCAGTTGAAGAATGTAT
	Iaf_E43A_R	ATACATTCTTCAACTGCTTCAATAGCTTTT
E46A	Iaf_E46A_F	ATTGAAGAAGTTGAAGCATGTATAGAACAT
	Iaf_E46A_R	ATGTTCTATACATGCTTCAACTTCTTCAAT
E49A	Iaf_E49A_F	TTGAAGAATGTATAGCACATCCAAATATAT
	Iaf_E49A_R	ATATATTTGGATGTGCTATACATTCTTCAA
Y73A	Iaf_Y73A_F	ATTAAGGCCGATGGCCGATAAGACCTGGGA
	Iaf_Y73A_R	TCCCAGGTCTTATCGGCCATCGGCCTTAAT
E78A	Iaf-E78A_R	CCATGGGATGAAGTGCCCAGGTCTTATCGT
	Iaf-E78A_F	ACGATAAGACCTGGGCACTTCATCCCATGG
L79A	Iaf-L79A_R	TAACCATGGGATGAGCTTCCCAGGTCTTAT
	Iaf-L79A_F	ATAAGACCTGGGAAGCTCATCCCATGGTTA
H80A	Iaf-H80A_R	TTATAACCATGGGAGCAAGTTCCCAGGTCT
	Iaf-H80A_F	AGACCTGGGAACTTGCTCCCATGGTTATAA
D118A	Iaf-D118A_F	AGCTCTTGGAACTGCTGATGAATATCAGAA
	Iaf-D118A_R	TTCTGATATTCATCAGCAGTTCCAAGAGCT
E120A	Iaf-E120A_F	TGGAACTGATGATGCATATCAGAAAACTAG
	Iaf-E120A_R	CTAGTTTTCTGATATGCATCATCAGTTCCA
Y154A	Iaf-Y154A_F	ATCCATATGAGTTTGCTAAGAAATGTGGTT
	Iaf-Y154A_R	AACCACATTTCTTAGCAAACTCATATGGAT
D175A	Iaf_D175A_F	AAAAAGGAAACCAGCTATATGGATGTGGAA
	Iaf_D175A_R	TTCCACATCCATATAGCTGGTTTCCTTTTT

Table 3-4. The expression vectors for AAC(6')-Iaf mutants.

Plasmids	Description
pSTV28	Cloning vector, Cm^r
pSTV-aac WT	Recombinant plasmid carrying ORF of *aac(6)-Iaf* gene and 185 upstream region inserted in the *PstI-EcoRI* site of pSTV28
pSTV-aac (TTG->ATG)	pSTV-aac WT replaced putative initiation codon TTG with ATG
pSTV-aacW34A	pSTV-aac (TTG->ATG) replaced Trp34 codon in AAC(6')-Iaf with Ala codon
pSTV-aacD36A	pSTV-aac (TTG->ATG) replaced Asp36 codon in AAC(6')-Iaf with Ala codon
pSTV-aacE43A	pSTV-aac (TTG->ATG) replaced Glu43 codon in AAC(6')-Iaf with Ala codon
pSTV-aacE46A	pSTV-aac (TTG->ATG) replaced Glu46 codon in AAC(6')-Iaf with Ala codon
pSTV-aacE49A	pSTV-aac (TTG->ATG) replaced Glu49codon in AAC(6')-Iaf with Ala codon
pSTV-aacY73A	pSTV-aac (TTG->ATG) replaced Try73 codon in AAC(6')-Iaf with Ala codon
pSTV-aacE78A	pSTV-aac (TTG->ATG) replaced Glu78 codon in AAC(6')-Iaf with Ala codon
pSTV-aacL79A	pSTV-aac (TTG->ATG) replaced Leu79 codon in AAC(6')-Iaf with Ala codon
pSTV-aacH80A	pSTV-aac (TTG->ATG) replaced His80 codon in AAC(6')-Iaf with Ala codon
pSTV-aacD118A	pSTV-aac (TTG->ATG) replaced Asp118 codon in AAC(6')-Iaf with Ala codon
pSTV-aacE120A	pSTV-aac (TTG->ATG) replaced Glu120 codon in AAC(6')-Iaf with Ala codon
pSTV-aacY154A	pSTV-aac (TTG->ATG) replaced Try154 codon in AAC(6')-Iaf with Ala codon
pSTV-aacD175A	pSTV-aac (TTG->ATG) replaced Asp175 codon in AAC(6')-Iaf with Ala codon

3-2-3. Preparation of anti-AAC(6')-Iaf polyclonal antibody

Rabbits were immunized with recombinant AAC(6')-Iaf protein emulsified in Freund's adjuvant. The animal experiments were approved by the ethical committee for animal experiments of the Research Institute of International Medical Center of Japan. Anti-AAC(6')-Iaf IgG, purified from rabbit sera on Protein G Sepharose (GE Healthcare), was coupled to NHS-activated Sepharose (GE Healthcare) according to the manufacturer's instructions.

3-2-4. Western blotting

E. coli JM109 carrying pSTV28, pSTV-*aac*WT or pSTV-*aac*(TTG→ATG) were cultivated for 16 h at 37ºC in LB broth containing 30 μg/ml chloramphenicol. *P. aeruginosa* IMCJ798 and IMCJ799 were cultivated for 16 h at 37ºC in LB broth containing 20 μg/ml amikacin. One milliliter of each culture was collected and centrifuged, and whole cell lysates were prepared in 200 μl of SDS-PAGE sample buffer. A 5 μl aliquot of each cell lysate was separated on 15% SDS-PAGE gels, and the proteins were transferred to PVDF membranes. The membranes were blocked with 5% skim milk in 20 mM Tris (pH8.0), 150 mM NaCl and 0.05% Tween20, and incubated with rabbit polyclonal anti-AAC(6')-Iaf antibodies, obtained by immunization with His-AAC(6')-Iaf. After incubation with secondary HRP-linked anti-rabbit IgG (GE Healthcare), bands were detected by chemiluminescence. The intensity of each band was quantified using Quantity One software (Bio-Rad Laboratories).

3-2-5. Construction of AAC(6')-Iaf-overexpressing strains

The *aac(6')-Iaf* gene was PCR amplified from *P. aeruginosa* IMCJ 798 DNA using the primer set, SphI-aac-F and PstI-aac-R (Table 2-3, above). The PCR product was digested with *Sph*I and *Pst*I and ligated into pQE2 (Invitrogen) that had been digested with the same restriction enzymes. These plasmids were used to transform DH5α, and the transformants were selected on LB agar containing 100 μg/ml ampicillin. The resulting plasmid, pQE-*aac(6')-Iaf*, was used to transform *E.coli* BL21(DE3), which was used for purification of recombinant protein.

3-2-6. Purification of recombinant AAC(6')-Iaf

E. coli BL21(DE3), carrying the plasmid pQE2-*aac(6')-Iaf*, was grown in LB medium containing 200 μg/ml ampicillin at 37°C until the A_{600} reached 0.3. IPTG was added to a concentration of 0.1 mM to induce the expression of AAC(6′)-Iaf, and the culture was incubated for 4 h at 37°C. The hexahistidine-tagged AAC(6')-Iaf was purified from the soluble fraction using Ni-NTA Agarose (QIAGEN, Tokyo, Japan) according to the manufacturer's instructions. The final concentration of protein was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA).

3-2-7. Gel filtration chromatography

The molecular mass of the purified native protein in solution was estimated by gel filtration chromatography on HiLoad 16/60 Superdex 200 (GE Healthcare Bio-Sciences KK, Tokyo, Japan) equilibrated in 25 mM NaCl, 10 mM Tris (pH 7.5). Chromatography was controlled using an AKTA purifier (GE Healthcare Bio-Sciences KK). The column was calibrated with aprotitnin (6,500 Da), ribonuclease A (13,700 Da), carbonic anhydrase (29,000 Da), ovalbumin (43,000 Da), and conalbumine (75,000 Da) standards, with blue dextrin used to determine the void volume. Molecular mass was determined by plotting log molecular mass of the standards versus K_{av}, where $K_{av}=(V_e-V_0)/(V_c-V_0)$ (V_e= elution volume; V_0= void volume; V_c= column volume).

3-2-8. Purification of native AAC(6')-Iaf from *P. aeruginosa*

Bacterial cells from overnight cultures of *P. aeruginosa* IMCJ798 were disrupted by sonication, and the cleared lysate was applied to the IgG-coupled Sepharose column. After

washing the column with phosphate buffered saline containing 0.05 % Tween20, protein was eluted with 0.1 M glycine-HCl (pH 2.5). The eluted fraction was immediately neutralized using 1M Tris buffer (pH9.0). The purified protein was dialyzed in Tris buffered saline (TBS; 50mM Tris, 150 mM NaCl, pH8.0) and separated by SDS-PAGE (15%).

3-2-9. N-terminal sequencing

Purified native AAC(6')-Iaf was analyzed using SDS-PAGE and transferred to PVDF membranes. The membranes were incubated with CBB, and the band containing AAC(6')-Iaf was removed. Its N-terminal sequence was determined by using Edman degradation method.

3-2-10. Thin-layer chromatography (TLC) of acetylated aminoglycosides

A mixture containing 2 mM aminoglycoside, 2 mM acetyl-CoA and 50 μg/ml AAC(6')-Iaf in 20 μl of phosphate buffer (pH 7.4) was incubated for 16 h at 37ºC, and 3 μl of each was spotted onto the surface of silica gel TLC plates 60 F_{254} (Merck Ltd., Japan), which were developed with 5% phosphate potassium solution. The aminoglycosides and their acetylated products were detected with 0.5 % ninhydrin in acetone (Zhu *et al.*, 1999).

3-2-11. Acetylation assay and kinetic study using spectrophotometer

AAC activity was determined spectrophotometrically by measuring the increase in A412 due to the formation of 5-thio-2-nitrobenzoate (TNB, 15,500 M–1 cm–1) resulting from the reaction between the sulfhydryl group of the product of the acetyltransfer reaction between CoA-SH and 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB). The reaction mixture (1 ml)

contained 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 100μM DTNB, 50μM AcCoA, and 100 nM proteins. After incubation for 1min, the reaction was initiated by the addition of 2.5 to 200 μM aminoglycoside. The reactions and monitoring were at 37°C. The mean value of initial velocity (V_0) of triplicate measurements was plotted against substrate concentration. Kinetic parameters were calculated using Prism software from GraphPad Inc.

The acetylation activity of AAC(6')-Iaf Y154A mutant was measured using 25 μM amikacin, the obtained result was compared to that of AAC(6')-Iaf WT measured under same condition.

3-2-12. Isothermal titration calorimetry (ITC)

ITC experiments were performed using NanoITC[2G] from TA instruments (Tokyo, Japan). The his-tagged AAC(6')-Iaf was extensively purified by using gel filtration chromatography on HiLoad 16/60 Superdex 200 (GE Healthcare Bio-Sciences KK, Tokyo, Japan) equilibrated in 50 mM Tris Buffer, pH7.5, containing 150 mM NaCl. All samples were prepared with buffer used to equilibrate gel filtration chromatography, and were degassed for 10 minnutes prior to being loaded on apparatus. In individual titrations, infections of 3 μl of the ligand solution were made via the computer-controlled microsyringe, at an interval of 240 seconds, into the enzyme solution (cell volume = 1.0 ml). During measurement, sample cell was stirred at 300 rpm. All measurement were carried out at 25 °C. The concentrations of enzyme and ligand were 25 μM and 0.8 mM, respectively. Dissociation constants (K_D) were determined by plotting heat per injection (μJ) versus the titrated stoichiometry between enzyme and ligend. The calcuration was performed with NanoAnalyze Software v2.1.9 from TA instruments.

Chapter 4. Development of an immunochromatographic assay targeting AAC(6') enzyme for the rapid detection of MDR *P. aeruginosa*

Multidrug-resistant (MDR) *P. aeruginosa* often causes nosocomial outbreaks, resulting in life-threatening infections in immunocompromised patients. Thus, early detection of these bacteria is crucial to early infection controls to prevent nosocomial infections.

The previous molecular epidemiology and the antibiotic gene screening in this study have shown that the *aac(6')-Iae* gene was an amikacin resistant factor in MDR *P. aeruginosa* clinical isolates in Japan. The *aac(6')-Iae* gene encodes an aminoglycoside 6'-*N*-acetyltransferase, and this gene, along with the bla_{IMP} and *aadA1* genes, were present in a class 1 integron In*113* of the MDR *P. aeruginosa* clone IMCJ2.S1 (Sekiguchi *et al*., 2007b). Two diagnostic methods have been developed to detect these strains: a loop-mediated isothermal amplification (LAMP) system using *aac(6')-Iae* specific primers, and an agglutination method using an anti-AAC(6')-Iae polyclonal antibody (pAb). Although these methods are highly sensitive, LAMP is time consuming and requires specialized tools and well-trained medical technicians. In contrast, the agglutination assay had several drawbacks, including human errors in assays of many samples.

Therefore an immunochromatographic assay using monoclonal antibodies that specifically recognize AAC(6')-Iae was designed to detect the prevalent AAC(6')-Iae-producing MDR *P. aeruginosa* (Fig. 4-1). In this chapter, I report its

effectiveness in clinical screening and molecular epidemiology as an alternative to the above

methods. To our knowledge, this is the first report of an immunochromatographic assay

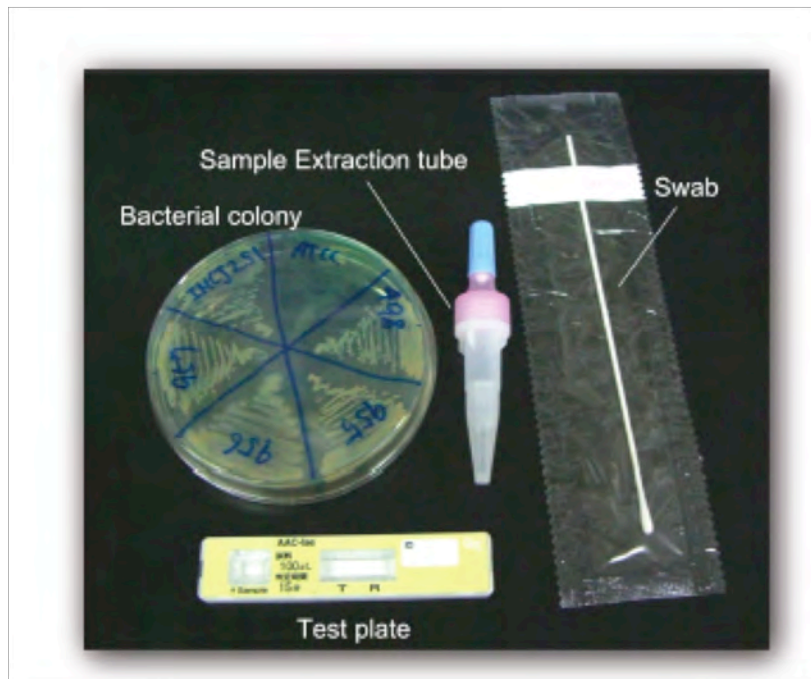using antibodies specific for a molecule that confers antibiotic resistance to bacteria.

Figure 4-1. Immunochromatographic detection kit targeting aminoglycside 6'-*N*-acetyltransferase AAC(6')-Iae developed in this study.

4-1. Results and discussion

4-1-1. Construction of immunochromatography

During screening for mAbs generated by hybridoma clones, we obtained three stable mAbs, 1H7, 3D4, and 3F12, all of which had high reactivity to recombinant His-AAC(6')-Iae from *P. aeruginosa* IMCJ2.S1. The specificities of these mAbs were characterized by western blotting analysis and ELISA using the His-tagged subtype AAC(6')-I proteins of AAC(6')-Iaf, -Ib, and -Iad identified in clinical isolates in Japan, as well as AAC(6')-Iae (Fig. 4-2). In both analyses, 1H7 and 3F12 specifically recognized AAC(6')-Iae, and 3D4 reacted with AAC(6')-Iaf and with AAC(6')-Iae, as well as did pAb against AAC(6')-Iae. The determined epitope sequence of mAbs were not conserved in AAC(6')-Iaf, -Ib, and –Iad (data not shown).

Thus, the 1H7 and 3F12 mAbs were utilized in the assembly of an immunochromatography assay based on a commercial influenza A/B kit, as described in Materials and Methods (see 4-2-4). In this assay, AAC(6')-Iae is detected by a sandwich reaction using mAbs 1H7 and 3F12. As a reference, rabbit gamma globulin labeled with colloidal gold was shown to react with goat anti-rabbit gamma globulin immobilized on membrane, a reaction that occurred in the presence or absence of AAC(6')-Iae. It was found that the assembled assay worked well in the preliminary experiments using 0.5-μg His-AAC(6')-Iae in PBS (Fig. 4-3).
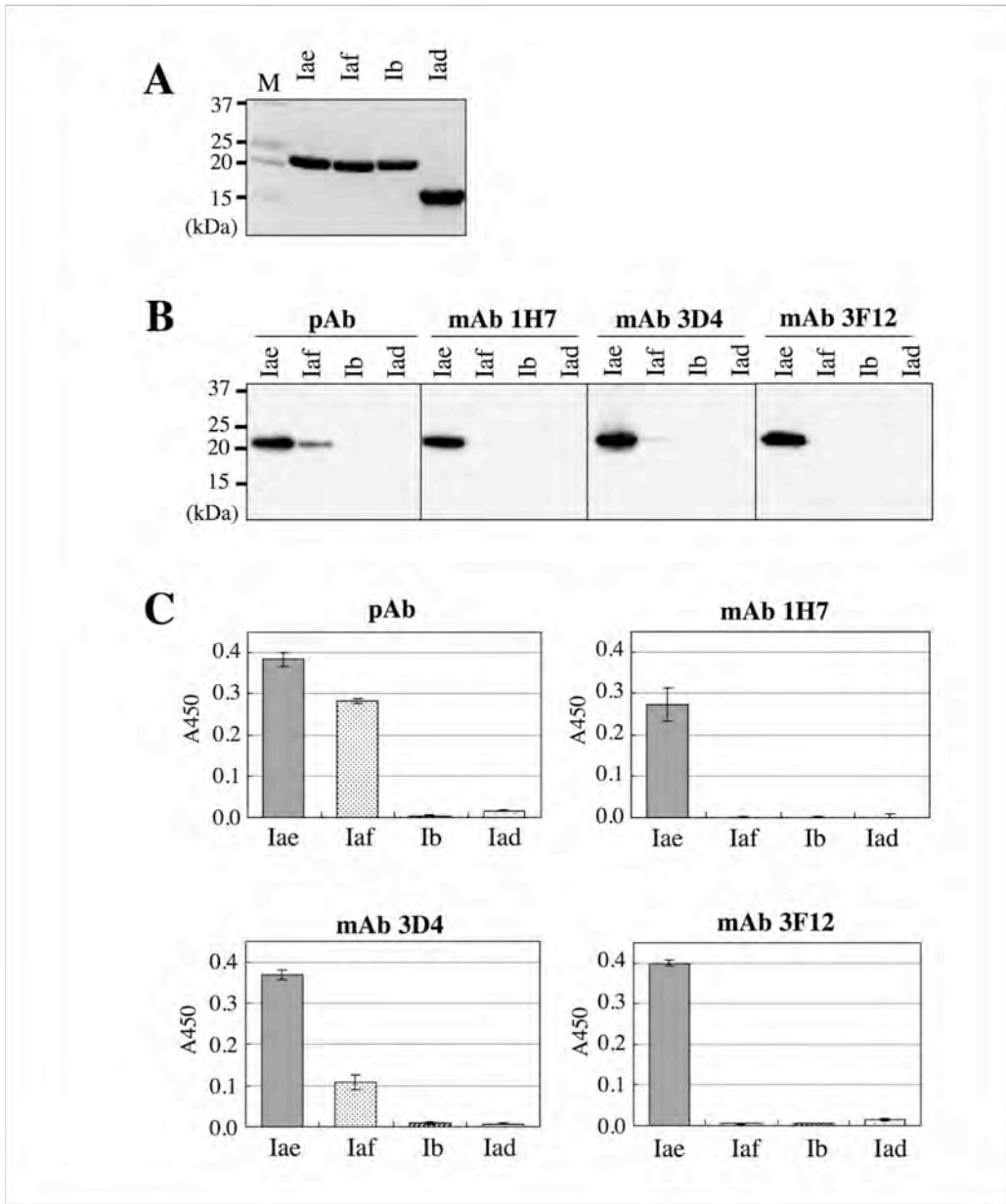
Figure 4-2. Specificity of anti-AAC(6')-Iae mAb for purified His-tagged AAC(6')-I proteins. Purified recombinant AAC(6')-I proteins were separated by SDS-PAGE (A), and the specificities of the rat anti-AAC(6')-Iae mAbs 1H7, 3D4, and 3F12 were analyzed by western blotting (B) and ELISA (C).
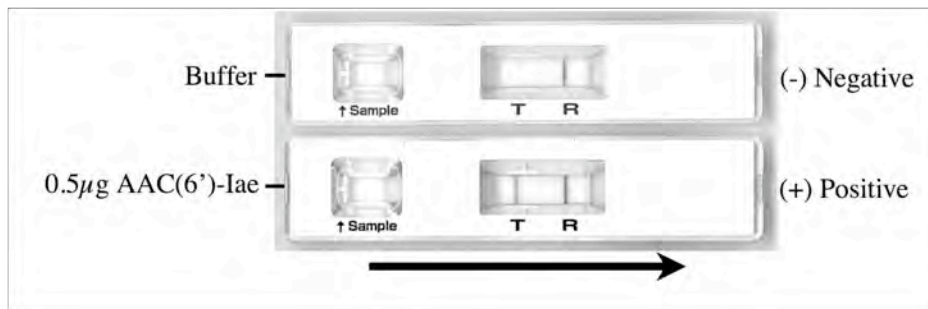
Figure 4-3. Immunochromatography using mAbs 1H7 and 3F12. Negative samples are indicated by a single line at the position of the reference line (R), whereas positive samples are indicted by a second line at the position of the test line (T) in addition to R. The arrow indicates the direction of lateral flow.

4-1-2. Sensitivity and specificity of the assay

The analytical sensitivity of this assay was determined using highly purified recombinant His-tagged AAC(6')-Iae protein and IMCJ2.S1 culture (Fig. 4-4). We observed a clear line with more than 3 ng of AAC(6')-Iae protein (Fig. 4-4A), whereas lines at the reference positions appeared in all samples. Plots of the mean intensity of triplicate measurements for each amount of AAC(6')-Iae were linear (Fig. 4-4B). The intensity of the test line was correlated with the quantity of AAC(6')-Iae, ranging from 1 to 100 ng, indicating a high degree of linearity (r^2 = 0.9755). The theoretical detection limit was 1.84 ng of AAC(6')-Iae per an assay.

In sensitivity testing using IMCJ2.S1 cultures, a clear line appeared with more than 1.2×10^5 cfu of bacteria (Fig. 4-4C), whereas lines at the reference positions appeared in all samples. The plots of the mean values of triplicate measurements for each quantity of bacteria were linear, similar to those for the recombinant protein (Fig. 4-4D). The intensity

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of the test line was correlated with the number of bacteria, ranging from 5.8×10^4 to 4.6×10^5 cfu, indicating a high degree of linearity ($r^2 = 0.9766$). The theoretical detection limit for bacteria was 1.0×10^5 cfu per assay. This was considered sufficient, since the number of bacteria per test ranged from 10^8 to 10^9 when nearly equal amounts were analyzed to determine cfu (data not shown). Most AAC(6')-Iae positive strains were isolated from urine and sputum. Further experiments are needed to evaluate this assay using such clinical specimens. In addition, since antibiotic resistance genes can be transmitted among different species of Gram-negative bacteria via plasmids and transposons, the assay may be able to detect AAC(6')-Iae in other Gram-negative species involved in nosocomial infections.

The assay was also evaluated using the AAC(6') isozymes, AAC(6')-Iaf, -Ib, and –Iad, and their parental strains, in addition to the sensitive strain of *P. aeruginosa* PAO1 (Fig. 4-4E and 4-4F). Using 2 μg of each purified protein, no reactions were observed for any strains, whereas reference lines appeared for all (Fig. 4-4E). Similar results were observed using bacterial lysates from $>10^8$ cfu of each strain (Fig. 4-4F).

Taken together, these results indicated that the assay constructed using the mAbs 1H7 and 3F12 was specific for AAC(6')-Iae and has a high sensitivity of detection.
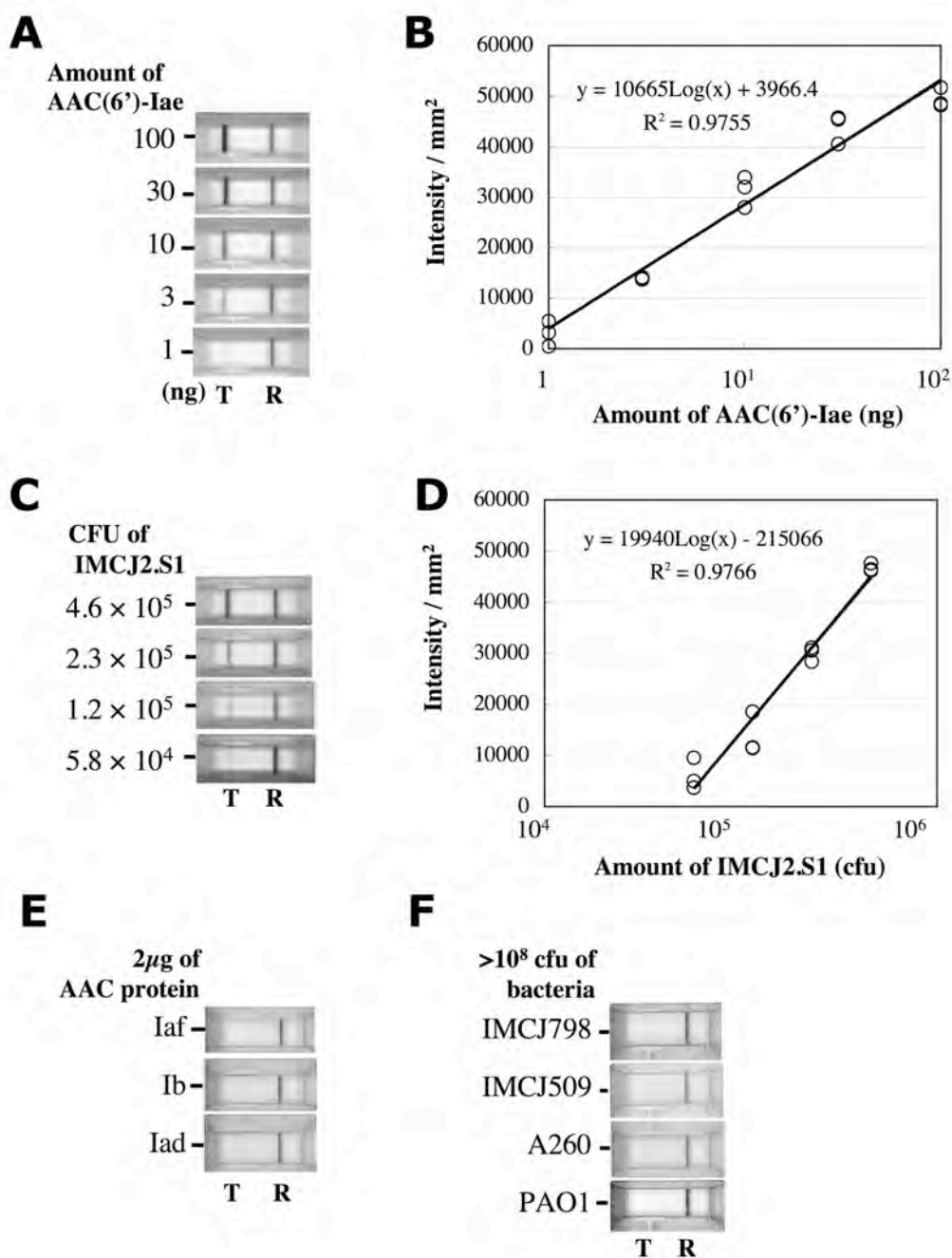
Figure 4-4. Sensitivity and linearity of the immunochromatographic assay. All tests were performed in triplicate. (A) Sensitivity testing using protein. (B) Relationship between amount of AAC(6')-Iae and the intensity of each test line. The intensity of each test line in Fig. 3A was plotted relative to the amount of AAC(6')-Iae. (C) Sensitivity testing using bacteria. (D) Relationship between amount of IMCJ2.S1 and the intensity of each test line. The intensity of each test line in Fig. 3D was plotted relative to the number of IMCJ2.S1 bacteria. (E) Preliminary specificity testing. Two µg of His-AAC(6')-Iaf, -Ib, or -Iad prepared in 100 µl extraction buffer were dropped onto the sample area. (F) Specificity testing using bacteria. Aliquots of 100 μL dilutions containing >10^8 cfu of *P. aeruginosa* IMCJ798 carrying *aac(6')-Iaf*, IMCJ509 carrying *aac(6')-Ib*, *A. baumannii* A260 carrying *aac(6')-Iad*, or sensitive strain *P. aeruginosa* PAO1 were dropped onto the sample.

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4-1-3. Assessment of the assay using clinical isolates

When tested the assay against 116 *P. aeruginosa* clinical isolates, it was found that 60 (52%) were positive for AAC(6')-Iae protein, findings fully consistent with those of *aac(6')-Iae* PCR analyses, indicating that our assay revealed no false positives or false negatives. The highest percentages of positive strains were from urine, followed by sputum.

4-1-4. Characterization of detected AAC(6')-Iae-positive isolates

Antimicrobial susceptibility testing showed that all positive strains had the MDR phenotype (Table 4-1). In addition to being resistant to imipenem, amikacin, and ofloxacin, for most AAC(6')-Iae positive strains showed increased MICs for piperacillin, piperacillin/tazobactam, ceftadizime, aztreonam, and meropenem. In contrast, most of these strains were less resistant to arbekacin, gentamycin, and polymixin-B.

Table 4-1. Antimicrobial susceptibility of detected AAC(6')-Iae-positive strains.

Antibiotics [a]	Positive strains (n= 60)			MIC of ATCC27853 (mg/L)	MIC of IMCJ2.S1 (mg/L)
	Range (mg/L)	MIC50 (mg/L)	MIC90 (mg/L)		
PIP	32 - >512	>512	>512	<4	256
TZP	16 - 512	64	256	4	256
CAZ	128 - >512	512	>512	<1	512
ATM	16 - >512	32	512	4	128
IPM	16 - >512	128	256	1	128
MEM	32 - >512	512	>512	2	>512
AMK	32 - 256	64	256	2	128
ABK	2 - 64	8	16	<0.5	2
GEN	<0.5 - 64	8	16	<1	16
OFX	16 - >128	128	>128	<0.5	128
PMB	2 - 4	4	4	2	2

[a] PIP, piperacillin; TZP, piperacillin-tazobactam; CAZ, cetazidime; IPM, imipenem; MEM, meropenem; ATM, aztreonam; AMK, amikacin; ABK, arbekacin; GEM, gentamicin; OFX, ofloxacin; PMB, polymyxin B.

The genetic environment of the *aac(6')-Iae* were determined by PCR and DNA sequencing. Of the 60 *aac(6')-Iae* positive strains, 48 (80%) carried bla_{IMP-1}, *aac(6')-Iae*, and *aadA1* in their integrons, making them identical to In*113* in IMCJ2.S1. In the remaining 12 (20%) positive strains, bla_{IMP-1} was altered to bla_{IMP-10}, due to a mutation from guanine to thymine at position 145 in bla_{IMP-1}. However, the 59 base element of the bla_{IMP-10} cassette was identical to that of bla_{IMP-1} in In113. These findings suggest that strains with similar genetic backgrounds acquired resistance via a small mobile element. For example, an analysis of class 1 integrons indicated that all positive strains carried In113 or In113-derived integrons. However, the mode of transmission of In113 has not been determined, and further analysis is needed to examine whether In113 is encoded onto the plasmid.

In addition to isolating *P. aeruginosa* strains carrying *aac(6')-Iae* from patients in Miyagi, Tokyo, and Hiroshima (Kouda *et al*., 2009, Sekiguchi *et al*., 2007b, Sekiguchi *et al*., 2007d), AAC(6')-Iae producing strains were found to be isolated from hospitals located in 5 prefectures of Gunma, Saitama, Kanagawa, Chiba, and Ibaraki in Japan (Fig. 4-5).

To clarify the genomic structure of these 116 *P. aeruginosa* isolates, the PFGE analysis was performed. The genetic lineages of AAC(6')-Iae positive strains were relatively similar to the IMCJ2.S1 strain previously isolated in Miyagi and to other outbreak-associated strains isolated from patients in Tokyo hospital A (Fig. 4-6). Although these findings suggested that some of these strains were spread clonally, it must be also noted that some negative strains of PFGE patterns were similar to positive strains.

All AAC(6')-Iae-positive strains had the serotype O:11, as did the strain previously described (Sekiguchi *et al*., 2007a).

Although these findings strongly demonstrate that AAC(6')-Iae is crucial as a marker molecule in MDR *P. aeruginosa* strains in Japan, not all MDR *P. aeruginosa* isolates could be detected using this assay. Actually, 21 of 56 (37%) negative strains had MDR phenotypes, similar to the number of strains resistant to amikacin (data not shown). Furthermore, 16 of 21 (76%) negative strains were positive for both *aac(6')-Ib*, which encodes aminoglycoside 6'-*N*-acetyltransferase Ib, and *bla*$_{IMP-1}$, which encodes metallo-β-lactamase IMP-1, with both of the latter associated with clonal nosocomial infections in hospital M (Fig. 4-5). These data suggested that AAC(6')-Ib and metallo-β-lactamase IMP-1 remain candidate markers of MDR *P. aeruginosa* in Japan. Nevertheless, further molecular-based surveillance of MDR *P. aeruginosa* is required.

Figure 4-5. Summary of the geographic distribution of AAC(6')-Iae-producing *P. aeruginosa* in Japan. The spread of AAC(6')-Iae-producing *P. aeruginosa* isolates in the Kanto area was newly discovered by evaluation of clinical isolates.
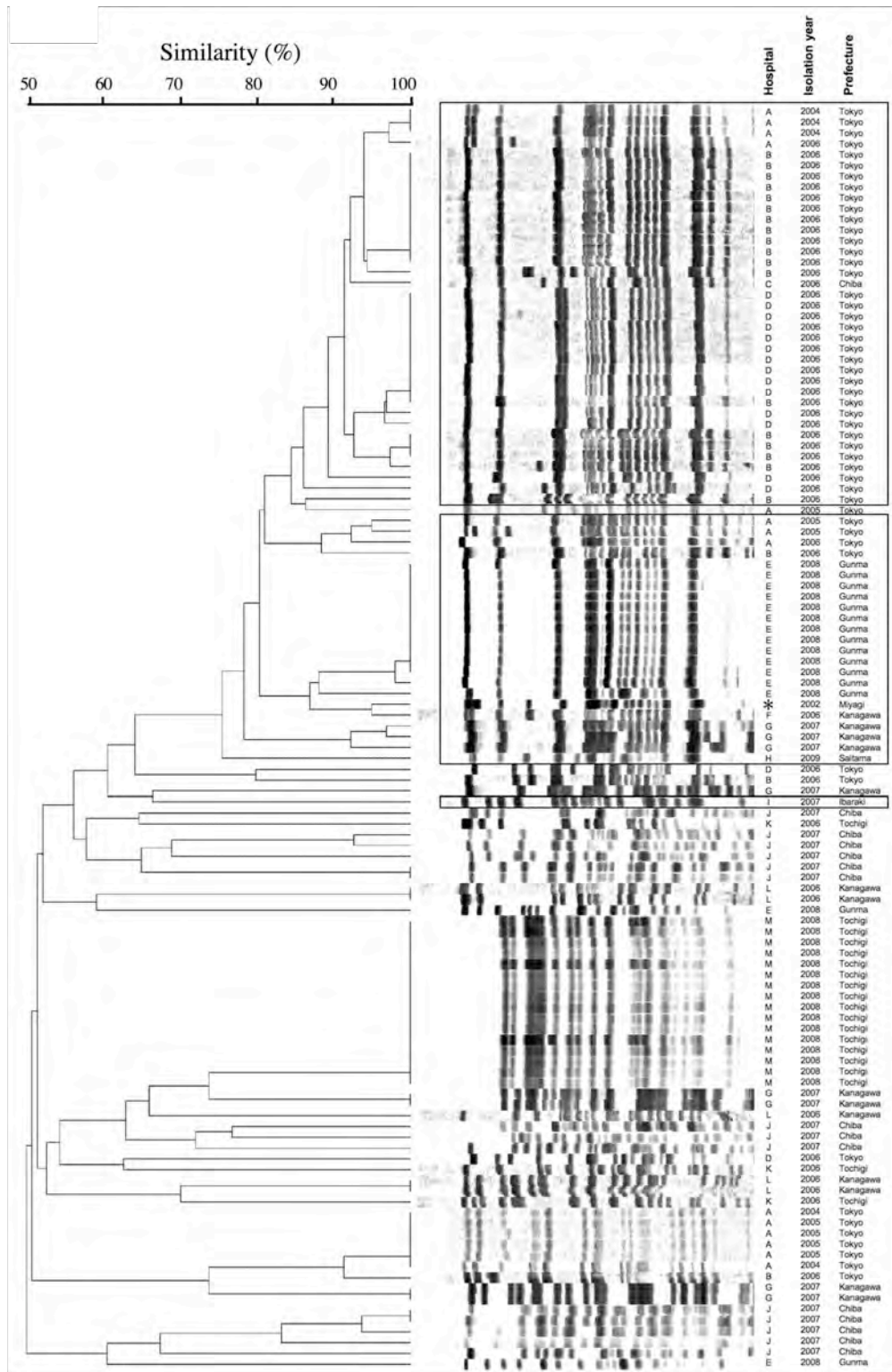
Figure 4-6. PFGE assay of 116 *P. aeruginosa* clinical isolates used in assessment of the development of immunochromatography. The asterisk indicates the P. aeruginosa IMCJ2.S1 strain previously found in Miyagi. The area enclosed in the square indicates the positive isolates detected.

4-2. Materials and Methods

4-2-1. Bacterial strains

P. aeruginosa IMCJ2.S1 was used as the positive control strain for *aac(6')-Iae*. *P. aeruginosa* IMCJ798 carrying *aac(6')-Iaf*, *P. aeruginosa* IMCJ509 carrying *aac(6')-Ib*, and *Acinetobacter baumannii* A260 carrying *aac(6')-Iad* (Doi *et al*., 2004) were used to test the specificity of the assay.

4-2-2. Purification of recombinant proteins

The *aac(6')-Iae, -Iaf, -Ib*, and *–Iad* genes were amplified using their specific primers from strains IMCJ2.S1, IMCJ798, IMCJ509, and A260, respectively (Table 4-2). Cloning and protein purification were performed as described (Materials and Methods 3-2-5).

Table 4-2. Primers used in this study (2)

Primer	Purpose	Sequence [a] (5' to 3')	Description
SphI-aac(6')Iae-F	AAC(6')-Iae amplification and sequencing	aaagcatgcgATGAAATACAACATTGTTA	Positions 1-19 in *aac(6')-Iae* with *Sph*I site
SalI-aac(6')Iae-R	AAC(6')-Iae amplification and sequencing	gaccgtcgacTTACATTATATTTTTCCACA	Positions 552-533 in *aac(6')-Iae* with *Sal*I site
SphI-aac(6')Iaf-F	AAC(6')-Iaf amplification	aaagcatgcgATGGACTATTCAATATGCGA	Positions 1-20 in *aac(6')-Iaf* with *Sph*I site [b]
PstI-aac(6')Iaf-R	AAC(6')-Iaf amplification	aactgcagCTAGCTAATATCTTTCCACA	Positions 552-533 in *aac(6')-Iaf* with *Pst*I site
SphI-aac(6')Ib-F	AAC(6')-Ib amplification	aaagcatgcgATGACCAACAGCACCGATTC	Positions 1-20 in *aac(6')-Ib* with *Sph*I site [c]
SalI-aac(6')Ib-R	AAC(6')-Ib amplification	gaccgtcgacTTAGGCATCACTGCGTGTTC	Positions 555-536 in *aac(6')-Ib* with *Sal*I site
SphI-aac(6')Iad-F	AAC(6')-Iad amplification	aaagcatgcgATGATTAGAAAAGCAACTGTCC	Positions 1-22 in *aac(6')-Iad* with *Sph*I site
SalI-aac(6')Iad-R	AAC(6')-Iad amplification	gaccgtcgacTTAAAGTTGCTTTGTAAAACAAAT	Positions 435-412 in *aac(6')-Iad* with *Sal*I site
5'-CS	Amplification and sequencing of class 1 integron	GGCATCCAAGCAGCAAG	5'-end common segment of class 1 integrons
3'-CS	Amplification and sequencing of class 1 integron	AAGCAGACTTGACCTGA	3'-end common segment of class 1 integrons
balIMP-F1	Sequencing of class 1 integron	AAAGGCAGCATTTCCTCTCA	Positions 262-243 in *bla*$_{IMP-1}$
balIMP-R1	Sequencing of class 1 integron	TTTTATAGCCACGCTCCACA	Positions 265-284 in *bla*$_{IMP-1}$
aac(6')-Iae-F1	Sequencing of class 1 integron	ATTATTGGTGTGATTCCTAA	Positions 481-500 in *aac(6')-Iae*
aac(6')-Iae-R1	Sequencing of class 1 integron	CCAAGAATCGAAATTTATGT	Positions 102-83 in *aac(6')-Iae*
aadA1-F1	Sequencing of class 1 integron	TGATTTGCTGGTTACGGTGA	Positions 114-163 in *aadA1*
aadA1-R1	Sequencing of class 1 integron	GGAGAATCTCGCTCTCTCCA	Positions 259-231 in *aadA1*

[a] Lower case letters represent restriction enzyme recognition sites attached to the 5' ends of the primers.

[b] The initiation codon TTG in *aac(6')-Iaf* was replaced with ATG.

[c] The initiation codon GTG in *aac(6')-Ib* was replaced with ATG.

4-2-3. Preparation of mAbs

Anti-AAC(6')-Iae monoclonal antibodies (mAbs) were prepared as described (Miyoshi-Akiyama *et al*., 2010). His-AAC(6')-Iae was used for immunization and screening of hybridomas by ELISA. The animal experiments were approved by the Ethical Committee for Animal Experiments at the Research Institute of the International Medical Center of Japan.

Epitopes of mAbs was also determined using 18 kinds of peptide that cover the entire sequence of AAC(6')-Iae, listed in Table 4-3. Briefly, the peptides (10 μg/mL) were immobilized onto the wells of a 96-well microtiter plate (Corning) by incubation in 50 mM carbonate buffer (pH 9.0) containing 1 mM of the chemical cross-linker disuccinimidyl suberate (DSS; Pierce) at 4 ℃ for 16 h. After blocking with Superblock (Pierce), the plate was incubated for 1 h with 10 μg/mL of monoclonal antibody (mAb) diluted with PBST, and washed three times with PBST. Binding of mAb to each peptide was detected with HRP-goat anti-rat IgG (GE Healthcare) and TMB (BioRad).

Table 4-3. Peptides used in epitope mapping of mAbs.

No.	amino acid sequence	Region	M.W.
1	MKYNIVNIKDSEKYI	Iae1-15	1858.2
2	SEKYITQAAEILFDV	Iae11-25	1726.96
3	ILFDVFSHINFDSWP	Iae21-35	1837.08
4	FDSWPSLQKATETVI	Iae31-45	1721.94
5	TETVIECISAENICI	Iae41-55	-
6	ENICIGILINDELCG	Iae51-65	1618.89
7	DELCGWVGLREMYKK	Iae61-75	1826.16
8	EMYKKTWELHPMVIK	Iae71-85	1933.38
9	PMVIKKKHQNKGFGK	Iae81-95	1740.16
10	KGFGKILIFETEKKA	Iae91-105	1709.08
11	TEKKAKERNLEGIVL	Iae101-115	1728.04
12	EGIVLGTDDETFRTT	Iae111-125	1653.78
13	TFRTTLSMSELNNEN	Iae121-135	1756.92
14	LNNENIFHEIKNIKN	Iae131-145	1840.08
15	KNIKNLKNHPFEFYE	Iae141-155	1921.2
16	FEFYEKCGYSIIGVI	Iae151-165	1768.07
17	IIGVIPNANGKNKPD	Iae161-175	1549.8
18	KNKPDILMWKNIM	Iae171-183	1631.05

4-2-4. Assembly of the assay

The assay was assembled based on a commercially available rapid diagnosis kit, Quick Chaser™ Flu A, B (Mizuho Medy, Saga, Japan), used to detect influenza viruses A and B, as described.(Miyoshi-Akiyama *et al.*). In our assay, test lines were prepared by coating 0.76 μg mAb 1H7 per test onto nitrocellulose membranes (Millipore, Billerica, MA) at a position 30 mm from the sample application area. To prepare reference lines, 0.2 μg of goat anti-rabbit IgG (Rockland Immunochemicals, Gilbertsville, PA) per test was coated onto the membrane

at a position 39 mm from the sample application area. Pads were prepared by soaking glass filters with mAb 3F12 and rabbit Ig, each conjugated with colloidal gold. The membranes and pads were assembled within a plastic housing, and the assembled assays were stored in a waterproof bag with desiccant at room temperature until use. The composition and principle of immunochromatographic assays are described in Fig. 4-7.
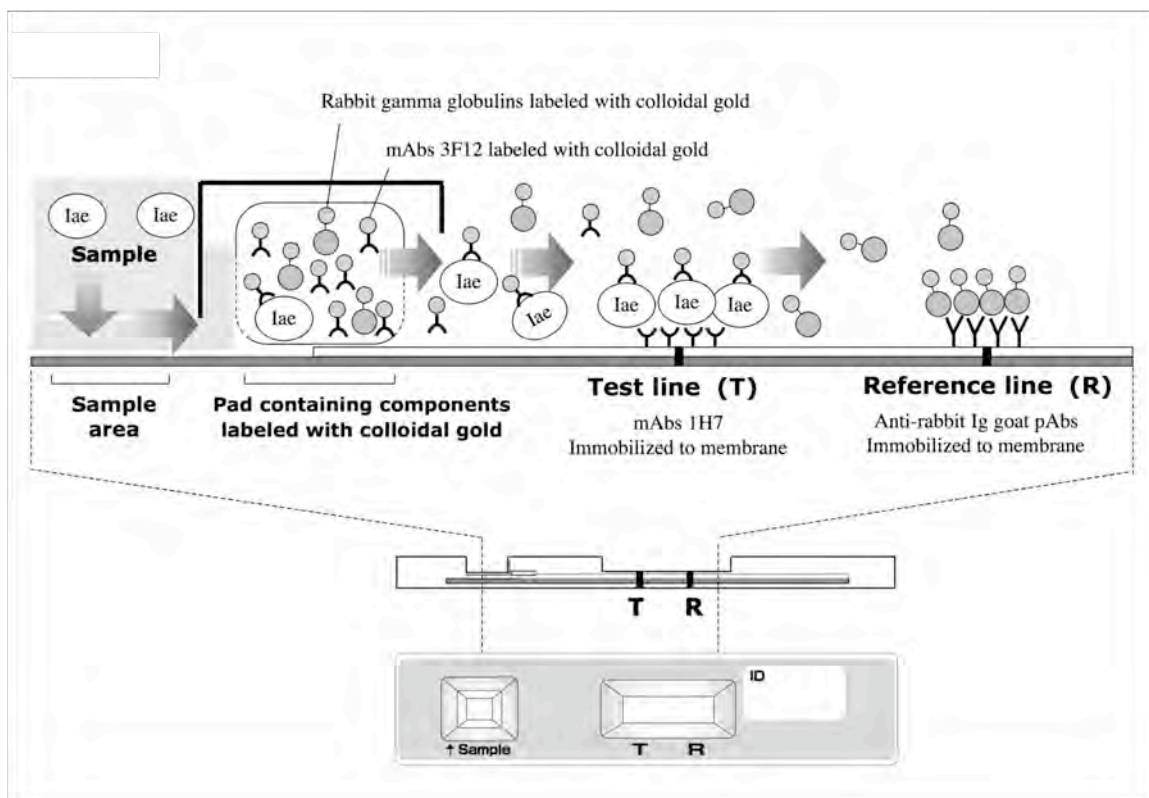

Figure 4-7. Composition and principle of the immunochromatographic assay targeting AAC(6')-Iae. The arrow indicates the direction of lateral flow. For the sample containing AAC(6')-Iae, target protein AAC(6')-Iae was first captured with mAbs 3F12 and then captured with mAbs 1H7.

4-2-5. Analytical sensitivity testing of the assay.

In the tests using purified recombinant proteins, 100 μL of serial dilutions, containing 100, 30, 10, 3, and 1 ng of AAC(6')-Iae proteins, were prepared with extraction buffer (20 mM Tris-HCl, pH 7.5, 1.0% Tton X-100), and aliquots were applied onto the sample area on the assay. In the tests using bacteria, *P. aeruginosa* IMCJ2.S1 strain was cultured in Mueller-Hinton (MH) broth at 37°C for 16 h, and 100-μl aliquots of twofold dilutions of bacterial cultures in MH broth were mixed with 900 μL extraction buffer to lyse the bacteria. Aliquots of 100 μL were applied onto the sample area on the assay. At the same time, 100 μL aliquots of bacterial dilutions were spread onto MH agar plates to determine the cfu. In both tests, the intensity of the test line was confirmed and quantified 10 min after applying the sample to the sample area. The intensity of the line was quantified using Quantity One software (Bio-Rad). Independent experiments were performed in triplicate for each sample, and the mean intensity of triplicate measurements at each point was plotted. The detection limit (y-axis) was defined as the intensity greater than the sum of the average values and $3 \times$ the standard deviation of the end point values in the linear standard curve. The cfu and protein amount (x-axis) required for theoretical detection limits were calculated from equations derived for each graph.

4-2-6. Assessment of the assay

To assess the accuracy of our assay, 116 *P. aeruginosa* clinical isolates were obtained from 13 hospitals located in the Kanto areas of Japan, where little has been known about AAC(6')-Iae producers. All of these isolates were associated with nosocomial infections from 2004 to 2009; they contain 14 strains from a Tokyo hospital (hospital A in Fig. 4-6) in our previous work (Sekiguchi *et al*., 2007d). Bacterial colonies on MH agar were directly picked up with a swab (Step 1) and suspended in soft test tubes containing extraction buffer (Step 2). After lysing the cells physically and chemically, four drops of lysate were dropped onto the assay (Step 3), and results were determined by visual inspection 10 min later (Fig. 4-8).

4-2-7. Antimicrobial susceptibility testing.

Antibiotic susceptibility testing was performed by the broth micro-dilution method recommended by the Clinical and Laboratory Standards Institute (CLSI, 2006). In this study, MDR *P. aeruginosa* was defined as showing resistance to imipenem (MIC≥16 mg/L), amikacin (MIC≥32 mg/L), and ofloxacin (MIC≥4 mg/L) based on the criteria of the Ministry of Health, Labor, and Welfare of Japan (Kirikae *et al*., 2008).

4-2-8. PCR amplification and DNA sequencing.

The *aac(6')-Iae* and class 1 integrons were amplified with specific primer sets (Table 4-2). All amplicons were sequenced to identify their contents, using the primers listed in Table 4-2.
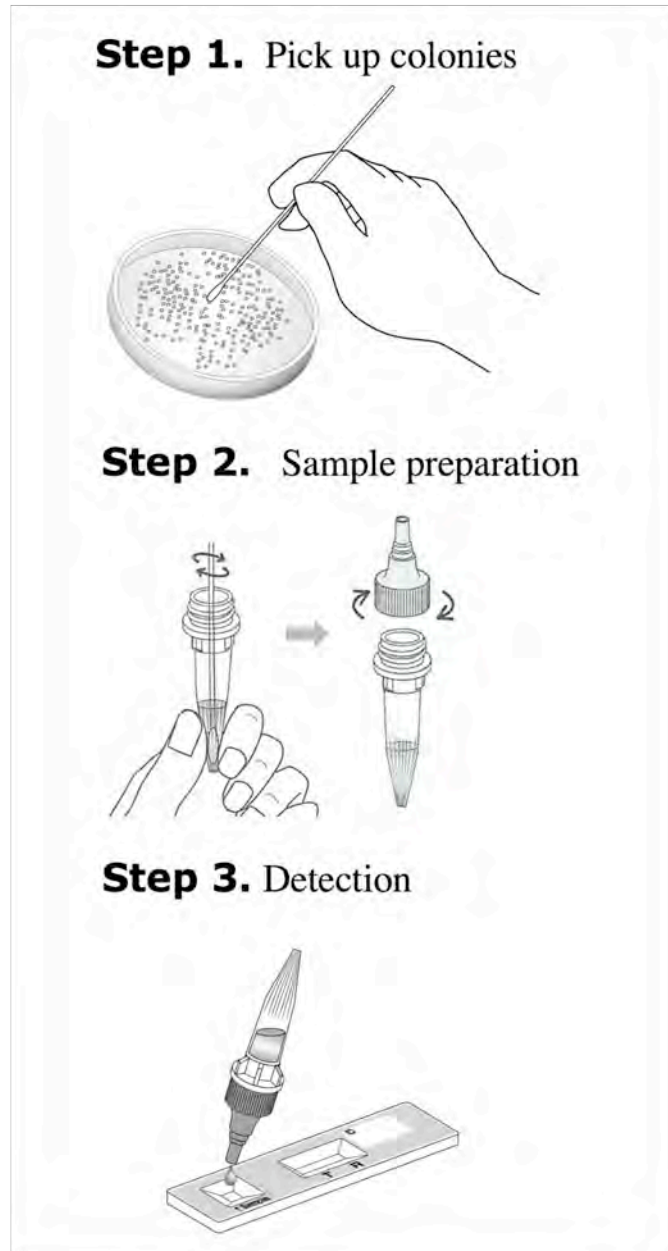
Figure 4-8. Procedure for immunochromatographic detection used in preliminary evaluation. Bacterial colonies on MH agar plates were picked with a swab (Step 1) and suspended in 500-µl extraction buffer (Step 2), and each bacterial lysate was placed onto the sample area for immunochromatography via a filter membrane in the cap (Step 3). Ten minutes after dropping the sample, the production or lack of production of AAC(6')-Iae was determined by the presence or absence of the test line, respectively.

Chapter 5. Conclusion and future prospects

This study aimed to clarify the mechanism of antibiotic resistance of MDR. *P. aeruginosa* strains isolated in Japan. Especially, an import system of bacterial gene that is called the class 1 integrons was focused to identify antibiotic resistance genes.

In the screening of antibiotic resistance gene in the variable region of class 1 integron, a novel gene encoding an aminoglycoside 6'-*N*-acetyltransferase isozyme, *aac(6')-Iaf*, was identified in two MDR *P. aeruginosa* clinical isolates, IMCJ798 and IMCJ799 (Chapter 2), while most of MDR *P. aeruginosa* tested carried the *aac(6')-Iae* gene. Both IMCJ798 and IMCJ799 were found to contain a novel chromosomal class 1 integron, In123, which included *aac(6')-Iaf* as the first cassette gene. The encoded protein AAC(6')-Iaf was found to consist of 183 amino acids, with 91% and 87% identity to AAC(6')-Iq and AAC(6')-Im, respectively. The modeling of predicted AAC(6')-Iaf 3D structure suggested that this protein could serve as an isozyme of aminoglycoside 6'-*N*-aminoglycoside acetyltransferases, as well as AAC(6')-Ii.

An important observation in the recent study for antibiotic resistant bacteria was the high prevalence of class 1 integrons with multiple resistance genes. This problem was widely assumed for Gram-negative bacteria including *P. aeruginosa,* and will be complicated by the combination of resistance factors.

However, it may be possible to predict the molecular determinants for antibiotic

resistance in the antibiotic resistant bacteria, because antibiotics are substances which have microbicidal action, and they are produced by microorganism. There are now nearly 1,000 completed bacterial and archaeal genomes available. Aminoglycosides were antibiotics produced by *Actinomycetes* and *Streptomyces*, and traditionally there is been a blossoming of searching for novel antibiotics using *Actinomycetes* and *Streptomyces*. Indeed, AAC(6')-Isa is a isozyme identified from *Streptomyces*. Thus, it may be possible to analyze the antibiotic resistance gene using genomic informations of such microorganisms (Woo PC, *et*. *al*., 2006). It will be also required to focus on their auto resistance mechanisms against self-produced antimictobial agents.

In functional dissection of AAC(6')-Iaf (Chapter 3), AAC(6')-Iaf was found to distribute highly resistant to amikacin, dibekacin and kanamycin, but not to gentamicin. The production of AAC(6')-Iaf by these strains was confirmed by western blot analysis. Thin layer chromatography showed that AAC(6')-Iaf is a functional acetyltransferase that specifically modifies the amino group at the 6' position of aminoglycosides. These findings indicated that AAC(6')-Iaf acts as an aminoglycoside resistance factor in MDR *P. aeruginosa*. Using gel filtration chromatography, the AAC(6')-Iaf was found to exsist as a dimer in solution. In addition, mutational analyses indicated that the Tyr154 residue of AAC(6')-Iaf may be the catalytic residue in the acetylation reaction catalyzed by this enzyme. The mutational analysis in predicted substrate pocket of AAC(6')-Iaf suggested that Trp34, Glu78, Asp118, and Asp175 in AAC(6')-Iaf were amino acid residues essential for aminoglycoside inactivation.

The proteins have features of both robustness and plasticity. AAC(6') enzymes are a member of GNAT superfamily with similar structural folding, and they are responsible for acetylation while they share low similarity of amino acid sequences. Structural basis for the interaction between AAC(6')s and substrates may shed light on the evolution of different enzyme classes. In addition, such studies may assist in the design of novel therapeutic agents that could circumvent antibiotic resistance. Thus, structural analyses of AAC(6')-Iaf-substrates complex are in progress to clarify the interaction between this enzyme and substrates.

Finally, by focusing on the mechanism of antibiotic resistance due to the production of antibiotic-inactivating enzymes, an easy-to-use and rapid detection method of antibiotic resistant bacteria was developed (Chapter 4). Given that AAC(6')-Iae may be a significant molecular marker in the aminoglycoside resistance of the most prevalent MDR *P. aeruginosa* isolates in Japan, an immunochromatographic assay specific for aminoglycoside 6'-*N*-acetyltransferase AAC(6')-Iae was designed. The assay system utilized monoclonal antibodies specific for AAC(6')-Iae. The analytical sensitivity tests indicated that the detection limit of this assay was 1.0×10^5 cfu. Preparation of sample with extraction buffer containing detergent enabled a determination of AAC(6')-Iae production by bacterial colonies within 15 min. Moreover, assays using clinical isolates of *P. aeruginosa* showed that our assay yielded no false positive or false negatives. Thus, the assay developed here is an easy-to-use, and reliable method for detecting AAC(6')-Iae-producing MDR *P. aeruginosa*.

Several immunochromatographic assays have been developed to identify various infectious agents, such as influenza virus (Miyoshi-Akiyama *et al*., 2010). Most of these target secretary proteins and cellular components. Immunological methods can utilize antibodies against antigens of interest, such as metallo-β-lactamase NDM-1 and KPC-1, which are emerging in Gram-negative bacteria. Therefore, this approach could serve as a model for other molecules involved in antibiotic resistance. Further work is in progress to design immunochromatography assays targeting other candidate markers of MDR strains in Japan, such as AAC(6')-Ib and metallo-β-lactamase IMP.

Nevertheless, not all MDR *P. aeruginoa* can be detected using the immunochromatography developed in this study. It was impossible to detect MDR *P. aeruginosa* lacking AAC(6')-Iae, the preliminary assessment of the assay indicated that 56 (48%) of 116 MDR strains are negative for AAC(6')-Iae. As described above, among all aminoglycoside-modifying enzymes, AAC(6')s have the one feature connected with resistance to amikacin, which provides an indication of MDR *P. aeruginosa* in Japan. If AAC(6') enzyme could account for a large share of determinants for amikacin resistance, the development of the rapid detection system based on the reaction mechanism of aminoglycoside acetylation may be required.

Abstract (In Japanese)

　　緑膿菌は臨床検体から比較的高頻度に分離されるグラム陰性桿菌である。医療現場では分離率のみならず耐性菌の割合も高く、複数の薬剤に耐性を示す多剤耐性緑膿菌による院内感染は近年深刻化している。本研究では、多剤耐性緑膿菌臨床分離株の中で薬剤耐性機序が不明な株から、アミノグリコシドアセチル基転移酵素AAC(6')のアイソザイムを新たに同定し、その機能解析を行った。また、多剤耐性緑膿菌が感受性菌にはない薬剤耐性に寄与する酵素群を産生していることに着目し、同酵素群を標的とする分子ベースの耐性菌検出法の開発に取り組んだ。

　　まず、薬剤耐性因子の遺伝子スクリーニングを行った結果、インテグロンと呼ばれる外来性遺伝子獲得エレメントの内部にTTGを開始コドンとする552塩基長のORFを同定した。配列解析から、183アミノ酸から成るAAC(6')の新規アイソザイムをコードすると予測されたことから、このORFを *aac(6')-Iaf* と命名した。次に、制限酵素I–*CeuI* を用いたゲノムタイピングと抗AAC(6')–Iaf 抗体を用いたウェスタン解析を行った結果、*aac(6')-Iaf* は緑膿菌染色体上に存在し、抗生剤の有無に関わらず構成的に発現していることが分かった。*aac(6')-Iaf* を導入した大腸菌は、広範囲のアミノグリコシド剤耐性を示し、耐性パターンは親株と相関があった。さらに、組換えAAC(6')–Iaf 蛋白質を用いた *in vitro* の生化学解析から、AAC(6')–Iaf は2量体を形成しアミノグリコシド剤6'位のアミノ基を特異的にアセチル化することが明らかになった。ITCを用いた相互作用解析は、AAC(6')–Iaf がアミノグリコシドと高い親和性を持って結合することを示した。さらに、AAC(6')–Iaf の予測活性中心における変異導入解析から、同酵素のTrp34、Glu78、Asp118およびAsp175はアミノグリコシドの不活化に寄与していることが示唆された。以上の結果から、AAC(6')–Iaf は多剤耐性緑膿菌においてアミノグリコシドアセチル基転移酵素として発現、機能し、アミノグリコシド剤耐性に寄与していることが明らかとなった。

　　耐性菌検出システムの開発では、薬剤耐性因子のスクリーニングで最も高頻度に検出されたAAC(6')–Iaeに対するモノクローナル抗体を調製し、イムノクロマト法を構築した。その結果、緑膿菌のコロニーあるいは培養液から15分以内にAAC(6')–Iae産生の有無を検出することに成功した。開発したシステムの検出限界は、1試験あたり1.0×10^5cfuであり、非常に高感度であった。陽性株は全て高度なアミノグリコシド剤耐性を示した。臨床分離株を用いた感度試験では偽陽性および偽陰性を示さないことから、本検出法はPCR法に代わる有効な薬剤耐性菌の

検出手段であることが示唆された。近年、緑膿菌だけでなく他の日和見感染菌も多様な外来性薬剤耐性因子を産生し耐性化している。イムノクロマト法は、目的標的蛋白質に対する抗体を複数適用できることから、今後、多剤耐性菌の検出に広く応用されることが期待される。

List of publications

[Publications and patent relating this thesis]

1. Kitao T., Miyoshi-Akiyama T., and Kirikae T.
   AAC(6')-Iaf, a novel aminoglycoside 6'-*N*-acetyltransferase from multidrug-resistant *Pseudomonas aeruginosa* clinical isolates.
   Antimicrob Agents Chemother. 53(6):2327-34. 2009

2. 特許出願 「緑膿菌の新規薬剤耐性遺伝子」
   発明者：切替 照雄、秋山 徹、安藤 公英.
   特願 2008-268799, 出願日：2008 年 10 月 17 日

3. Kitao T., Miyoshi-Akiyama T., Shimada K., Tanaka M., Narahara K., Saito N., and Kirikae T.
   Development of immunochromatographic assay for the rapid detection of AAC(6')-Iae-producing multidrug-resistant *Pseudomonas aeruginosa*.
   J. Antimicrob. Chemother. 65(7):1382-6. 2010

[Other publications]

1. Sekiguchi J, Morita K, Kitao T, Watanabe N, Okazaki M, Miyoshi-Akiyama T, Kanamori M, Kirikae T.
   KHM-1, a novel plasmid-mediated metallo-β-lactamase from a *Citrobacter freundii* clinical isolate.
   Antimicrob Agents Chemother. 52(11):4194-7. 2008

2. Takahashi S, Leiss M, Moser M, Ohashi T, Kitao T, Heckmann D, Pfeifer A, Kessler H, Takagi J, Erickson HP, Fassler R.
   The RGD motif in fibronectin is essential for development but dispensable for fibril assembly.
   J Cell Biol. 178(1):167-78. 2007

3. Yasui N, Nogi T, <u>Kitao T</u>, Nakano Y, Hattori M, Takagi J.
   Structure of a receptor-binding fragment of reelin and mutational analysis reveal a recognition mechanism similar to endocytic receptors.
   Proc Natl Acad Sci USA. 104(24):9988-93. 2007

4. Suzuki K, Okuno T, Yamamoto M, Pasterkamp RJ, Takegahara N, Takamatsu H, <u>Kitao T</u>, Takagi J, Rennert PD, Kolodkin AL, Kumanogoh A, Kikutani H.
   Semaphorin 7A initiates T-cell-mediated inflammatory responses through alpha1beta1 integrin.
   Nature. 446(7136):680-4. 2007

5. Narita J, Okano K, <u>Kitao T</u>, Ishida S, Sewaki T, Sung MH, Fukuda H, Kondo A.
   Display of alpha-amylase on the surface of *Lactobacillus casei* cells by use of the PgsA anchor protein, and production of lactic acid from starch.
   Appl Environ Microbiol. 72(1):269-75. 2006

6. <u>Kitao T</u>, Kuroishi C, Tahirov TH.
   Crystallization and preliminary crystallographic analysis of the nickel-responsive regulator NikR from *Pyrococcus horikoshii*.
   Acta Crystallogr Sect F Struct Biol Cryst Commun. 61(Pt 1):43-5. 2005

7. Rehse PH, <u>Kitao T</u>, Tahirov TH.
   Structure of a closed-form uroporphyrinogen-III C-methyltransferase from *Thermus thermophilus*.
   Acta Crystallogr D Biol Crystallogr. 61(Pt 7):913-9. 2005

8. Tahirov TH, Inagaki E, Ohshima N, <u>Kitao T</u>, Kuroishi C, Ukita Y, Takio K, Kobayashi M, Kuramitsu S, Yokoyama S, Miyano M.
   Crystal structure of purine nucleoside phosphorylase from *Thermus thermophilus*.
   J Mol Biol. 337(5):1149-60. 2004

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References

Ahmed, A. M., T. Nakagawa, E. Arakawa, T. Ramamurthy, S. Shinoda & T. Shimamoto, (2004) New aminoglycoside acetyltransferase gene, *aac(3)-Id*, in a class 1 integron from a multiresistant strain of *Vibrio fluvialis* isolated from an infant aged 6 months. *J. Antimicrob. Chemother*. **53**: 947-951.

Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller & D. J. Lipman, (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic. Acids. Res*. **25**: 3389-3402.

Altschul, S. F., J. C. Wootton, E. M. Gertz, R. Agarwala, A. Morgulis, A. A. Schaffer & Y. K. Yu, (2005) Protein database searches using compositionally adjusted substitution matrices. *FEBS J*. **272**: 5101-5109.

Baraniak, A., R. Izdebski, M. Herda, J. Fiett, W. Hryniewicz, M. Gniadkowski, I. Kern-Zdanowicz, K. Filczak & U. Lopaciuk, (2009) Emergence of Klebsiella pneumoniae ST258 with KPC-2 in Poland. *Antimicrob Agents Chemother* **53**: 4565-4567.

Bissonnette, L. & P. H. Roy, (1992) Characterization of In0 of *Pseudomonas aeruginosa* plasmid pVS1, an ancestor of integrons of multiresistance plasmids and transposons of Gram-negative bacteria. *J. Bacteriol*. **174**: 1248-1257.

Burk, D. L., N. Ghuman, L. E. Wybenga-Groot & A. M. Berghuis, (2003) X-ray structure of the AAC(6')-Ii antibiotic resistance enzyme at 1.8 A resolution; examination of oligomeric arrangements in GNAT superfamily members. *Protein Sci*. **12**: 426-437.

Burk, D. L., B. Xiong, C. Breitbach & A. M. Berghuis, (2005) Structures of aminoglycoside acetyltransferase AAC(6')-Ii in a novel crystal form: structural and normal-mode analyses. *Acta. Crystallogr. D Biol. Crystallogr*. **61**: 1273-1279.

Carter, A. P., W. M. Clemons, D. E. Brodersen, R. J. Morgan-Warren, B. T. Wimberly & V. Ramakrishnan, (2000) Functional insights from the structure of the 30S ribosomal subunit and its interactions with antibiotics. *Nature* **407**: 340-348.

Casse, F. B., C.; Julliot, J. S.; Michel, M.; Denarie, J., (1979) Identification and characterization of large plasmids in *Rhizobium meliloti* using agarose gel electrophoresis. *J. Gen. Microbiol*. **113**: 229-242.

Centron, D. & P. H. Roy, (1998) Characterization of the 6'-*N*-aminoglycoside acetyltransferase gene *aac(6')-Iq* from the integron of a natural multiresistance plasmid. *Antimicrob. Agents Chemother*. **42**: 1506-1508.

CLSI, (2006) Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 7th ed. Approved standard *M07-A7*. Clinical and Laboratory Standards Institute., Wayne, Pa.

Collis, C. M. & R. M. Hall, (1992) Site-specific deletion and rearrangement of integron insert genes catalyzed by the integron DNA integrase. *J. Bacteriol*. **174**: 1574-1585.

Costa, Y., M. Galimand, R. Leclercq, J. Duval & P. Courvalin, (1993) Characterization of the chromosomal *aac(6')-Ii* gene specific for *Enterococcus faecium*. *Antimicrob. Agents Chemother*. **37**: 1896-1903.

Daigle, D. M., D. W. Hughes & G. D. Wright, (1999) Prodigious substrate specificity of AAC(6')-APH(2"), an aminoglycoside antibiotic resistance determinant in enterococci and staphylococci. Chem Biol 6: 99-110.

Doi, Y., J. Wachino, K. Yamane, N. Shibata, T. Yagi, K. Shibayama, H. Kato & Y. Arakawa, (2004) Spread of novel aminoglycoside resistance gene *aac(6')-Iad* among Acinetobacter clinical isolates in Japan. *Antimicrob Agents Chemother* **48**: 2075-2080.

Draker, K. A. & G. D. Wright, (2004) Molecular mechanism of the enterococcal aminoglycoside 6'-*N*-acetyltransferase': role of GNAT-conserved residues in the chemistry of antibiotic inactivation. *Biochemistry* **43**: 446-454.

Dyda, F., D. C. Klein & A. B. Hickman, (2000) GCN5-related *N*-acetyltransferases: a structural overview. *Annu Rev Biophys Biomol Struct* **29**: 81-103.

Erol, S., U. Altoparlak, M. N. Akcay, F. Celebi & M. Parlak, (2004) Changes of microbial flora and wound colonization in burned patients. *Burns* **30**: 357-361.

Fourmy, D., M. I. Recht, S. C. Blanchard & J. D. Puglisi, (1996) Structure of the A site of Escherichia coli 16S ribosomal RNA complexed with an aminoglycoside antibiotic. *Science* **274**: 1367-1371.

Gouet, P., X. Robert & E. Courcelle, (2003) ESPript/ENDscript: Extracting and rendering sequence and 3D information from atomic structures of proteins. *Nucleic Acids Res* **31**: 3320-3323.

Grundmann, H., C. Schneider, D. Hartung, F. D. Daschner & T. L. Pitt, (1995) Discriminatory power of three DNA-based typing techniques for *Pseudomonas aeruginosa*. *J. Clin. Microbiol.* **33**: 528-534.

Hall, R. M., H. J. Brown, D. E. Brookes & H. W. Stokes, (1994) Integrons found in different locations have identical 5' ends but variable 3' ends. *J. Bacteriol.* **176**: 6286-6294.

Hall, R. M. & C. M. Collis, (1995) Mobile gene cassettes and integrons: capture and spread of genes by site-specific recombination. *Mol. Microbiol.* **15**: 593-600.

Hamano, Y., Y. Hoshino, S. Nakamori & H. Takagi, (2004) Overexpression and characterization of an aminoglycoside 6'-N-acetyltransferase with broad specificity from an epsilon-poly-L-lysine producer, Streptomyces albulus IFO14147. J. Biochem. 136: 517-524.

Hannecart-Pokorni, E., F. Depuydt, L. de wit, E. van Bossuyt, J. Content & R. Vanhoof, (1997) Characterization of the 6'-*N*-aminoglycoside acetyltransferase gene *aac(6')-Im* [corrected] associated with a *sul*I-type integron. *Antimicrob. Agents Chemother.* **41**: 314-318.

Harris, A. A., L. Goodman & S. Levin, (1984) Community-acquired *Pseudomonas aeruginosa* pneumonia associated with the use of a home humidifier. *West J Med* **141**: 521-523.

Head, N. E. & H. Yu, (2004) Cross-sectional analysis of clinical and environmental isolates of *Pseudomonas aeruginosa*: biofilm formation, virulence, and genome diversity. *Infect Immun* **72**: 133-144.

Jenney, A., L. Liolios, D. Spelman, P. Russo, J. Wilson, S. Wesselingh & T. Kotsimbos, (2003) Use of pulsed-field gel electrophoresis in infection control issues concerning Burkholderia cepacia. *Infect Control Hosp Epidemiol* **24**: 624-626.

Kado, C. I. & S. T. Liu, (1981) Rapid procedure for detection and isolation of large and small plasmids. *J. Bacteriol.* **145**: 1365-1373.

Kato, T., Y. Sato, S. Iyobe & S. Mitsuhashi, (1982) Plasmid-mediated gentamicin resistance of *Pseudomonas aeruginosa* and its lack of expression in *Escherichia coli*. *Antimicrob. Agents Chemother*. **22**: 358-363.

Kiefer, F., K. Arnold, M. Kunzli, L. Bordoli & T. Schwede, (2009) The SWISS-MODEL Repository and associated resources. *Nucleic Acids Res* **37**: D387-392.

Kirikae, T., Y. Mizuguchi & Y. Arakawa, (2008) Investigation of isolation rates of *Pseudomonas aeruginosa* with and without multidrug resistance in medical facilities and clinical laboratories in Japan. *J. Antimicrob. Chemother*. **61**: 612-615.

Kitchel, B., J. K. Rasheed, J. B. Patel, A. Srinivasan, S. Navon-Venezia, Y. Carmeli, A. Brolund & C. G. Giske, (2009) Molecular epidemiology of KPC-producing *Klebsiella pneumoniae* isolates in the United States: clonal expansion of multilocus sequence type 258. *Antimicrob Agents Chemother* **53**: 3365-3370.

Kouda, S., M. Ohara, M. Onodera, Y. Fujiue, M. Sasaki, T. Kohara, S. Kashiyama, S. Hayashida, T. Harino, T. Tsuji, H. Itaha, N. Gotoh, A. Matsubara, T. Usui & M. Sugai, (2009) Increased prevalence and clonal dissemination of multidrug-resistant *Pseudomonas aeruginosa* with the *bla*IMP-1 gene cassette in Hiroshima. *J Antimicrob Chemother* **64**: 46-51.

Kumarasamy, K. K., M. A. Toleman, T. R. Walsh, J. Bagaria, F. Butt, R. Balakrishnan, U. Chaudhary, M. Doumith, C. G. Giske, S. Irfan, P. Krishnan, A. V. Kumar, S. Maharjan, S. Mushtaq, T. Noorie, D. L. Paterson, A. Pearson, C. Perry, R. Pike, B. Rao, U. Ray, J. B. Sarma, M. Sharma, E. Sheridan, M. A. Thirunarayan, J. Turton, S. Upadhyay, M. Warner, W. Welfare, D. M. Livermore & N. Woodford, (2010)

Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. *Lancet Infect Dis* **10**: 597-602.

Larkin, M. A., G. Blackshields, N. P. Brown, R. Chenna, P. A. McGettigan, H. McWilliam, F. Valentin, I. M. Wallace, A. Wilm, R. Lopez, J. D. Thompson, T. J. Gibson & D. G. Higgins, (2007) Clustal W and Clustal X version 2.0. *Bioinformatics* **23**: 2947-2948.

Levesque, C., S. Brassard, J. Lapointe & P. H. Roy, (1994) Diversity and relative strength of tandem promoters for the antibiotic-resistance genes of several integrons. *Gene* **142**: 49-54.

Levesque, C., L. Piche, C. Larose & P. H. Roy, (1995) PCR mapping of integrons reveals several novel combinations of resistance genes. *Antimicrob. Agents Chemother.* **39**: 185-191.

Li, X. Z., D. M. Livermore & H. Nikaido, (1994) Role of efflux pump(s) in intrinsic resistance of Pseudomonas aeruginosa: resistance to tetracycline, chloramphenicol, and norfloxacin. *Antimicrob Agents Chemother* **38**: 1732-1741.

Lister, P. D., D. J. Wolter & N. D. Hanson, (2009) Antibacterial-resistant Pseudomonas aeruginosa: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. *Clin Microbiol Rev* **22**: 582-610.

Liu, S. L., A. Hessel & K. E. Sanderson, (1993) Genomic mapping with I-*Ceu* I, an intron-encoded endonuclease specific for genes for ribosomal RNA, in *Salmonella spp.*, *Escherichia coli*, and other bacteria. *Proc. Natl. Acad. Sci. USA* **90**: 6874-6878.

McKay, G. A., P. R. Thompson & G. D. Wright, (1994) Broad spectrum aminoglycoside phosphotransferase type III from Enterococcus: overexpression, purification, and substrate specificity. Biochemistry 33: 6936-6944.

Magalhaes, M. L., M. W. Vetting, F. Gao, L. Freiburger, K. Auclair & J. S. Blanchard, (2008) Kinetic and structural analysis of bisubstrate inhibition of the Salmonella enterica aminoglycoside 6'-N-acetyltransferase. Biochemistry 47: 579-584.

Magnet, S. & J. S. Blanchard, (2005) Molecular insights into aminoglycoside action and resistance. *Chem Rev* **105**: 477-498.

Mauch, L., V. Bichler & R. Brandsch, (1990) Functional analysis of the 5' regulatory region and the UUG translation initiation codon of the *Arthrobacter oxidans* 6-hydroxy-D-nicotine oxidase gene. *Mol. Gen. Genet.* **221**: 427-434.

Mazel, D., (2006) Integrons: agents of bacterial evolution. *Nat Rev Microbiol* **4**: 608-620.

Mingeot-Leclercq, M. P., Y. Glupczynski & P. M. Tulkens, (1999) Aminoglycosides: activity and resistance. *Antimicrob Agents Chemother* **43**: 727-737.

Mingeot-Leclercq, M. P. & P. M. Tulkens, (1999) Aminoglycosides: nephrotoxicity. *Antimicrob Agents Chemother* **43**: 1003-1012.

Miyoshi-Akiyama, T., K. Narahara, S. Mori, H. Kitajima, T. Kase, S. Morikawa & T. Kirikae, (2010) Development of an immunochromatographic assay specifically detecting pandemic H1N1 (2009) influenza virus. *J Clin Microbiol* **48**: 703-708.

Morrison, A. J., Jr. & R. P. Wenzel, (1984) Epidemiology of infections due to Pseudomonas aeruginosa. *Rev Infect Dis* **6 Suppl 3**: S627-642.

Neuwald, A. F. & D. Landsman, (1997) GCN5-related histone *N*-acetyltransferases belong to a diverse superfamily that includes the yeast SPT10 protein. *Trends Biochem. Sci.* **22**: 154-155.

Ogawa, A. & T. Takeda, (1993) The gene encoding the heat-stable enterotoxin of Vibrio cholerae is flanked by 123-base pair direct repeats. *Microbiol Immunol* **37**: 607-616.

Ohara, T. & K. Itoh, (2003) Significance of Pseudomonas aeruginosa colonization of the gastrointestinal tract. *Intern Med* **42**: 1072-1076.

Poirel, L., O. Menuteau, N. Agoli, C. Cattoen & P. Nordmann, (2003) Outbreak of extended-spectrum β-lactamase VEB-1-producing isolates of *Acinetobacter baumannii* in a French hospital. *J Clin Microbiol* **41**: 3542-3547.

Pournaras, S., E. Protonotariou, E. Voulgari, I. Kristo, E. Dimitroulia, D. Vitti, M. Tsalidou, A. N. Maniatis, A. Tsakris & D. Sofianou, (2009) Clonal spread of KPC-2 carbapenemase-producing Klebsiella pneumoniae strains in Greece. *J Antimicrob Chemother* **64**: 348-352.

Riccio, M. L., J. D. Docquier, E. Dell'Amico, F. Luzzaro, G. Amicosante & G. M. Rossolini, (2003) Novel 3-*N*-aminoglycoside acetyltransferase gene, *aac(3)-Ic*, from a *Pseudomonas aeruginosa* integron. *Antimicrob. Agents Chemother.* **47**: 1746-1748.

Rowe-Magnus, A. D., J. Davies & D. Mazel, (2002) Impact of integrons and transposons on the evolution of resistance and virulence. *Curr Top Microbiol Immunol* **264**: 167-188.

Rowe-Magnus, D. A. & D. Mazel, (2002) The role of integrons in antibiotic resistance gene capture. *Int J Med Microbiol* **292**: 115-125.

Sambrook, J., and D. W. Russell. , (2001) Molecular cloning: a laboratory manual, 3rd ed. . *Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.*

Schwocho, L. R., C. P. Schaffner, G. H. Miller, R. S. Hare & K. J. Shaw, (1995) Cloning and characterization of a 3-*N*-aminoglycoside acetyltransferase gene, *aac(3)-Ib*, from *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **39**: 1790-1796.

Sekiguchi, J., T. Asagi, T. Miyoshi-Akiyama, T. Fujino, I. Kobayashi, K. Morita, Y. Kikuchi, T. Kuratsuji & T. Kirikae, (2005) Multidrug-resistant *Pseudomonas aeruginosa* strain that caused an outbreak in a neurosurgery ward and its *aac(6')-Iae* gene cassette encoding a novel aminoglycoside acetyltransferase. *Antimicrob. Agents Chemother.* **49**: 3734-3742.

Sekiguchi, J., T. Asagi, T. Miyoshi-Akiyama, A. Kasai, Y. Mizuguchi, M. Araake, T. Fujino, H. Kikuchi, S. Sasaki, H. Watari, T. Kojima, H. Miki, K. Kanemitsu, H. Kunishima, Y. Kikuchi, M. Kaku, H. Yoshikura, T. Kuratsuji & T. Kirikae, (2007a) Outbreaks of multidrug-resistant *Pseudomonas aeruginosa* in community hospitals in Japan. *J. Clin. Microbiol.* **45**: 979-989.

Sekiguchi, J., K. Teruya, K. Horii, E. Kuroda, H. Konosaki, Y. Mizuguchi, M. Araake, A. Kawana, H. Yoshikura, T. Kuratsuji, H. Miyazaki & T. Kirikae, (2007b) Molecular epidemiology of outbreaks and containment of drug-resistant *Pseudomonas aeruginosa* in a Tokyo hospital. *J. Infect. Chemother.* **13**: 418-422.

Shaw, K. J., P. N. Rather, R. S. Hare & G. H. Miller, (1993) Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. *Microbiol. Rev.* **57**: 138-163.

Siregar, J. J., K. Miroshnikov & S. Mobashery, (1995) Purification, characterization, and investigation of the mechanism of aminoglycoside 3'-phosphotransferase type Ia. Biochemistry 34: 12681-12688.

Stokes, H. W. & R. M. Hall, (1989) A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons. *Mol Microbiol* **3**: 1669-1683.

Stokes, H. W., D. B. O'Gorman, G. D. Recchia, M. Parsekhian & R. M. Hall, (1997) Structure and function of 59-base element recombination sites associated with mobile gene cassettes. *Mol. Microbiol.* **26**: 731-745.

Tenover, F. C., D. Filpula, K. L. Phillips & J. J. Plorde, (1988) Cloning and sequencing of a gene encoding an aminoglycoside 6'-*N*-acetyltransferase from an R factor of *Citrobacter diversus. J. Bacteriol.* **170**: 471-473.

Thuong, M., K. Arvaniti, R. Ruimy, P. de la Salmoniere, A. Scanvic-Hameg, J. C. Lucet & B. Regnier, (2003) Epidemiology of *Pseudomonas aeruginosa* and risk factors for carriage acquisition in an intensive care unit. *J Hosp Infect* **53**: 274-282.

Vaisvila, R., R. D. Morgan, J. Posfai & E. A. Raleigh, (2001) Discovery and distribution of super-integrons among pseudomonads. *Mol Microbiol* **42**: 587-601.

Vakulenko, S. B. & S. Mobashery, (2003) Versatility of aminoglycosides and prospects for their future. *Clin. Microbiol. Rev.* **16**: 430-450.

Vanhoof, R., E. Hannecart-Pokorni & J. Content, (1998) Nomenclature of genes encoding aminoglycoside-modifying enzymes. *Antimicrob. Agents Chemother.* **42**: 483.

Vetting, M. W., S. d. C. LP, M. Yu, S. S. Hegde, S. Magnet, S. L. Roderick & J. S. Blanchard, (2005) Structure and functions of the GNAT superfamily of acetyltransferases. *Arch Biochem Biophys* **433**: 212-226.

Watanabe, M., S. Iyobe, M. Inoue & S. Mitsuhashi, (1991) Transferable imipenem resistance in *Pseudomonas aeruginosa. Antimicrob. Agents Chemother.* **35**: 147-151.

Woo PC, Lau SK, Huang Y, & Yuen KY. (2006) Genomic evidence for antibiotic resistance genes of actinomycetes as origins of antibiotic resistance genes in pathogenic bacteria simply because actinomycetes are more ancestral than pathogenic bacteria.

Med Hypotheses. **67**:1297-304.

Wright, G. D., (1999) Aminoglycoside-modifying enzymes. *Curr. Opin. Microbiol.* **2**: 499-503.

Wright, G. D. & P. Ladak, (1997) Overexpression and characterization of the chromosomal aminoglycoside 6'-*N*-acetyltransferase from *Enterococcus faecium*. *Antimicrob. Agents Chemother*. **41**: 956-960.

Yong, D., M. A. Toleman, C. G. Giske, H. S. Cho, K. Sundman, K. Lee & T. R. Walsh, (2009) Characterization of a new metallo-β-lactamase gene, bla(NDM-1), and a novel erythromycin esterase gene carried on a unique genetic structure in Klebsiella pneumoniae sequence type 14 from India. *Antimicrob Agents Chemother* **53**: 5046-5054.

Yoshida, S., (2007) Toda's New Bacteriology*, 33th ed*. Nanzando, Tokyo.

Zhu, C. B., A. Sunada, J. Ishikawa, Y. Ikeda, S. Kondo & K. Hotta, (1999) Role of aminoglycoside 6'-acetyltransferase in a novel multiple aminoglycoside resistance of an actinomycete strain #8: inactivation of aminoglycosides with 6'-amino group except arbekacin and neomycin. *J. Antibiot. (Tokyo)* **52**: 889-894.