ANTIMICROBIAL RESISTANCE

Characterisation of drug resistance gene cassettes associated with class 1 integrons in clinical isolates of *Escherichia coli* from Taiwan, ROC

CHUNG-YU CHANG, LIN-LI CHANG, YU-HUNG CHANG, TSONG-MING LEE* and SHUI-FENG CHANG

Department of Microbiology, School of Medicine, Kaohsiung Medical University, Kaohsiung 807 and

*Department of Food Sanitation, Tajen Institute of Technology, Ping Tung 907, Taiwan, ROC

The presence of class 1 integrons in clinical isolates of *Escherichia coli* was detected by PCR. Of 104 *E. coli* isolates from Kaohsiung, 54 (52%) carried class 1 integrons, with inserted DNA regions of 1–3 kb. These integrons were located on plasmids, as demonstrated by Southern hybridisation. DNA sequencing was used to identify the genetic content of the integron-variable regions. Different class 1 integrons contained various numbers, kinds and combinations of gene cassettes within their variable regions. These gene cassettes included those encoding resistance to trimethoprim (*dfrIa*, *dfrV*, *dfr12* and *dfr17*), aminoglycosides (*aadA1a*, *aadA2*, *aadA4* and *aadB*), chloramphenicol (*cmlA*), erythromycin (*ereA2*) and β -lactams (*blaPI*). An integron carrying three inserted cassettes – *dfr12-orfF-aadA2* – was present in 33 (61%) of the 54 isolates with class 1 integrons. Gene cassettes encoding resistance were expressed phenotypically. The results indicate that class 1 integrons are widespread in clinical *E. coli* isolates in Taiwan. The types, combinations and frequency of the gene cassettes in integrons may reflect the specific selective pressures to which the isolates were exposed and could provide useful surveillance data for relation to antibiotic usage information.

Introduction

The rapid dissemination of drug-resistant bacteria is an alarming and increasing problem, complicating the treatment of infections. Much of the problem is the result of antibiotic resistance genes transferring among bacterial species, carried by plasmids and transposons [1]. In recent years, a third mechanism for the dissemination of antibiotic resistance genes has been discovered; this involves integrons, novel DNA elements that mediate the integration of antibiotic resistance genes through site-specific recombination [1–3]. This recombination system depends on two determinants: an *int* gene encoding a site-specific DNA integrase (Int) [1, 2], and an adjacent site, *attI*, which is recognised by the integrase and acts as the receptor for the inserted genes [3]. The inserted genes, which mostly encode antibiotic resistances, are contained in mobile elements called gene cassettes [4]. Each gene cassette consists of a coding region and an integrase-

specific recombination site known as a 59-base element [4, 5]. Most of the gene cassettes lack their own promoters, and integrons act as natural expression vectors by supplying a common promoter, P_{ant} , located in the conserved sequences upstream of the inserted genes [2–4].

Four types of integrons, with different *int* genes, have been identified. Most integrons from clinical isolates belong to class 1 [6] and consist of two conserved segments. A 5'-conserved segment contains the *int* gene (*intI1*), the *attI* site and the common promoters P_{ant} [3, 4], whereas the 3'-conserved segment includes an antiseptic resistance gene (*qacE Δ 1*), a sulphonamide resistance gene (*sul1*) and an open reading frame (*orf5*) of unknown function [2]. The central variable region includes different combinations of inserted gene cassettes. More than 40 different antibiotic resistance cassettes have been characterised from integrons, which consequently can allow their bacterial hosts to become resistant to broad spectra of antimicrobial agents [4].

Several studies have investigated the prevalence of integrons in clinical isolates and have found them to be widespread [7–9]. However, the kinds of gene cassettes

Received 18 Feb. 2000; revised version accepted 26 April 2000.

Corresponding author: Dr S-F. Chang (e-mail: shfech@cc.kmu.edu.tw).

inserted into the integrons were not identified, except in the work of Sallen *et al.* [7]. In Taiwan, resistance to various antibiotics is very common in clinical isolates, often more so than in Western countries [10]. However, the frequency, characteristics, and roles of integrons and gene cassettes have not yet been investigated. In this study, *Escherichia coli* isolates from Kaohsiung, Taiwan, were investigated to demonstrate the prevalence and characteristics of class 1 integrons and the gene cassettes in these organisms, relating the results to the phenotypic expression of resistance.

Materials and methods

Bacterial strains

A total of 104 *E. coli* isolates was collected consecutively in 1993 and 1994 from the Kaohsiung Medical University Hospital, a medical centre in southern Taiwan. They were isolated from various clinical specimens from diverse units of the hospital. Once identified, the isolates were preserved at -70°C in Tryptic Soy Broth (Difco Laboratories, Detroit, MI, USA) containing glycerol 15% v/v.

PCR analysis

Integrons were detected by PCR with primers specific for the 5'-conserved segment (5'-CS; 5'-GGCATCC AAGCAGCAAG-3') and the 3'-conserved segment (3'-CS; 5'-AAGCAGACTTGACCTGA-3') [11]. The DNA templates for PCR were prepared as described by Bass *et al.* [12]. The PCR itself was performed in 20- μl volumes containing 200 μM dNTP, 1 U Taq DNA polymerase, 10 $\times$ PCR buffer (TaKaRa, Shiga, Japan), 0.3 μM of each primer and template DNA in a Gene Amp 9700 PCR system (Perkin-Elmer, Norwalk, CT, USA). The PCR conditions consisted of denaturation at 95°C for 1 min, then 30 cycles of denaturation for 30 s at 95°C , annealing for 30 s at 55°C and extension for 1.5–2 min at 72°C . The PCR products were visualised after electrophoresis through an agarose 1.5–2% gel containing ethidium bromide. Plasmid pUB2401, which harbours transposon Tn21 containing the In2 class 1 integron [2–4], was used as a positive control. Plasmid ColE1::Tn7, which harbours transposon Tn7 with a class 2 integron [2–4], was included as a negative control.

Southern hybridisation

Plasmid DNA was extracted by the method of Kado and Liu [13], subjected to electrophoresis through an agarose 0.8% gel (Boehringer-Mannheim GmbH, Mannheim, Germany), stained with ethidium bromide and photographed under UV transillumination. The gels were then partially depurinated in 0.25 M HCl and denatured in 0.5 M NaOH, after which the DNA was transferred to a nylon membrane. This was prehybridised, then hybridised with an oligonucleotide probe (5'-GCCTCGACTTCGCTGCTGCCC-3') specific for the *intI1* gene [14]. This probe was labelled with DIG-ddUTP. A positive hybridisation result was

detected with a DIG Luminescent Detection Kit (Boehringer-Mannheim), used according to the manufacturer's recommendations. Luminescence was documented by exposure to Kodak BioMax Light film (Kodak, Rochester, NY, USA).

DNA sequencing and computer analysis of sequence data

The genetic material inserted within the integron-variable regions was identified by direct nucleotide sequencing. The template amplicons were purified with a QIAquick PCR Purification Kit (QIAGEN GmbH, Hilden, Germany). When two amplicons of different lengths were obtained from a single isolate, each fragment was excised from the agarose gel and purified with a QIAquick Gel Extraction Kit (QIAGEN). Nucleotide sequencing was performed with the ABI PRISMTM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer), and the reactions were analysed on an ABI PRISM 377 DNA sequencer. Similarity searches with nucleotide sequences in the GenBank database were performed with the BLASTN program (National Center for Biotechnology Information, USA).

Amplicon cloning

To further explore the expression of the integron-borne gene cassettes, an amplicon representative of each of the integrons found with different gene cassettes was ligated into the pGEM-T Easy Vector (Promega, Madison, WI, USA) according to the manufacturer's instructions and was then transformed into *E. coli* JM101 [*supE thi Δ (lac-proAB) F' traD36 proAB⁺ lacI^q lacZΔM15*] [15]. Transformants were selected on Luria-Bertani (LB) agar containing ampicillin 100 mg/L, 0.5 mM isopropylthiogalactoside and X-Gal 80 $\mu\text{g}/\text{ml}$.

Antimicrobial susceptibility testing

Antimicrobial susceptibility tests were performed by the disk diffusion method recommended by the National Committee for Clinical Laboratory Standards [16].

Results

Presence and location of class 1 integrons in *E. coli* isolates

Class 1 integrons were sought in 104 *E. coli* isolates from clinical specimens by PCR with the 5'-CS and 3'-CS primers, which correspond to the sequences flanking the integron-variable regions; 54 (52%) of the isolates carried detectable class 1 integron structures. The amplicon lengths, corresponding to the approximate sizes of the inserted cassette DNA, varied from 1 to 3 kb (Table 1 and Fig. 1). Among the 54 isolates, 46 yielded one amplicon and eight yielded two amplicons of different sizes (Table 1 and Fig. 1). None of the *E. coli* isolates had only the integron conserved segments without inserted DNA; if such 'empty' integron structures were present, the resulting amplicons would be c. 150 bp and would have been resolved

Table 1. Sizes and numbers of amplicons amplified from *E. coli* isolates with primers 5'-CS and 3'-CS

Isolates yielding single amplicons (n = 46)		Isolates yielding two amplicons (n = 8)	
Approximate length (kb)	Number of isolates (group)	Approximate lengths (kb)	Number of isolates (group)
1.0	9 (1)	1.0, 1.9	3 (6)
1.6	6 (2)	1.0, 2.1	1 (7)
1.9	29 (3)	1.6, 1.9	1 (8)
2.0	1 (4)	1.6, 2.0	3 (9)
3.0	1 (5)	...	...

Fig. 1. PCR amplification of the integron-variable regions with the 5'-CS and 3'-CS primers. The amplicons were separated by electrophoresis through an agarose 1.5% gel. Lane M, size markers; lane 1, Tn21 (positive control); 2, ColE1::Tn7 (negative control); 3–11, amplicons from clinical *E. coli* isolates; 3, 1.0-kb amplicon; 4, 1.6-kb amplicon; 5, 1.9-kb amplicon; 6, 2.0-kb amplicon; 7, 3.0-kb amplicon; 8, 1.0-kb and 1.9-kb amplicons; 9, 1.0-kb and 2.1-kb amplicons; 10, 1.6-kb and 1.9-kb amplicons; 11, 1.6-kb and 2.0-kb amplicons.

by the electrophoresis conditions used. The PCR analysis showed that different insert sizes were present, indicating heterogeneity in the gene cassettes; nevertheless, 33 of the 54 isolates with class 1 integrons had 1.9-kb inserts.

Southern hybridisation with an oligonucleotide probe specific for the *intI1* gene consistently revealed that the integrons were located on plasmids (Fig. 2).

Identification of integron-borne gene cassettes

Purified PCR products were subjected to DNA sequencing and the gene cassettes thereby identified are listed in Table 2. The integrons were classified into nine groups according to the length and the numbers of amplicons yielded by a single isolate (Table 1). Groups 1 and 2 were further divided into two subgroups, a and b, based on the kinds of gene cassettes identified (Table 2). The gene cassettes encoded antibiotic resistance determinants with the possible exception of one open reading frame encoding an unknown product, *orfF*. These antibiotic resistance gene cassettes included four trimethoprim resistance determinants (*dhfr1a*, *dhfrV*, *dhfr12* and *dhfr17*) and three spectinomycin/streptomycin resistance determinants (*aadA1a*, *aadA2* and *aadA4*).

Other gene cassettes found included *aadB*, encoding gentamicin resistance; *cmlA*, encoding non-enzymic chloramphenicol resistance [17]; *ereA2*, encoding erythromycin resistance and *blaP1*, encoding PSE-1, a class A β -lactamase [18].

The integrons were found to contain one-to-three gene cassettes and the combinations of these are shown in Table 2. The 1.9-kb amplicon present in 33 isolates carried *dhfr12*, *orfF* and *aadA2* (group 3, 6 and 8). An integron containing two newly described gene cassettes, *dhfr17* and *aadA4* [19], was found in the five isolates yielding a 1.6-kb amplicon (groups 2b and 8). The entire sequences of the *dhfr17* and *aadA4* cassettes were deposited in GenBank, under accession no. AF170088. Eight isolates yielded two amplicons of different sizes with primers 5'-CS and 3'-CS; sequencing analysis confirmed the co-existence of two distinct integrons carrying different gene cassettes in these isolates (group 6, 7, 8 and 9).

Antimicrobial resistance in relation to integron carriage

When isolates contained a gene cassette, the corresponding antibiotic-resistant phenotype was detected

Fig. 2 Agarose gel electrophoresis (a) and Southern hybridisation (b) of plasmid DNA from clinical *E. coli* isolates. Southern hybridisation was performed with a probe specific for the *intI1* gene. Lanes 1–11, *E. coli* clinical isolates; 12, ColE1::Tn7 (negative control).

consistently (Table 2). Further cloning and expression of the amplicons containing the cassette regions demonstrated that the transformants containing an amplicon expressed phenotypic antibiotic resistance encoded by the gene cassettes present in this amplicon. For instance, the transformants containing the 1.9-kb amplicon, comprising *dfr12*, *orfF* and *aadA2* cassettes, were resistant to trimethoprim, spectinomycin and streptomycin. Nevertheless, the integron-carried genes did not account for all the phenotypic resistances of the *E. coli* isolates. Thus, although 39 isolates were resistant to chloramphenicol and 22 were resistant to gentamicin, only one contained an integron carrying the *cmlA* and *aadB* cassettes (group 5 in Table 2). A few isolates (*c.* 2–7%) were resistant to cefmetazole, ceftazidime, moxalactam, aztreonam and netilmicin, drugs to which no integron-associated resistance determinants were found.

Discussion

Of 104 *E. coli* isolates, 54 (52%) had class 1 integron structures, as detected by PCR with primers specific for the CS regions flanking the inserted gene cassettes.

Other reports have shown the prevalence of class 1 integrons in gram-negative clinical isolates to be *c.* 43% in Western and Central Europe [8], >50% in the Netherlands [20] and 59% in France [7]. Among aminoglycoside-resistant isolates from the USA, nearly 75% had integrons [11]. These results, together with those obtained in the present study, indicate that class 1 integrons are widespread in clinical isolates. International travel may contribute to their dissemination and that of their host strains. Most (61%) of the class 1 integrons found in this study carried *dfr12*, *orfF* and *aadA2* cassettes. This pattern was also reported in a urinary *E. coli* isolate from Turku, Finland [21], and in *Shigella* strains isolated in Finland, but originating from Asia [21]. The present study also revealed an integron carrying two newly characterised gene cassettes, *dfr17* and *aadA4*. These cassettes were also found in a recent urinary *E. coli* isolate from Australia [22] and the same *aadA* cassette (although designated as *aadA5*) was found in a class 1 integron in a porcine *E. coli* isolate from Denmark [23]. These data reveal that the same cassettes can disseminate in different bacterial species, in different human or animal hosts and in different areas of the world. Such observations underscore the concern that human, animal and

Table 2. Amplicons and corresponding gene cassettes inserted in class 1 integrons, and antibiotic resistance phenotype of gene cassettes and *E. coli* isolates

Integron group	Approximate length of amplicons (kb)	Identity and order of inserted gene cassettes	Resistance phenotype	Number of isolates	Number of <i>E. coli</i> isolates with antibiotic resistance (n = 54)													
					Su	Sm	Sp [†]	Tp	Ap	Cm	Gm	Mi	Cmz	Caz	Mox	Atm	Net	
1a	1.0	<i>aadA1a</i>	SmSp	7	7	7	7	3	5	6	4	6						
1b	1.0	<i>aadA2</i>	SmSp	2	2	2	2											
2a	1.6	<i>dfr1a-aadA1a</i>	TpSmSp	2	2	2	2	2	2	2	1	2						
2b	1.6	<i>dfr17-aadA4</i>	TpSmSp	4	4	4	4	4	4	3	3	4		1	1			
3	1.9	<i>dfr12-orfF-aadA2</i>	TpSmSp	29	29	29	29	29	20	20	8	16	1	1			3	
4	2.0	<i>blaP1-aadA2</i>	ApSmSp	1	1	1	1		1	1								
5	3.0	<i>aadB-aadA1a-cmlA</i>	GmSmSpCm	1	1	1	1	1	1	1	1	1						
6	1.0	<i>aadA1a</i>	SmSp	3	3	3	3	3	3	2	3	1	1	1			1	
	&1.9	<i>dfr12-orfF-aadA2</i>	TpSmSp															
7	1.0	<i>aadA1a</i>	SmSp	1	1	1	1	1	1	1								
	&2.1	<i>dfrV-ereA2</i>	TpErm															
8	1.6	<i>dfr17-aadA4</i>	TpSmSp	1	1	1	1	1	1	1	1	1						
	&1.9	<i>dfr12-orfF-aadA2</i>	TpSmSp															
9	1.6	<i>dfr1a-aadA1a</i>	TpSmSp	3	3	3	3	3	2	2	1	3						
	&2.0	<i>blaP1-aadA2</i>	ApSmSp															
Total	...	...	...	54	54	54	54	47	40	39	22	34	2	1	2	1	4	

^{*} Ap, ampicillin; Atm, aztreonam; Caz, ceftazidime; Cm, chloramphenicol; Cmz, cefmetazole; Erm, erythromycin; Gm, gentamicin; Mi, minocycline; Mox, moxalactam; Net, netilmicin; Sm, streptomycin; Sp, spectinomycin; Su, sulphonamide; Tp, trimethoprim. [No inhibition zone around a spectinomycin disk (25 µg) was seen for these 54 isolates.]

* Ap, ampicillin; Atm, aztreonam; Caz, ceftazidime; Cm, chloramphenicol; Cmz, cefmetazole; Erm, erythromycin; Gm, gentamicin; Mi, minocycline; Mox, moxalactam; Net, netilmicin; Sm, streptomycin; Sp, spectinomycin; Su, sulphonamide; Tp, trimethoprim. † No inhibition zone around a spectinomycin disk (25 µg) was seen for these 54 isolates.

environmental microbial ecosystems are inextricably intertwined, with antibiotic resistance readily crossing ecological boundaries and spreading widely [24]. This makes control much more difficult.

Although the integrons contained different combinations of gene cassettes, some combinations in gene cassettes were frequently conserved, such as *dfr1a-aadA1a* and *dfr12-orfF-aadA2*. This observation may indicate that these gene cassettes have become stably integrated over a long period of time. Although integrase-mediated insertion and excision of gene cassettes has been demonstrated [2–4], Martinez-Freijo *et al.* also proposed that many inserted gene cassettes may be preserved and stable [6]. Moreover, the transfer of the entire integron, *via* a plasmid or transposon, is more frequent than single gene mobilisation or integration within the integron [6]. In this study, integrons were located on plasmids, and conjugation experiments revealed that strain-to-strain transfer could occur.

All the 54 isolates with class 1 integrons contained at least one kind of *aadA* cassette (*aadA1a*, *aadA2* or *aadA4*), alone or in combination with other cassettes. *aadA* genes confer resistance to spectinomycin and streptomycin and have been found frequently in integrons [6, 7]. Moreover, despite minimal use as therapeutic agents, resistances to streptomycin and spectinomycin remain prevalent among *E. coli* isolates [12, 25]. It seems likely that, due to the lack of direct selection pressure, different *aadA* variants have not been selected to the extent that has occurred with genes conferring resistance to trimethoprim and β -lactams. However, the finding of a novel streptomycin/spectinomycin resistance gene indicated that variations in the *aadA* gene do arise. Moreover, streptomycin is still used extensively in animal husbandry [26], perhaps selecting new *aadA* gene variants that may spread to pathogenic bacteria isolated from man.

Some researchers propose that the *aadA* gene may be the first cassette acquired by integrons [27, 28]. In the present study, most *aadA* cassettes were combined with other kinds of gene cassettes, adjacent to the 5'-conserved segment and thus upstream of the *aadA* cassettes. To this extent the results could support the suggestion that *aadA* is the first cassette acquired by integrons, because a gene cassette preferentially recombines into the *attI* site, rather than into the *attC* sites at the 3'-ends of cassettes already present in the integron [3]. On the other hand, *aadA* genes were rarely the sole cassettes in integrons. This finding is different from that of Rosser and Young [28], who found that *aadA* was often the sole cassette in the variable region of integrons from coliform bacteria, *Pseudomonas*-like and *Vibrio*-like isolates from a natural estuarine environment. The disagreement might reflect the fact that clinical isolates are exposed to more antibiotic selection than environmental bacteria, promoting accumulation of multiple gene cassettes.

Besides *aadA*, other frequently found cassettes were those encoding resistance to trimethoprim (*dfr*), which were present in 43 (80%) of the isolates. Four *dfr* gene types cassettes were identified. All the 54 *E. coli* isolates with class 1 integrons showed resistance to sulphonamides, perhaps reflecting the fact that co-trimoxazole is frequently used to treat *E. coli* infection. Such specific selection pressure may favour the acquisition and maintenance of a trimethoprim resistance cassette by class 1 integrons also containing *sulI* in the 3' region [23]. This selection pressure also may explain the increasing number of different types of cassette-encoded dihydrofolate reductase. For example, *dfr17* is a novel trimethoprim resistance gene cassette. Furthermore, *dfr* cassettes were located directly behind the 5'-conserved segment. This site is the closest to the promoter, giving high-level expression and contingent resistance.

Antimicrobial susceptibility tests confirmed that the gene cassettes in the integrons contributed to resistance, as also found by others [7, 28]. Many isolates also had other antibiotic resistances not encoded by integron-associated resistance gene cassettes. These resistances may have resulted from chromosomal mutation, plasmid acquisition or the presence of other integrons besides class 1 types.

The present study revealed that class 1 integrons are widespread in clinical isolates. Their location on plasmids may contribute to horizontal dissemination of the antibiotic resistance gene cassettes. All the integrons contained antibiotic resistance gene cassettes. The study of integrons and their associated gene cassettes could provide information about which antibiotics should be used most carefully to prevent further accumulation of resistance.

This study was supported by grants from the Department of Health, ROC (DOH88-TD-1009 and DOH89-TD-1005) and partially from the Kaohsiung Medical University (M8725).

References

1. Stokes HW, Hall RM. A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons. *Mol Microbiol* 1989; **3**: 1669–1683.
2. Hall RM, Stokes HW. Integrons: novel DNA elements which capture genes by site-specific recombination. *Genetica* 1993; **90**: 115–132.
3. Hall RM, Collis CM. Mobile gene cassettes and integrons: capture and spread of genes by site-specific recombination. *Mol Microbiol* 1995; **15**: 593–600.
4. Recchia GD, Hall RM. Gene cassettes: a new class of mobile element. *Microbiology* 1995; **141**: 3015–3027.
5. Hall RM, Brookes DE, Stokes HW. Site-specific insertion of genes into integrons: role of the 59-base element and determination of the recombination cross-over point. *Mol Microbiol* 1991; **5**: 1941–1959.
6. Martinez-Freijo P, Fluit AC, Schmitz F-J, Verhoef J, Jones ME. Many class I integrons comprise distinct stable structures occurring in different species of *Enterobacteriaceae* isolated from widespread geographic regions in Europe. *Antimicrob Agents Chemother* 1999; **43**: 686–689.
7. Sallen B, Rajoharison A, Desvarenne S, Mabilat C. Molecular epidemiology of integron-associated antibiotic resistance genes in clinical isolates of *Enterobacteriaceae*. *Microb Drug Resist* 1995; **1**: 195–202.
8. Martinez-Freijo P, Fluit AC, Schmitz F-J, Grek VSC, Verhoef J, Jones ME. Class I integrons in Gram-negative isolates from different European hospitals and association with decreased susceptibility to multiple antibiotic compounds. *J Antimicrob Chemother* 1998; **42**: 689–696.
9. Gonzalez G, Sossa K, Bello H, Dominguez M, Mella S, Zemelman R. Presence of integrons in isolates of different biotypes of *Acinetobacter baumannii* from Chilean hospitals. *FEMS Microbiol Lett* 1998; **161**: 125–128.
10. Chang SC, Hsieh WC. Current status of bacterial antibiotic resistance in Taiwan. *J Infect Dis Soc ROC* 1996; **7**: 83–88.
11. Lévesque C, Piché L, Larose C, Roy PH. PCR mapping of integrons reveals several novel combinations of resistance genes. *Antimicrob Agents Chemother* 1995; **39**: 185–191.
12. Bass L, Liebert CA, Lee MD *et al.* Incidence and characterization of integrons, genetic elements mediating multiple-drug resistance, in avian *Escherichia coli*. *Antimicrob Agents Chemother* 1999; **43**: 2925–2929.
13. Kado CI, Liu S-T. Rapid procedure for detection and isolation of large and small plasmids. *J Bacteriol* 1981; **145**: 1365–1373.
14. Schwocho LR, Schaffner CP, Miller GH, Hare RS, Shaw KJ. Cloning and characterization of a 3-*N*-aminoglycoside acetyltransferase gene, *aac(3)-Ib*, from *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 1995; **39**: 1790–1796.
15. Yanisch-Perron C, Vieira J, Messing J. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* 1985; **33**: 103–119.
16. National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial disk susceptibility test, 5th edn. Approved Standard: M2-A5. Villanova, PA, NCCLS. 1993.
17. Bissonnette L, Champetier S, Buisson J-P, Roy PH. Characterization of the nonenzymatic chloramphenicol resistance (*cmlA*) gene of the In4 integron of Tn696: similarity of the product to transmembrane transport proteins. *J Bacteriol* 1991; **173**: 4493–4502.
18. Huovinen P, Jacoby GA. Sequence of the PSE-1 β -lactamase gene. *Antimicrob Agents Chemother* 1991; **35**: 2428–2430.
19. Chang C-Y, Chang L-L, Chang Y-H, Lee T-M, Li Y-H, Chang S-F. Two new gene cassettes, *dfr17* (for trimethoprim resistance) and *aadA4* (for spectinomycin/streptomycin resistance), inserted in an *Escherichia coli* class 1 integron. *J Antimicrob Chemother* 2000 (in press).
20. Jones ME, Peters E, Weersink A-M, Fluit A, Verhoef J. Widespread occurrence of integrons causing multiple antibiotic resistance in bacteria. *Lancet* 1997; **349**: 1742–1743.
21. Heikkilä E, Skurnik M, Sundström L, Huovinen P. A novel dihydrofolate reductase cassette inserted in an integron borne on a Tn21-like element. *Antimicrob Agents Chemother* 1993; **37**: 1297–1304.
22. White PA, McIver CJ, Deng Y-M, Rawlinson WD. Characterisation of two new gene cassettes, *aadA5* and *dfrA17*. *FEMS Microbiol Lett* 2000; **182**: 265–269.
23. Sandvang D. Novel streptomycin and spectinomycin resistance gene as a gene cassette within a class 1 integron isolated from *Escherichia coli*. *Antimicrob Agents Chemother* 1999; **43**: 3036–3038.
24. Witte W. Medical consequences of antibiotic use in agriculture. *Science* 1998; **279**: 996–997.
25. Chiew Y-F, Yeo S-F, Hall LMC, Livermore DM. Can susceptibility to an antimicrobial be restored by halting its use? The case of streptomycin versus *Enterobacteriaceae*. *J Antimicrob Chemother* 1998; **41**: 247–251.
26. Liebert CA, Hall RM, Summers AO. Transposon Tn21, flagship of the floating genome. *Microbiol Mol Biol Rev* 1999; **63**: 507–522.
27. Bissonnette I, Roy PH. Characterization of In0 of *Pseudomonas aeruginosa* plasmid pVS1, an ancestor of integrons of multiresistance plasmids and transposons of gram-negative bacteria. *J Bacteriol* 1992; **174**: 1248–1257.
28. Rosser SJ, Young H-K. Identification and characterization of class I integrons in bacteria from an aquatic environment. *J Antimicrob Chemother* 1999; **44**: 11–18.