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A NOVEL INTEGRON IN THE GENOME OF *ESCHERICHIA COLI* ISOLATED FROM INDONESIAN MONITOR LIZARD *(VARANUS* SPP).

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ABSTRACT

The genotype of antibiotic resistance in natural isolates of *Escherichia coli* was determined through integron detection and characterization of the associated antibiotic resistance. *E. coli* SG2 isolated from *Varanus salvator* of Java demonstrated resistance to spectinomycin (50ng/ml) and streptomycin (SOng/ml). Integron detection indicated that eight isolates out of nine *E. coli* isolates possessed a conserved segment of the integron. Amplification of the inserted cassette of the integron in this SG2 isolate yielded a 1-kb DNA fragment. Sequence analyses indicated that this fragment was homologous with *aad* gene, which confirmed the resistance to spectinomycin/streptomycin. This is the first report on the presence of integron in the *E. coli* isolated from the environment.

Key words: Integron / antibiotic resistance / *Escherichia coli*

INTRODUCTION

Antibiotic is a substance produced by a microorganism as protection from another microorganism (Madigan *et al.* 1997). Antibiotics have a specific target site in the cell. For example, in trying to get hold of protein synthesis targeted to 30S ribosom, the presence of tetracycline, spectinomycin and streptomycin results in an invalid codon-anticodon reaction and therefore, the synthesized amino acid is also invalid (Schumm 1992).

Currently, there is concern on the spread of antibiotic resistance among bacteria. Such dissemination is partly a consequence of the antibiotic-resistant genes mobility which reside in mobile genetic elements such as plasmids, transposons, and integrons (Francia *et al.* 1999).

Integron is a new type of mobile element which has evolved by a site-specific recombination mechanism. Integrons consist of two conserved segments of DNA separated by a variable region containing one or more genes integrated as cassettes (Levesque *et al.* 1995). The 5'conserved segment contained in the *int* gene, which encodes a polypeptide of 337 amino acids, has been shown to be homologous to other members of the *integrase* family and on the opposite strand, a common promoter region *P1-P2* is directed toward the site of integration. The 3' conserved segment contains the *qacE&*, *suR* genes, and an open reading frame (Fig.l).

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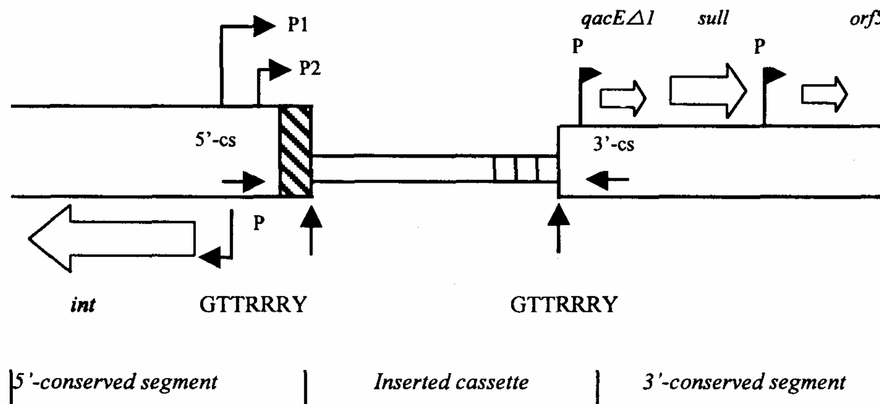

Figure 1. Integron structure (Levesque et al. 1995)

Genetic changes in bacterial genome could contribute to antibiotic resistance in some bacteria. There are three mechanisms of antibiotic inactivation: (i) modification of antibiotic structure; (ii) prevention of antibiotic to get into the target site; or (iii) change in antibiotic target site (Neu 1992).

Resistance to aminoglycoside antibiotics such as spectinomycin and streptomycin, could be due to the penetration failure, the low affinity of antibiotic to ribosom, or the inactivation of the antibiotic by enzymes such as phosphorylase, adenylase and acetylase produced by the bacteria (Gian and Gian 1995).

Desselberger (1998) reported that data on antibiotic-resistant genes in the environment were rare, whereas antibiotic-resistant genes among bacterial strains were increasing at an alarming rate. Biawak monitors (Varanus spp.) are reptilians lacking venomous gland, although there are some reports on Biawak bites resulting in bacterial infection (Auffenberg 1981). Probably, this infection is caused by toxic substances produced by microbiota living in the oral of Varanus spp. Auffenberg (1981) identified five bacteria species that could cause infection. Yogiara (1998) reported that many of E. coli isolates living in the buccal of Varanus spp. are resistant to ampicillin. This study was conducted in order to understand the mechanism of the antibiotic resistance of E. coli in the digestive system of Varanus spp. In this experiment, an antibiotic-resistant gene from E. coli was isolated and further characterized by DNA sequence analysis.

MATERIAL AND MOTHODS

Bacterial Strains and Plasmids

The bacterial strains and p;asmids used in this research are shown in Table 1.

Table 1. Bacterial strains plasmida

Bacteria and plasmids	Characteristics	References
Escherichia coli TOP 10	F⁻ *mcrA* Δ(*mrr-hsd*/RMS-*mcr* BC) *ϕ*80 *lacZ* MIS*Δ lac recAI deoR araD*139 *Δ(ara-leu)* 7697 *galU galK rpsL* (Str^R) *endAI napG*	Invitrogen, Branchburg, California
Escherichia coli isolates code: P2;PG1;30.1;5;7;2;SG2;SG 1	Wild type (isolated from buccal of *Varanus*), Ap^R	Yogiara 1998
pAS900	Generated from pCR® 2.1-TOPO (Invitrogen, co). Multiple cloning site one site of *Eco*RI, Km^R(Tn5 derivative), ΔAp^R with *Xma*I site dan *Sca*I site deletions	A. Suwanto (unpublished)
pAS901	Km^R; 11,2-kb fragment encodes Ap^R from *E. coli* 2-2-*Eco*RI cloned in pAS900-*Eco*RI	Yogiara 1998
pCRAS	Km^R; 1-kb PCR product from SG2 isolate - *Not*I cloned in pAS900-*Not*I	This study
pS2T	Ap^R; 1-kb PCR product from SG2 isolate cloned in pGEM-T Easy	This study
pEP10	Ap^R; 10-kb fragment encodes Ap^R from *E. coli* 2-2-*Hin*dIII cloned in pAS900-*Hin*dIII	This study

Growth Conditions

Antibiotic-resistant bacteria were cultured aerobic (100 rpm) at 37°C and kept overnight in Luria Bertani (LB) medium supplemented with one, or a combination of the following antibiotics: ampicillin (100 u.g/ml), streptomycin (50 ng/ml), spectinomycin (50 ng/ml).

DNA Extraction

Bacterial isolates were grown in 10 ml LB in the presence of a selective antibiotic at 37°C overnight. Alkaline lysis method (Sambrook *et al.* 1989) was used for plasmid DNA extraction, and Wizard®Genomic DNA Purification Kit (Promega, Madison, WI) was used according to manufacturer protocol for genomic DNA extraction.

PCR Amplification

PCR were performed in Gene Amp® PCR System 2400 (Perkin Elmer, Branchburg, New Jersey). Ready To Go™ PCR Beads (Pharmacia Biotech, Uppsala, Sweden) was used for each PCR reaction. Each of 25 ul reaction mixtures contained PCR beads, 4 ng/ul of genomic DNA, 10 ng/ul of primers (5'cs: GGCATCCAAGCAGCA AG, 3'cs: AAGCAGACTTGACCTGA, *qac:* ATCGCA-ATAGTTGGCGAAGT, *sul:* GCAAGGCGGAAACCCGCGCC) and distilled water up to 25 ul.

PCR condition for the integron was amplified in 35 thermal cycles at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 3 minutes. A final extension step for 7 minutes at 72°C was also included. The primers used were 5'cs and 3'cs that amplify variable regions of integron, *qac* and *sitll* primers to amplify the conserved region. PCR product was purified using Gene Clean Kit (Bio 101, La Jolla, California).

Cloning and Transformation

Amplified DNA was ligated into pAS900 (Km^R) and pGEM-T Easy (Ap^R) vector. The approximate ratio of pAS900 vector to insert DNA were 1:4 and that of pGEM-T Easy to insert DNA was 1 to 16. Ligation reaction consisted of vector-insert mixture 1 ul of T4- ligase, Ix ligation buffer, and distilled water up to 20 ul. Ligation mixture was incubated at 7°C for 16-18 h.

The ligation mixture was transformed into 250 ul of chemically competent *E. coli* TOP10, followed by heat shocked at 42°C for 45 seconds. After the addition of 3 ml LB, the culture was incubated with vigorous shaking at 37°C for 90 minutes (Sambrook *et al.* 1989). Transformants were then selected on Luria Bertani Agar (LBA) supplemented with X-gal (40ug/ml) and appropriate antibiotic/s.

DNA Sequencing

pS2T double - stranded templates were derived from cloning of STI in pGEM-T Easy. Sequencing reaction consisted of 8 ul Big Dye-Terminator, 360 ng of DNA template, 8 ng/ul of primer and distilled water up to 20 ul Cycle sequencing was performed using PCR machine with three-step profile for 25 cycles: a 10-second denaturation at 96°C, a 5-second annealing at 50°C, and a 4-minute extension at 60°C.

Cycle sequencing product was purified by ethanol-sodium acetate precipitation method. A 1.5 ml microcentrifuge tube was filled with 2 ul of sodium acetate (pH 4.6), 50 ul of ethanol 95%, and 20 ul of the cycle sequencing mixture, and then incubated at room temperature for 30 minutes. The mixture was centrifuged at maximum speed for 20 minutes. The supernatant was removed from the tube, and 250 ul of 70% ethanol was added into the tube. The mixture was centrifuged again for 5 minutes. The supernatant was removed and the DNA pellet was vacuum-dried at 50 cm Hg for 10 minutes.

The DNA was electrophoresed according to Sambrook *et al.* (1989). Prior to electrophoresis, DNA was denatured with 6 ul of loading buffer containing Blue Dextran and 25 mM of EDTA in formamide at 95°C for 2 minutes, and quickly placed on ice. Approximately 1.5 ul of DNA sample was loaded to each well and ran for 10 hours. The DNA was compared with those in the GenBank and EMBL databases.

RESULTS AND DISCUSSIONS

Integron Detection by PCR

Amplification of DNA sequence experiments using *qac-sul* primers indicated that there were eight out of nine *E.coli* isolates possessing 3'- conserved segment of the integron (Table 2). *E. coli* TOP 10 containing recombinant plasmid pEPIO did not show integron amplification, although it carries a 10-kb DNA fragment originated from genomic DNA of *E.coli* SG2 isolated from *Varanus* spp. (Table 1). We suspected that parts of the integron have been deleted when it was digested by restriction enzymes. PCR product using 5'cs-3'cs primers generated a single amplified DNA band of about 1-kb. It was generated from genomic DNA of *E. coli* SG2 isolate, which was designated as STI (Table 2 and Fig.2 lane 3).

At the downstream end of each resistant gene cassette inserted in the variable region of integrons, there is a short imperfect inverted repeat element called the 59-base element. Each inserted gene has its own version of this element (Levesque *et al.* 1995). The STI was also thought to have a 59-base element, although it was not demonstrated in this study. These 59-base elements are known to be important in the recombination events observed in the evolution of the integron. For example, in plasmid pVSI, which possesses the 5' and 3' conserved segment but no inserted gene between the conserved segments, there is no 59-base element (Levesque *et al.* 1995).

The other isolates used in this study were found to be similar to pVSI (Table 2). It was reported that pVSI, a plasmid derived from *Pseudomonas aeruginosa,* possessed integron type InO which has an unoccupied integration site and hence may be an ancestor of the more complex integrons (Bissonnette and Roy 1992).

Cloning of STI in pAS900 (Km^R) vector yielded recombinant plasmid called pCRAS. The purpose of cloning was to detect ampicillin-resistant gene in STI. *E. coli* TOP 10 harbouring the recombinant plasmid pCRAS was sensitive to ampicillin. This result indicated that STI was not an ampicillin-resistant gene, or it was an ampicillin-resistant gene which could not be expressed.

Expression of antibiotic-resistant genes in the integrated cassettes of integrons depends on cassette position. In all cases, the resistance level was the highest when the gene was present in the first cassette (Collis and Hall 1995). Therefore, there is a need to sequence the STI for gene characterization.

Table 2. Integron detection by PCR

Isolates	PCR product using qac- sul primers	PCR product using 5'cs– 3'cs primers
PVHA1 (control 3'-conserved segment of integron)	+	-
P2	+	-
PEP10	-	-
PG1	+	-
30.1	+	-
5	+	-
7	+	-
2	+	-
SG2	+	+
SG1	+	-

+ : amplified
- : not amplified

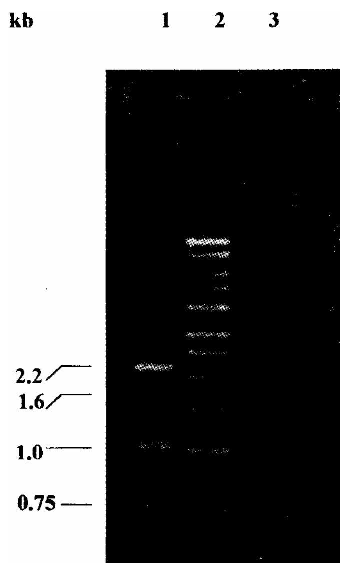

Figure 2. Integron PCR product from total DNA of pVHAl isolate (lane 1), SG2 isolate (lane 3), and *Kb-Ladder* as marker (lane 2).

Sequencing

Cloning of STI in pGEM-T Easy vector yielded recombinant plasmid designated as pS2T. Transformant was isolated, and then sequenced. The result indicated that the STI sequence was homologous to *aadA* gene encoding aminoglycoside adenyltransferase, which was responsible for streptomycin and spectinomycin resistance in *E. coli. BLAST Search* analysis of the STI sequence indicated that the 700 nucleotides of 3'STI are integron type 3 (Int 3) as in the IncL/M plasmid from *Salmonella typhimurium* (96% identity) and pNCCSOl plasmid from *Enterococcus faecalis* (96% identity).

The other 600 nucleotides of 5'STI were similar to the pNCCSOl plasmid from *E, faecalis* (97% identity) and the R100.1 plasmid from bacteriophage T4 (97% identity).

In summary, there were eight isolates out of nine *E. coli* isolates possessing a conserved segment of integron and only one possessed inserted gene between the conserved segments of the integron. The sequencing analysis indicated that spectinomycin- and streptomycin-resistant gene was present in the 1-kb DNA fragment.

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