Original article

Polymorphism of gene cassette promoter variants of class 1 integron harbored in S. Choleraesuis and Typhimurium isolated from Taiwan

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ABSTRACT

Integrons, mobile genetic units, capture and incorporate antibiotic resistance gene cassette by site-specific recombination. Class 1 integrons are widespread and associated with dispersion of antibiotic resistance among Gram-negative bacteria. The expression of gene cassette in Class 1 can vary, based on the Pc promoter but seldom from another promoter downstream of Pc, called P2. To probe distribution and prevalence of gene cassette promoter variants, we analyzed 169 S. Choleraesuis and 191 S. Typhimurium isolates from humans and animals, finding 95.27% occurrence of integron among S. Choleraesuis, 83.25% among S. Typhimurium. PCR-RFLP analysis identified four promoters (PcS+P2, PcW TGN-10+P2, PcH1+P2, and PcW TGN-10+P2-GGG) in said integron-positive isolates; major types in S. Choleraesuis and S. Typhimurium were PcS+P2 and PcW TGN-10+P2, respectively. Likewise, β-galactosidase assay rated promoter strength of variants by transcriptional fusion constructs to show extended -10 promoter (TGN/-10 promoter) in Pc and three-nucleotide insertion (GGG) between -35 and -10 region of P2 improving promoter strength of gene cassette.

1. Introduction

Salmonellosis ranks among the most common bacterial infections worldwide [1]. Salmonella species are rod-shaped, aerobic, and Gram-negative bacteria, all major food-borne pathogens in the world [2]. Until 2004, over 2,500 Salmonella serotypes were identified [3]. Among these serovars, Salmonella enterica serovar Choleraesuis and Typhimurium are common non-typhoidal serotypes that pose global concern [4,5]. The USA diagnoses over 4 million cases of Salmonella infection per annum [6], about 500 fatal [2,7]. While mild and self-limited in adults, salmonellosis can require drugs, especially antibiotics, to treat infant, elderly, or immunocomprised patients [8]. Abuse of antibiotics in many locales nowadays spurs development of resistant strains. Studies show ever more multidrug resistance by Salmonella, causing serious public health hazards [5,9,10]. Such mechanisms entail obtaining genes or point mutation in genomes [11], resistance dispersed by [1] clonal expansion of drug-resistant strains or [2] horizontal transfer of determinants. Multidrug resistant genes transmitted between human and animal pathogens [12] mean mobile genetic elements playing a key role in dispersion of drug resistance among bacterial population [13-16].

Plasmids, transposons, and integrons are well known mobile genetic elements that mediate drug resistance genes disseminating via horizontal or vertical transfer [2]. Quantity of integron research has grown recently, with five classes of identified by sequences of integrases. Class 1 is most prevalent and closely linked with multidrug resistance in Gram-negative bacteria [11,17,18]. Typical Class 1 integron consists of intI gene encoding integrase, recombination specific site attI, major promoter Pc, and gene cassettes [18-21]. Over 100 gene cassettes harbored in Class 1 integron have been identified [22]; Pc is thought responsible for expression of gene cassettes [23]. Several Pc variants are described based on strength [24]: PcS for “strong”, PcW for “weak”, PcH1 for Hybrid 1, and PcH2 for Hybrid 2, the last two containing -35 and -10 hexamers raising transcription efficacy of σ70 promoters in E. coli [24,25]. Occasionally, Pc combines with a second promoter designated P2, located 119 bp downstream of Pc in 10% of Class 1 integrons [24,26,27]. A rare P2 type was described by Tenover [28] and Tae-Eun Kim [29]: three G residue insertion optimizes

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spacing (17 bp) between potential -35 and -10 hexamer sequences. Strength of four Pc types has been detailed in previous studies [24,30-32], several of which show a great polymorphism among variant Pc-P2 combinations. To evaluate dissemination of integron-driven drug resistance, this study examined 360 Salmonella isolates for prevalence of Pc variants and strengths of Pc-P2 variant combinations in Taiwan.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Salmonella isolates in this study had been described in previous report [5]. A total of 360 Salmonella isolates (169 S. Choleraesuis and 191 S. Typhimurium) were amassed from human and animal hosts. For serovar identification of Salmonella enterica, antisera of O and H antigen detection were purchased from Denka Seiken Co., Ltd. in Japan and S&A Reagents Lab Limited in Thailand, respectively. Analysis based on the Kaufmann-White scheme and protocols for serotyping established by the Centers for Disease Control and Prevention in Atlanta, GA [33]. Salmonella isolates were maintained in 25 % frozen glycerol stock and inoculated on Salmonella-Shigella (SS) agar (Difco, USA) at 37 °C.

2.2. PCR detection of Class 1 integrons in Salmonella isolates

All Salmonella isolates were probed for integrons by polymerase chain reaction (PCR) and nucleotide sequencing. After culturing bacteria in Luria-Bertani (LB) broth to log-stationary phase at 37 °C with vigorous shaking, genomic DNA were extracted from isolates, as per manufacturer’s instructions for Tissue & Cell Genomic DNA Purification Kit (Genemark, Taiwan). Specific primers IntegronA and IntegronB [34] (Table 1) screened intI1, Class 1 integrase gene, within bacterial isolates. PCR mixture was in a total volume of 25 μl containing 3 μl genomic DNA as template, 1 μl of each primer (10 μM), 5 μl of 5x PCR Plus Master Mix II solution (Genemark, Taiwan), and 15 μl of distilled water. PCR mixture used T1 Thermocycler (Biometra, USA). Template was initially denatured at 95 °C for 5 min followed by 35 cycles at 95 °C for 30 sec, 57°C for 30 sec, and 72°C for 30 sec. Final extension for 10 min was done at 72 °C, PCR products confirmed via 1 % agarose gel electrophoresis.

2.3. Characterization of gene cassette promoter (Pc-P2) variants in Salmonella isolates by PCR-Restriction fragment length polymorphism (PCR-RFLP)

To ferret out promoter variants of Class 1 integrons in Salmonella intI1-positive isolates, PCR-RFLP method served for analysis: 330-bp fragment of Pc-P2 regions in Class 1 integrons amplified by PCR with SC-RGA-F1 and SC-RGA-R specific primers (Table 1). Preparation of PCR mixture as detailed above proceeded as follows: after initial denaturation (5 min at 95°C), DNA fragment was amplified for 35 cycles of 30 sec at 95°C, annealing at 60°C for 30 sec, and 30 sec for extension at 72°C, with a final extension step at 72°C for 7 min. Before performing RFLP, all PCR products were purified by PCR clean-up kit (Genemark, Taiwan). To screen promoter variants, HincII or AluI restriction enzymes identified Pc variants; BerGII restriction enzyme was also applied to analyze three nucleotide insertions between -35 and -10 region of P2. Each digestion reaction containing 2 μl of 10X NEBuffer 4, 0.2 μl of 100X BSA, 1 μl of restriction enzyme (10U; New England BioLabs, Inc.), 10 μl of purified PCR products, and added distilled water to 20 μl. Mixture was incubated at 37°C for 6 hrs, after which treatments were analyzed on 1.5 % agarose gel electrophoresis.

2.4. Plasmid constructions for promoter activity assay

After characterizing types of gene cassette promoter, one bacterial strain stood for each type was picked randomly from Salmonella intI1-positive isolates. To study relative strength of a gene cassette promoter, transcriptional fusion with both Pc and P2 were cloned into the promoterless lacZ gene upstream in a reporter vector (pCB267, [35]). Extracting genomic DNA from bacterial strains representing promoter types, we amplified Pc-P2 region by specific SC-RGA-F1 and SC-RGA-R primers (Table 1). To gauge effect of three nucleotide insertions in -35 and -10 region of P2 on promoter strength, only P2 region with or without insertion was amplified by SC-RGA-F2 and SC-RGA-R primer pairs (Table 1). PCR were run for 5 min at 95°C followed by 35 cycles of 30 sec at 95°C, 30 sec at 60°C, 30 sec at 72°C and final extension of 15 min at 72°C. PCR products purified by kit (Genemark, Taiwan) were digested with BamH I and HindIII, then ligated to BamH I- and HindIII-digested pCB267. These constructs could assess strength of promoters.

Table 1 - Sequences of oligonucleotide primers used in this study.

| Name | Sequence (5’-3’) | Product size (bp) | References |
|------|-----------------|------------------|------------|
| IntegronA | GCCTTGCTGTTCCTTCTACGG | 558 | [34] |
| IntegronB | GATGCCCTCCTGTTCCTCAGG | | |
| SC-RGA-F1 | ATGGATCCATCCGGTACGCGCAACCCGTGGAAACGGAT | 328 | This study |
| SC-RGA-F2 | ATGGATCCACCTCCTGACGCGCAACCCGTGGAAACGGAT | 218 | |
| SC-RGA-R | ATTAAGCTTTGAGTTCATATGGCTAACTTTGTTTT | | |

a. IntegronA and IntegronB primers detected Class I integron. SC-RGA-F1 and SC-RGA-R primers for Pc-P2 region in integrons (328 bp); SC-RGA-F2 and SC-RGA-R for only P2 region in integron (218 bp).

b. Nucleotide sequences recognized by BamH I restriction enzyme underlined

c. Nucleotide sequences recognized by HindIII restriction enzyme underlined
3.1. Detection of Class 1 integrons among Salmonella isolates

All isolates in this study were characterized in previous work [5], including the Class 1 integron presence and antimicrobial resistance patterns. A total of 360 isolates, belonging to either S. Typhimurium or S. Choleraesuis, were collected from humans and animals during 1997-2009 in Taiwan. Among S. Choleraesuis isolates, 111 are from pigs and 50 from humans; among S. Typhimurium isolates, 17 are from pigs, 115 from humans, 1 from pigeons, 9 from turtles, 12 from chickens, 1 from snakes, 4 from ducks. Using PCR, we showed 95.27% (161/169) of S. Choleraesuis and 83.25% (159/191) of S. Typhimurium isolates harbor Class 1 integron (Table 2).

3.2. Molecular characterization of gene cassettes’ common promoters in intI1-positive Salmonella isolates

Pc is the major promoter located upstream of gene cassettes in Class 1 integron. Occasionally, a second promoter (P2) is located 119 bp downstream of Pc. Based on nucleotide sequence of -35 and -10 Pc regions, it can be classified into strong, hybrid, and weak types [24,27,29,32]. By driving the expression of CAT reporter with Pc variants, relative strengths of PcS and PcW + P2 to PcW were measured, with PcS and PcW + P2 promoters about 30- and 15-fold higher than PcW, respectively [24,31].

PCR-RFLP characterized Pc-P2 variants. Promoter regions were amplified by primers SC-RGA-F1 and SC-RGA-R (Table 1), then amplicons subjected to enzyme digestion. The -35 region of PcS (TTGACA) and -10 region of PcW (TAAGCT) were digested by HincII and AluI, respectively. Digestion of fragment containing -35 region of PcS with HincII would yield two fragments, 277bp and 51 bp in length; digestion of fragment containing -10 region of PcW with AluI would produce 252bp and 76 bp fragments. Those not fitting this restriction enzyme digestion pattern were subject to sequence and classified as PcW-TGNN-10. Three guanine (GGG) insertions between -35 and -10 region of P2 were studied to create a new promoter (P2-GGG) occasionally found in Class 1 integrons [26]. For quicker P2

| Promoter Type | Variant | -35 Region | Spacing | -10 Region |
|---------------|---------|------------|---------|-----------|
|               |         | Sequence   | HincII (GTY/RAC) | No. of nt | N14-TCN (TGN) | Sequence | AluI (AG/CYT) |
| Pc            | PcS     | TTAGACA    | +        | 17        | TCN         | TAAACT   | -          |
|               | PcW-TGNN-10 | TGGACA  | -        | 17        | TGN         | TAAGCT   | +          |
|               | PcH1    | TGGACA     | -        | 17        | TCN         | TAAGCT   | -          |

| Promoter Type | Variant | -35 Region | Spacing | -10 Region |
|---------------|---------|------------|---------|-----------|
|               |         | Sequence   | No. of nt | 3-nt-insertion | BglII (T/GTACAT) | Sequence |
| Pc            | P2      | TTTGTTA    | 14       | -          | +            | TACAGT   |
| P2            | P2-GGG  | TTTGTTA    | 17       | +          | -            | TACAGT   |
characterization, PCR amplified fragments were digested by BsrG1. P2-GGG promoter could not be digested, but P2 promoter yielded fragments (188 and 140 bp) after digestion. Not only PCR-RFLP analysis but also DNA sequence was applied to variants, confirming promoters’ molecular characterization. Major Pc-P2 variants identified (Table 2) were PcS+P2, PcW\textsubscript{TGN-10}+P2, PcH1+P2, and PcW\textsubscript{TGN-10}+P2-GGG. PcS+P2 was the major variant in S. Choleraesuis (143/161, 88.82%) and PcW\textsubscript{TGN-10}+P2 for S. Typhimurium (131/159, 82.39%). Surprisingly, multiple variants appeared in PcS + P2/PcH1 + P2/PcW\textsubscript{TGN-10} + P2 combination in S. Choleraesuis and PcW\textsubscript{TGN-10} + P2/PcS + P2/PcW\textsubscript{TGN-10} + P2-GGG combination in S. Typhimurium. Of six isolates, two of S. Choleraesuis and four of S. Typhimurium harbored more than one Pc-P2 combination in one strain (Table 3).

### Table 3 - Occurrence of each promoter variants detected in this study.

| Serotype       | Total no. | No. of integrons (%) | Promoter variant (occurrence, %) |
|----------------|-----------|----------------------|----------------------------------|
| S. Choleraeuis | 169       | 161 (95.27 %)        | PcS + P2 (143/161, 88.82 %)      |
|                |           |                      | PcH1 + P2 (16/161, 9.94 %)       |
|                |           |                      | PcS + P2/PcH1 + P2/PcW\textsubscript{TGN-10} + P2 (2/161, 1.24 %) |
| S. Typhimurium | 191       | 159 (83.25 %)        | PcW\textsubscript{TGN-10} + P2 (131/159, 82.39 %) |
|                |           |                      | PcS + P2 (15/159, 9.43 %)        |
|                |           |                      | PcW\textsubscript{TGN-10} + P2-GGG (9/159, 5.66 %) |
|                |           |                      | PcW\textsubscript{TGN-10} + P2/PcS + P2/PcW\textsubscript{TGN-10} + P2-GGG (4/159, 5.52 %) |

#### 3.3. Relative strength of gene cassette promoter variants

To rate promoter strength, Pc-P2 each variant identified was cloned into a promoterless vector (pCB267) to drive lac\textsubscript{Z} reporter gene expression. β-galactosidase activities were compared between variants; PcS+P2 proved strongest (Fig. 1). Strength of PcW\textsubscript{TGN-10} + P2 was 31% greater than PcH1+P2. In prior studies, strength of hybrid Pc had intermediate activity between PcS and PcW [24]. Promoter activity of PcW rises with PcW carrying TGN-10 motif between -35 and -10 region (PcW\textsubscript{TGN-10}). Strength of P2-GGG region alone was 4.5-fold that of P2 without insertion. Our data concurred with earlier studies [28, 29]: promoter strength of PcW\textsubscript{TGN-10} combined with P2-GGG was about 2-fold that of PcW\textsubscript{TGN-10} combined with P2. Therefore, 3-G insertion can enhance promoter strength in general.

![Graph showing LacZ activity](image)

**Fig. 1 - Strengths of Pc-P2 variants.** Promoter strengths of Pc-P2 combination and P2 variants were estimated by β-galactosidase activity. At least three independent assays were performed for each construct and in each experiment. Bars indicate standard error of the mean.
4. Discussion

S. Typhimurium DT104, a bacterial strain isolated during the early 1980s in the United Kingdom, is resistant to multiple antibiotics: e.g., ampicillin, chloramphenicol, streptomycin, sulfonamides, tetracycline (ACSSuT). After that, multi-drug resistance to ACSSuT is a common Salmonella trait [5, 39-41]. ACSSuT resistance genes are mostly disseminated through Class 1 integron [5]. In our study, 88.9% of Salmonella isolates harbored Class 1 integron in S. Choleraesuis (95.27%) was higher than in S. Typhimurium (83.25%), suggesting Class 1 integron already widespread in Taiwan.

PCR-RFLP analysis identified four Pc-P2 combinations: Pc-S+P2, PcW TGN-10>P2, PcH1+P2, and PcW TGN-10>P2-GGG, the first two predominant in S. Choleraesuis and S. Typhimurium, respectively. These variants in our study are also the most prevalent forms in silico study [24]. Based on previous research on gene cassettes in Class 1 integron [5], for isolates carrying more than one set of Pc-P2 combination, some only carry one kind of gene cassette. Data portend more than one copy of Class 1 integrons in one strain, albeit with variant promoter combination.

Transcriptional fusion constructs were used to monitor promoter strength among Pc-P2 variants. Our data agreed with prior study that strengths of promoter variants is PcS > PcS+P2, PcW TGN-10>P2, PcH1+P2, and PcW TGN-10>P2-GGG, showing more than one set of Pc-P2 combination, some only carry one kind of gene cassette. Data portend more than one copy of Class 1 integrons in one strain, albeit with variant promoter combination.

Promoter polymorphism might alter levels of expression. Promoter polymorphism might alter levels of antibiotic resistance in response to environmental stress.

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Declaration of Interest: Authors declare no conflicts of interest for this work.

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