A plasmid-encoded class 1 integron carrying sat, a putative phosphoserine phosphatase gene and aadA2 from enterotoxigenic Escherichia coli O159 isolated in Japan

Ashraf M. Ahmed, Tadashi Shimamoto *

Laboratory of Food Microbiology and Hygiene, Graduate School of Biosphere Science, Hiroshima University, 1-4-4 Kagamiyama, Higashi-Hiroshima, Hiroshima 739-8528, Japan

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Abstract

A class 1 integron was detected in a single multidrug-resistant strain of enterotoxigenic Escherichia coli (ETEC) O159 after examination of 23 clinical E. coli isolates. This isolate was resistant to streptomycin, kanamycin, gentamicin, chloramphenicol and ampicillin. Sequencing of the class 1 integron identified three-gene cassettes. The first is the streptothricin acetyltransferase gene, sat, which confers resistance to streptothricin. The second is an ORF whose product is a putative phosphoserine phosphatase (PSP), and the last is an aminoglycoside adenyltransferase gene, aadA2, which confers resistance to streptomycin and spectinomycin. The putative PSP gene product was found to be 39%, 38%, 28%, and 27% identical to PSP gene products of Vibrio vulnificus CMCP6, V. vulnificus YJ016, Pseudomonas syringae, and P. aeruginosa, respectively. Southern-blot hybridization showed that this integron is located on a 90 kb plasmid. This is the first report identifying a putative PSP gene in an integron.

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1. Introduction

Diarrhea caused by multidrug-resistant bacteria is an important public health problem among children and adults in developing countries, and is a research priority of the Diarrheal Disease Control program of the World Health Organization. Enterotoxigenic Escherichia coli (ETEC) are among the most common enteropathogens responsible for endemic diarrhea in children and adults in countries with poor environmental hygiene as well as travelers to these regions [1]. One of the more common ETEC O-serogroups is E. coli O159, which was first isolated from a case of infantile diarrhea and described by Ørskov et al. [2]. It has been found to be the most common ETEC isolate from British troops in Saudi Arabia [3] and in a community-based study in Egypt [4].

Reversible protein phosphorylation is a widespread mechanism of intracellular signal transduction that relies upon the regulated tension between protein kinases and protein phosphatases [5]. Protein phosphatases are classified in three groups, Ser/Thr phosphatases, Ser/Thr/Tyr phosphatases, and Tyr phosphatases, depending on their phosphoamino acid specificity [6]. In addition to its important role in the phosphorylated pathway of serine biosynthesis [7], phosphoserine phosphatase (PSP) shares homology with other protein phosphatases in the dephosphorylation processes that are associated with signal transduction in many bacterial species. In Bacillus subtilis PSPs, RsbU and RsbX, control the activity of the general stress transcription factor sigma B [8]. The sigma B transcription factor of B. subtilis is activated by growth-limiting energy or environmental challenge to direct the synthesis of more than 100 general stress proteins [9]. In Methanococcus jannaschii, the
high-resolution crystal structure of PSP showed typical catalytic domains and structural similarity with E. coli CheY [7,10]. CheY is the central regulator of bacterial chemotaxis that uses a signaling cascade of protein phosphorylation and dephosphorylation reactions to control its flagellar motors in response to environmental chemical changes [11]. The similar properties of M. jannaschii PSP and E. coli CheY have led researchers to postulate a common reaction mechanism [10].

The ability of bacteria to acquire and disseminate exogenous genes via mobile genetic elements such as plasmids and transposons has been the major factor in the development of multiple drug resistance over the last 50 years. In recent years, the matter became more complicated after the discovery of a third mechanism for the dissemination of antibiotic resistance genes in bacteria involving integrons. Integrons are novel DNA elements that mediate the integration of antibiotic resistance genes by a site-specific recombination system [12]. Class 1 integrons are widespread among multiresistant Gram-negative bacteria [13], and the most notable gene cassettes identified within integrons are those conferring resistance to antibiotics. To date, only the super-integron of Vibrio cholerae has harbored many open reading frames (ORFs) with a variety of adaptive functions, including metabolic activities and virulence traits, in addition to potential antibiotic resistance determinants [14]. Numerous studies have focused on the epidemiology and the virulence determinants of ETEC, but little is known about the mechanism of antibiotic resistance in ETEC, especially the multidrug-resistant strains isolated from humans.

2. Materials and methods

2.1. Bacterial strains

A total of 23 E. coli isolates were isolated from diarrheic patients in Tokushima, Japan. E. coli isolates were identified by standard procedures [15]. All isolates were stored in LB broth containing 25% glycerol at −80 °C until use.

2.2. Antimicrobial susceptibility testing

The MIC values of the antibiotics were determined by the NCCLS broth microdilution method. The MIC values for E. coli O159 were determined for the following antibiotics supplied by different commercial companies: ampicillin, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, streptomycin, tetracycline, trimethoprim and neomycin. MIC breakpoints were evaluated according to NCCLS guidelines [16].

2.3. Bacterial DNA preparation, PCR, and DNA sequencing

Preparation of bacterial DNA templates and the PCR conditions used for detection of class 1 integron were carried out as previously described [17], with some modifications. Briefly, 200 μl of overnight bacterial culture was mixed with 800 μl of distilled water and then boiled for 10 min. The resulting solution was centrifuged and the supernatant used as the DNA template. Class 1 integron primers (5′-CS and 3′-CS) [18], which amplify the region between the 5′-CS and the 3′-CS, were used. Amplification reactions were carried out with 10 μl of boiled bacterial suspensions, 250 μM deoxynucleoside triphosphate, 2.5 mM MgCl₂, 50 pmol of primers, and 1 U of AmpliTaq Gold DNA Polymerase (Applied Biosystems, Roche, NJ, USA). Distilled water was added to bring the final volume to 50 μl. The PCR cycle included an initial denaturation at 94 °C for 10 min, followed by 30 cycles of denaturation for 1 min at 94 °C, primer annealing for 1 min at 55 °C, extension for 2 min at 72 °C, and a final extension at 72 °C for 10 min. The reaction product was then analyzed by electrophoresis in 1.0% agarose gels stained with ethidium bromide and visualized under UV light. The PCR fragment was then purified from the agarose gel using a QIAquick gel extraction kit (Qiagen K. K., Japan). Both DNA strands of the entire integron segment were sequenced using an ABI automatic DNA sequencer (Model 373; Perkin–Elmer).

2.4. Plasmid isolation, preparation of probe, and Southern-blot hybridization

Plasmids were isolated from E. coli by the alkaline lysis method as described by Sambrook and Russell [19]. After agarose gel electrophoresis, DNA fragments in the gel were transferred onto Hybond-N⁺ nylon membranes (Amersham Biosciences Inc.) according to the manufacturer’s instructions. The DNA fragment containing the whole integron (2.5 kb) was amplified with PCR by using integron primers (5′-CS and 3′-CS), and was purified as described above. The purified fragment was labeled with alkaline phosphatase by using the AlkPhos Direct Labeling System (Amersham Bioscience, Inc.), and then used as a DNA probe. All hybridization steps were carried out according to the manufacturer’s protocols. The hybridization was performed at 55 °C for 12 h. The hybridized DNA was detected by using the CDP-Star chemiluminescent signal generation system (Amersham Bioscience, Inc.) according to the manufacturer’s instructions.

2.5. Computer analysis of the sequenced data

A similarity search was carried out using the BLAST program available at the NCBI BLAST homepage.
3. Results and discussion

3.1. Multidrug-resistance phenotypes and class 1 integrons

Class 1 integrons, located on plasmids and transposons, make up the majority of the integrons found in clinical isolates and these integrons are associated with the multidrug resistance (MDR) seen in hospital environments [21]. To examine the association between MDR and the presence of class 1 integrons, PCR was used to test 23 clinical isolates of \textit{E. coli} isolated from diarrheic patients. The resulting PCR product was visualized by agarose gel electrophoresis (data not shown), and the presence of a class 1 integron with an amplicon size of 2.5 kb was detected in only one isolate, ETEC O159. This strain showed MDR phenotypes against streptomycin (MIC 64 \(\mu\)g/ml), gentamicin (64 \(\mu\)g/ml), kanamycin (128 \(\mu\)g/ml), chloramphenicol (64 \(\mu\)g/ml), and ampicillin (128 \(\mu\)g/ml). On the other hand, it was susceptible to tetracycline (0.5 \(\mu\)g/ml), trimethoprim (0.5 \(\mu\)g/ml), neomycin (2 \(\mu\)g/ml), nalidixic acid (1 \(\mu\)g/ml) and ciprofloxacin (0.125 \(\mu\)g/ml). Sequencing of both DNA strands of the purified PCR fragment identified three gene cassettes inside the class 1 integron (Fig. 1). The first was a streptothricin acetyltransferase gene, \textit{sat}, which confers resistance to streptothricin. The second was an ORF whose product is a putative PSP, and the last was an aminoglycoside adenyltransferase gene, \textit{aadA2}, which confers resistance to streptomycin and spectinomycin. These results show independent association between the class 1 integron and the resistance to kanamycin, gentamicin, chloramphenicol and ampicillin in ETEC O159. Previous studies characterizing integrons in multidrug resistant clinical isolates of \textit{E. coli} showed that inserted gene cassettes predominantly confer resistance to trimethoprim and aminoglycosides, in addition to resistance to spectinomycin, chloramphenicol and erythromycin. Of note, the most prevalent gene cassettes confer resistance to the older aminoglycosides, such as streptomycin and kanamycin [21–24].

Southern-blot hybridization was used to determine the location of the class 1 integron in \textit{E. coli} O159. Many plasmids of different sizes were detected in \textit{E. coli} O159 (Fig. 2), but a positive hybridization signal was detected only in the largest plasmid (90 kb) band (Fig. 2).

3.2. Streptothricin acetyltransferase gene (\textit{sat}) and its significance

The first gene cassette in the class 1 integron of ETEC O159 is a streptothricin acetyltransferase gene, \textit{sat}, which confers resistance to streptothricin. This gene showed 94\% identity with a recently identified \textit{sat} gene associated with a class 1 integron in a single isolate of \textit{Shigella sonnei} isolated from western Ireland [25]. Resistance to streptothricin antibiotics has been reported in \textit{E. coli} containing a transferable plasmid mediating resistance following the use of nourseothricin as an antimicrobial feed additive for industrial animal farms in Germany [26]. This plasmid-containing strain was found to spread from pigs to pig farmers and their families, and was found in \textit{E. coli} from gut flora.

3.3. The putative phosphoserine phosphatase gene

3.3.1. A putative PSP gene in the class 1 integron

The second gene cassette in the class 1 integron is a putative PSP gene. Its protein product sequence was found to be 39\%, 38\%, 28\%, and 27\% identical to PSP gene products of \textit{Vibrio vulnificus} CMCP6 (Accession No. NP_763165), \textit{V. vulnificus} YJ016 (Accession No. BAC96116), \textit{Pseudomonas syringae} (Accession No. ZP_00128168), and \textit{P. aeruginosa} (Accession No. ZP_00140384), respectively. It is well known that the most notable gene cassettes identified within integrons are those conferring resistance to antibiotics. Only the...
super-integron of V. cholerae harbors a number of open reading frames (ORFs) with a variety of adaptive functions, including metabolic activities, virulence traits, in addition to potential antibiotic resistance determinants [14]. Super-integrons have also been identified recently in the genomes of diverse Gram-negative proteobacteria [27]. The detection and identification of a putative PSP, a metabolic enzyme not related to antibiotic resistance to streptomycin and spectinomycin. This gene contains the three conserved active catalytic domains of other phosphatases, and also shares other motifs with the PSP of M. jannaschii (Fig. 3), we predict that this PSP may play a role in signal transduction and chemotaxis of E. coli. Especially since this putative PSP is encoded by a plasmid, and this denotes that it is not an essential metabolic gene in E. coli O159.

3.3.2. The catalytic active domains of PSP

Previously, sequence analysis has shown that PSP belongs to the haloacid dehalogenase (HAD) superfamily of hydrolases, which comprises phosphatases, epoxide hydrolases, and L-2-haloacid dehalogenases [28]. Enzymes in this superfamily contain three highly conserved sequence motifs: motif I: DXDX [TV][LV]; motif II: [S/T]XX; and motif III: K-[G/S][D/S][X][X][D/N] [28,29]. These three conserved motifs play an important role in the mechanism of action of PSP [28]. In comparing of E. coli O159 PSP with that of M. jannaschii (Accession No. NP_248603), we found that both enzymes share these three highly conserved sequence motifs, in addition to other multiple conserved motifs (Fig. 3). This may indicate similarity in their functions.

3.4. Biological functions of PSP in bacteria

PSP shares homology with other protein phosphatases in the dephosphorylation processes that are associated with signal transduction in many bacterial species. For example, in B. subtilis PSPs, RsbU and RsbX, control the activity of the general stress transcription factor sigma B [8]. In addition, the catalytic domains and structures seen in the high-resolution crystal structure of PSP from M. jannaschii are similar to those seen in the structure of E. coli CheY [7,10], a response regulator of a two-component signal transduction pathway involved in bacterial chemotaxis [11]. These common properties suggest common reaction mechanisms [7,10]. As the PSP in the class 1 integron of ETEC O159 contains the three conserved active catalytic domains of other phosphatases, and also shares other motifs with the PSP of M. jannaschii (Fig. 3), we predict that this PSP may play a role in signal transduction and chemotaxis of E. coli. Especially since this putative PSP is encoded by a plasmid, and this denotes that it is not an essential metabolic gene in E. coli O159.

3.5. Aminoglycoside adenyltransferase gene, aadA2

The third ORF in the class 1 integron is the aminoglycoside adenyltransferase gene, aadA2, which confers resistance to streptomycin and spectinomycin. This gene shows 100% identity with the recently detected plasmid mediated aadA2 in class 1 integrons of diverse bacterial species, such as clinical isolates of E. coli from China [30], Aeromonas salmonicida isolated in Japan [31] and Corynebacterium glutamicum [32].

4. Conclusion

In this report, we recorded the first instance of the presence of a putative metabolic enzyme, PSP, in a class 1 integron that is not related to antibiotic resistance mechanisms. Our results support the notion that the
recently discovered super-integrons are likely the ancestors of other integrons.

## 5. Nucleotide sequence accession numbers

The nucleotide sequences of ETEC O159 class 1 integron, including sat, PSP, and aadA2 genes, have been assigned the DDBJ/GenBank/EMBL Accession No. AB121039.

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## References


