ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 2011, p. 943–945 Vol. 55, No. 2
0066-4804/11/$12.00 doi:10.1128/AAC.00988-10

Use of Inverse PCR for Analysis of Class 1 Integrons Carrying an Unusual 3′ Conserved Segment Structure▽

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Received 20 July 2010/Returned for modification 1 September 2010/Accepted 11 November 2010

By using inverse PCR and DNA sequencing, 13 *sul3*-associated mutational integrons, 2 defective class 1 integrons, and 1 *qnrB2*-associated complex *sul1*-type class 1 integrons were identified in *Salmonella enterica* serovar Choleraesuis, *Pseudomonas aeruginosa*, and *Enterobacter cloacae*, respectively. In addition, conjugation and Southern hybridization demonstrated that unusual class 1 integrons were located on plasmids or integrated into chromosomal DNA. Thus, an inverse PCR assay can be a valuable tool for the analysis of unusual structures of the 3′ conserved region of class 1 integrons.

Integrons are genetic elements comprised of a gene cassette capture system, which is the most important resistance acquisition mechanism for Gram-negative pathogens, by means of site-specific recombination catalyzed by specific integrase genes (9). To date, class 1 integrons are the major type of integrons obtained from clinical isolates (7, 15). Typically, a class 1 integron structure contains a 5′ conserved segment (5′CS), a 3′ conserved segment (3′CS), and a variable region with resistance gene cassettes (14). PCR analyses of class 1 integrons commonly use the specific 5′CS and 3′CS primers to anneal to the conserved segment of class 1 integrons and to amplify the gene cassette variable regions (14). In recent years, class 1 integrons with unusual 3′ regions have been described (17). However, little information is available to determine the genetic organization of the 3′ region in unusual class 1 integrons. Because of the limitations of conserved segment PCR (CS-PCR) in mapping of integrons by using 5′CS and 3′CS primers, the gene cassette array in novel, complex unusual class 1 integrons may not have been amplified. Therefore, a practical tool for the study of 3′ ends of unusual class 1 integrons is necessary.

The inverse PCR is a first-generation PCR assay for rapid amplification, by means of inversely oriented specific primers, of both unknown sequences flanking a specified fragment and genome-walking sequences (3, 16). Inverse PCR involves generating circular DNA fragments constructed from DNA digestion and ligation and requires specific primers complementary to both termini of the target DNA with a known sequence (11).

In this study, we applied an inverse PCR assay to characterize unusual class 1 integrons without CS-PCR products in 13 isolates of *Salmonella enterica* serovar Choleraesuis, 2 isolates of *Pseudomonas aeruginosa*, and 1 isolate of *Enterobacter cloacae*, which were negatively amplified by 5′CS- and 3′CS-specific primer pairs for class 1 integron detection, as previously described (12, 13). Furthermore, based on plasmid profiles and antibiotic phenotypes, no clonal relatedness was found among the 13 isolates of *S.* Choleraesuis or 2 isolates of *P. aeruginosa*. For inverse PCR assay, the following steps were performed. For step 1 (inverse PCR primer design), the inverse primers of *intI1* and *sul1* gene were designed using an inverse PCR primer design program (Primo Inverse 3.4; Chang Bioscience [available at http://www.changbioscience.com/primo/primoinv.html]) (Table 1). These specific primers could amplify ligation-mediated ring-like DNA. For step 2 (digestion), because neither *intI1* nor *sul1* genes had BamHI restriction sites, the BamHI restriction enzyme was used (NEB, Ipswich, MA) to manipulate genomic DNA, and 3- to 6-kb DNA fragments were selected for further study. For step 3 (ligation), the digested DNA fragments were further diluted and ligated under suitable conditions that facilitate the construction of monomeric circles (6). The intramolecular ligation products were then used as substrates for PCR analysis using specific primers (Table 1) for amplification of regions that flank the known sequence. For step 4 (inverse PCR amplification), inverse PCR amplification was performed using 1.25 units TaKaRa Ex *Taq* DNA polymerase (TaKaRa Bio Inc., Otsu, Shiga, Japan) for amplification of longer products. Optimal conditions for inverse PCR cycling are an initial denaturation step at 94°C for 1 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 1 min, extension at 72°C for 5 to 10 min (1 min/kbp), and a final extension step at 72°C for 10 min. Finally, the inverse PCR products were purified using the QIAquick PCR purification kit (Qiagen Inc., Valencia, California) for primer-walking-strategy DNA sequencing and then analyzed using the BLAST suite of programs.

In addition, conjugation experiments and Southern hybridization were carried out to demonstrate genetic localization of class 1 integron with unusual 3′ ends. Plasmid DNA was isolated using an alkaline lysis method (10). Plasmid DNA sizes were determined according to the reference strain, *Escherichia coli* 39R861. Southern hybridization was carried out with a

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▽ Published ahead of print on 22 November 2010.

TABLE 1. Primers and probes used in this study

Primer or probe	DNA sequence (5′–3′)	Nucleotide position	GenBank accession no.[a]	Target gene/ genetic region[a]
Primers				
IntI1F	GGGTCAAGGATCTGGATTTCG	786–766	U49101	*intI1*
IntI1R	ACATGCGTGTAAATCATCGTCG	303–324		
IntI2A	ATGTCTAACAGTCCATTTTTAAATTCTA	1474–1495	AJ002782	*intI2*
IntI2B	AAATCTTTAACCCGCAAACGC	1917–1887		
IntI3A	GTGGCGCAGGGTGTGGAC	194–211	D50438	*intI3*
IntI3B	ACAGACCGAGAAGGCTTATG	959–939		
5′-CS	GGCATCCAAGCAGCAAG	1190–1206	M73819	Class 1 integron variable region
3′-CS	AAGCAGACTTGACCTGA	1342–1326		
sul1F	ATG GTG ACG GTG TTC GGC ATT CTG A	1–25	EF592571	*sul1*
sul1R	CTA GGC ATG ATC TAA CCC TCG GTC T	840–816		
sul2F	GAA TAA ATC GCT CAT CAT TTT CGG	3–26	AF542061	*sul2*
sul2R	CGA ATT CTT GCG GTT TCT TTC AGC	812–798		
sul3F	GAG CAA GAT TTT TGG AAT CG	3–22	AJ459418	*sul3*
sul3R	CATCTGCAGCTAACCTAGGGCTTTGGA	792–775		
qnrB2-F	GTGATTTTTCAGGTGCCGAC	83–102	AB281054	*qnrB2*
qnrB2-R	AATGTGTGAAGTTTGCTGCT	520–501		
sul1-invF	GCGTCTGGAGATCTGCGAAGCGCAATC	3590–3616	EF592571	*sul1* (inverse PCR)
sul1-invR	ACGACGTCTGATCCGACTCGCAGCATTTC	2970–2942		
intI1-invF	CGAACCCAGTTGACATAAGCCTGTTCGG	4756–4783	EF138817	*intI1* (inverse PCR)
intI1-invR	GGCCATTCCGACGTCTCTACGACGATG	3977–3951		
qnrB2inF	CGGCGCGTTGATTTACAAGGCGTTAAGTTGG	559–589	AB281054	*qnrB2* (inverse PCR)
qnrB2inR	GACAGCCGATAAATTCAGTGCCGCTCAGG	130–102		
Probes for *intI1* gene				
Int11	CGCTGAAAGGTCTGGTCATA	471–490	U49101	*intI1*
Int12	GCCCAGCTTCTGTATGGAAC	838–819		

[a] Accession numbers and target genes/genetic region shown in the rows for the first listed primer or probe correspond to the primer/probe pair.

digoxigenin (DIG)-labeled probe for integrase gene *intI1* by using a DIG system described previously (12).

In this study, three types of class 1 integrons with variable 3′ ends, namely, *sul3*-associated mutational integrons, defective class 1 integrons, and *qnrB2*-associated complex *sul1*-type integrons, were characterized (Fig. 1). The *sul3*-associated complex integron presented a distinct gene cassette array in 13 strains of *S.* Choleraesuis (Fig. 1A). Moreover, defective class 1 integrons containing *sul3* (*sul1*-like) and a sequence of unknown function (*orf5*) (GenBank accession no. EU930362) were present in two strains of *P. aeruginosa* (Fig. 1B). The genetic organization of the *qnrB2*-containing complex integron was present in one strain of *E. cloacae* (Fig. 1C).

The present study describes the use of inverse PCR combined with *sul1*, *sul2*, and *sul3* gene screening to detect *sul3*-like gene-containing elements linked to class 1 integrons in *S.* Choleraesuis isolates (12). It is interesting to note that *sul3*-like and *qacH* genes had replaced *sul1* and *qacEΔ1* genes, respectively. These findings are similar to those reported by Antunes et al. (2) and Bischoff et al. (4). These *sul3*-like associated integrons join two aminoglycoside genes in the same unusual class 1 integron structures, but one of the two aminoglycoside genes may act as a pseudogene coming from the insertion of an internal stop codon. This may explain the presence of two *aadA*-type aminoglycoside genes observed in the same integron-associated structure. Moreover, phosphoserine phosphatase, a metabolic enzyme not related to antibiotic resistance in *sul3*-associated class 1 integrons, may play a role in signaling transduction and chemotaxis of *S.* Choleraesuis (1). Furthermore, spread of a *sul3*-associated integron in human and animal *S.* Choleraesuis isolates in Taiwan is an increasingly significant threat to human health. In short, the application of inverse PCR can rapidly demonstrate the presence of mutational integrons (e.g., *sul3*-like associated integron) in *S.* Choleraesuis isolates.

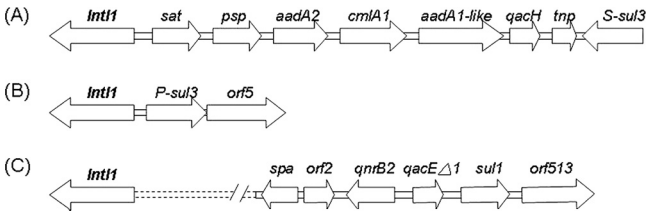

FIG. 1. Schematic representation of three different types of gene cassette arrays presented in class 1 integrons with unusual 3′ ends. (A) The *sul3*-type complex integron (GenBank accession no. EU834941) in 13 *S.* Choleraesuis isolates. (B) Defective class 1 integrons containing *sul3* and a sequence of unknown function (*orf5*) (GenBank accession no. EU930362) in two *P. aeruginosa* isolates. (C) Genetic organization of the *qnrB2*-containing complex integron.

Based on our findings, *sul1*-like genes containing defective class 1 integrons, which locate on plasmids of *P. aeruginosa* isolates (Fig. 2), may result in a horizontal distribution of antimicrobial resistance. Moreover, we discovered that *sul1*-like genes in two *P. aeruginosa* strains are similar to the *sul3* gene (GenBank accession no. AJ294721) but differs from the *sul3* gene corresponding to GenBank accession number AJ459418. Therefore, we have identified the *sul1*-like gene as a P-*sul3* gene deriving from *P. aeruginosa* isolates, whereas the *sul3* gene found in *S.* Choleraesuis is designated S-*sul3*. By

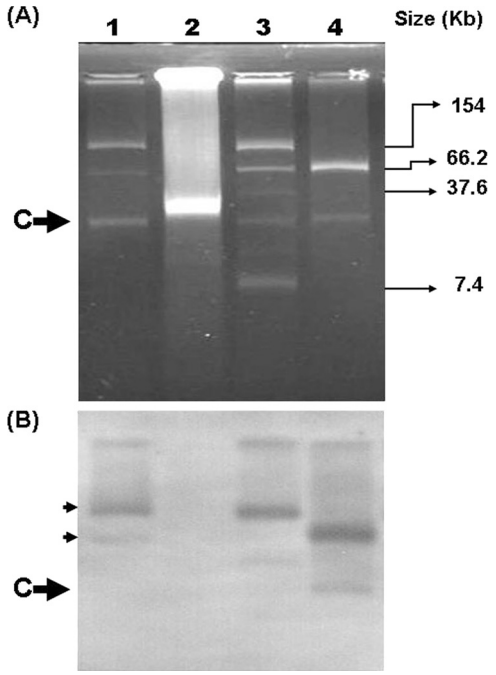

FIG. 2. Plasmid DNA isolation and Southern hybridization with the *intI1* probe to determine the location of *intI1* genes in defective class 1 integrons. (A) Plasmid DNA profiles. Lanes: 1, *P. aeruginosa* 9425 isolate (containing 160-kb and 50-kb plasmid DNAs); 2, *E. cloacae* 9555 isolate carrying bla_{IMP-8} (154-kb plasmid DNA); 3, standard plasmid sizes of *E. coli* 39R861 (154, 66.2, 37.6, and 7.4 kb); 4, plasmid pUB2401::Tn*21* carrying the Tn*21* integron, which was used as the positive control for the presence of the *intI1* gene. (B) Southern hybridization with *intI1* probe. Hybridization of the DNAs described for panel A with specific *intI1* gene probe. For lane 1, *intI1* genes are present on 160-kb and 50-kb plasmid DNAs, as indicated by arrows. Lane 3 shows *E. coli* 39R861 harboring an *intI1* gene located on the 154-kb plasmid. C, chromosomal DNA.

using inverse PCR, we can identify the defective class 1 integron more easily. It has been noticed that the two *P. aeruginosa* isolates containing the same *sul1*-like genes have different phenotypes of sulfonamide resistance. It will be indeed useful to characterize these mechanisms in further investigations.

The increasing frequency of plasmid-mediated quinolone resistance among the *Enterobacteriaceae* is an emerging worldwide challenge (5). *qnrA* and *qnrB 2* have been identified in IS*CR1*-associated complex integrons (18). In an earlier report, we discussed the presence of the *qnrB2* gene in a complex *sul1*-type integron located on chromosomes in *E. cloacae* isolates whose results are shown in Fig. 2 (13). The genetic organization of *qnrB2* in *E. cloacae* isolates is similar to that of the plasmid-borne *qnrB2*-associated complex *sul1*-type integron in *Salmonella enterica* serovar Keurmassar isolates (8). However, further studies are needed to investigate the genetic environment surrounding of the *qnrB2* gene. To our knowledge, this is the first report about the *qnrB2*-containing complex *sul1*-type integron found on chromosomes in clinical isolates in Taiwan. We believe that inverse PCR is a valuable tool for the detection of complex *sul1*-type integrons.

Indeed, inverse PCR is a useful and powerful tool for rapid assay of class 1 integrons with atypical 3′ ends, including mutational, defective, and large complex *sul1*-type class 1 integrons.

This study was financially supported by grant NSC 98-2320-B-037-023 from the National Science Council, Taiwan, Republic of China.

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