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The Lengths of attI Sites Have No Effect on the Transcription and Expression of the Cassette and Integrase Genes

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Abstract: Class 1 integron is a genetic element with the ability to capture and express of the exogenous drug-resistance genes embedded in gene cassettes, offering bacteria with multi-drug resistance. The structures of the integron itself can regulate the transcription and express levels of the cassette genes. In this study, the effects of the attI sites on the transcription and express of the cassette and integrase genes were reported. pACYC184 plasmids, containing cloned integron fragments which differed only with respect to the lengths of attI sites were transferred into *E. coli* JM109. Then, the transcription and expression levels of the integrase and the first cassette gene after Pc promoter were detected in the corresponding recombinant strains. The results showed that the lengths of attI sites had no effect on the transcription and expression of the gene cassettes and integrase genes. This research was reported for the first time.

Key words: Class 1 integron, attI site, transcription and expression, gene cassette, integrase gene

INTRODUCTION

Currently, antibiotics are widely used all over the world, resulting in the emergence of multidrug-resistant strains. Bacteria acquire exogenous resistance genes through horizontal gene transfer which is one reason for the accelerated emergence of drug-resistant clinical strains. Genetic structures closely associated with the horizontal transfer of bacterial resistance genes include plasmids, transposons, integrative bacteriophages and integrons which have been discovered in recent years (Hall and Collis, 1995). Integrons capture exogenous genes and convert them into functional gene expression units. Using horizontal transfer catalyzed by integrase, integrons capture exogenous resistance genes and cause them to be expressed, thus conferring antibiotic resistance on pathogens. Integrons can transfer resistance-gene cassettes by themselves or plasmids and transposons, resulting in the spread of resistance genes (Collis and Hall, 1992; Di Conza and Gutkind, 2010; Gravel *et al*., 1998). Integrons can be divided into four classes, according to the different amino acid sequences of the associated integrases (Mazel, 2006). Class 1 integrons are the predominant integrons currently found in bacteria, so research into the drug resistance mechanisms of integron gene cassettes has focused on them.

Integrons consist of a 5' Conserved Segment (5'CS) and a 3' Conserved Segment (3'CS) with a Variable region (VS) between them. The 5'CS is the basic structure of the integron and includes a gene encoding integrase (intI), a gene recombination site (attI) and a variable-region promoter Pc (Rodriguez *et al*., 2006). IntI catalyzes the integration of the gene cassette between the integron recombination site, attI and the gene cassette recombination site, attC. It has been reported that intI expression is controlled by the SOS response (Fluit and Schmitz, 2004; Guerin *et al*., 2011), so that the insertion of gene cassettes is directional and each newly inserted gene cassette causes the genes after the attI site to shift in turn. The variable region is composed of zero or several inserted foreign gene cassettes and each gene cassette consists of a structural gene and attC (also called a "59-base element") and the length of the attC site varies from 57-141 bp. The 3'CS varies across the different classes of integrons and is missing in some integrons (Partridge *et al*., 2009).

The transcription and expression of gene cassettes must be driven by Pc promoters because the cassettes themselves usually do not have a promoter (Collis and Hall, 1995). Many factors including the strengths of Pc and P2 promoters (Guerin *et al*., 2011; Wei *et al*., 2011), the distances between a Pc promoter and a given cassette

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gene (Collis and Hall, 1995), the copy numbers of cassette genes (Szczepanowski *et al*., 2004), the structures of attC (Jacquier *et al*., 2009) and so on can regulate the transcription and expression levels of the cassettes genes. The varying strength of each individual promoter is the most critical determining factor.

According to the previous report when multiple cassettes were present, the expression level was highest when the gene was in the first cassette after the Pc promoter, i.e., closest to Pc (Collis and Hall, 1995). The attI site is between Pc promoter and the first gene cassette and the lengths of attI sites are variable. It means that the distances between a Pc promoter and the first cassette gene are different. Until now, the issue of whether the length of attI site affects the transcription and expression of gene cassettes has not been published. In this study, pACYC184 plasmids containing cloned integron fragments which differ only with respect to the lengths of attI sites were used to examine the transcription and expression levels of integron-associated cassette genes and the integrase genes. It is the first report about this concern.

MATERIALS AND METHODS

Bacterial strains, plasmids and positive sera: The 88 field strains of *Escherichia coli* (*E. coli*) and *Riemerella anapestifer* (RA) were isolated from pigs and ducks in March 2014. *E. coli* DH5α and JM109 and pMD 19-T vector (simple) were purchased from TaKaRa (Dalian, China). The plasmid pACYC184 was kept at our laboratory. Strains were grown in nutrient broth (LB) or on 1.5% agar supplemented plate when required with ampicillin (100 μg mL^{-1}) or tetracycline (30 μg mL^{-1}).

The anti-aadA2 serum, anti-integrase serum and anti-tetracycline serum were prepared by inoculation of rabbits using the corresponding protein expressed in *E. coli*.

PCR amplification of integron-gene cassette: The complete integron-gene cassette sequences containing integrase gene, attI site, cassette genes and attC sites were amplified using the primers IntF (5' CCA TGG CTA CCT CTC ACT AGT GAG 3') and 3CS (5' AGT ACT AAG CAG ACT TGA CCT GA 3') from the field strains of *E. coli* and RA. Underlined letters indicated NcoI and ScaI restriction sites, respectively. The PCR products were purified and sequenced and five integron-gene cassettes including Int-aadA2, Int-aadA5, Int-aacA4-aadA1, Int-dfrA12-orfF-aadA2 and Int-dfrA17-aadA5 were amplified. The sequences of Pc promoters and attI sites of the above five integron-gene cassettes were showed in Fig. 1. These research were previously performed.

As shown in Fig. 1, four Pc promoters named PcS (strong), PcW (weak), PcH1 (hybrid 1) and PcH2 (hybrid 2) and four attI sites (145, 144, 157 and 196 bp) were found in the five integron-gene cassettes. Int-aadA2 and Int-aadA5 had the same attI of 145 bp. Int-dfrA12-orfF-aadA2, Int-dfrA17-aadA5 and Int-aacA4-aadA1 had the attI of 144, 157 and 196 bp, respectively.

Design and synthesis of integron-gene cassette carrying different attI sites: The integron-gene cassette Int-aadA2 naturally carried PcS promoter and the attI of 145 bp. Based on this, the attI site (145 bp) of Int-aadA2 was replaced with those of Int-dfrA12-orfF-aadA2 (144 bp), Int-dfrA17-aadA5 (157 bp) and Int-aacA4-aadA1 (196 bp),

Fig. 1: Four Pc promoters and four attI sites found in the five integron-gene cassettes

respectively to produce new DNA fragments with the same integrase genes, PcS promoters, *aadA2* gene cassettes and the attC sites naturally carried by Int-aadA2. The new gene fragments were synthesized by Nanjing GenScript Company (Nanjing China). The four DNA fragments carrying different attI sites were named Int-aadA2-144, Int-aadA2-145, Int-aadA2-157 and Int-aadA2-196 and cloned into pUC-57 (simple) vector, respectively. The positive plasmids named pUC-Int-aadA2-144, pUC-Int-aadA2-145, pUC-Int-aadA2-157 and pUC-Int-aadA2-196 were sequenced to ensure correct insertion.

Construction of JM/pAC-Int-aadA2-144, JM/pAC-Int-aadA2-145, JM/pAC-Int-aadA2-157 and JM/pAC-Int-aadA2-196 stains: The plasmids of pACYC184, pUC-Int-aadA2-144, pUC-Int-aadA2-145, pUC-Int-aadA2-157and pUC-Int-aadA2-196 were each digested with ScaI at 37°C for 4 h. The fragments resulting from the digestion of pACYC184 plasmid and the other four plasmids were approximately 4200 and 3000 bp, respectively. The DNA fragments purified using an agarose gel DNA purification kit were digested with NcoI at 37°C for 2 h. Subsequently, the approximately 4100 bp fragments resulting from the digestion of pACYC184 fragments and the approximately 2100 bp fragments resulting from the digestion of pUC-Int-aadA2-144, pUC-Int-aadA2-145, pUC-Int-aadA2-157and pUC-Int-aadA2-196 fragments were purified again. The purified fragments of Int-aadA2-144, Int-aadA2-145, Int-aadA2-157 and Int-aadA2-196 were connected with the purified fragments of pACYC184, respectively and the mixtures were transformed into *E. coli* JM109 competent cells. The cells were grown on LB plates with tetracycline (30 μg mL^{-1}) at 37°C overnight. Then, the single clones were cultured in LB broth with tetracycline (30 μg mL^{-1}) at 37°C with 200 rpm overnight. The clones were identified by PCR using the primers IntF and 3CS and the PCR products were sequenced. The positive plasmids were named pAC-Int-aadA2-145, pAC-Int-aadA2-157 and pAC-Int-aadA2-196, respectively and the corresponding positive strains were named JM/pAC-Int-aadA2-144, JM/pAC-Int-aadA2-145, JM/pAC-Int-aadA2-157 and JM/pAC-Int-aadA2-196.

Primers of quantitative real-time PCR: Quantitative real-time PCR primers for amplification of the aadA2, integrase and tetracycline gene fragments were listed in Table 1.

RNA extraction and reverse transcription: The strain JM/pAC-Int-aadA2-145 was grown to an optical density at 600 nm (OD_{600}) of 0.5~1.0 and total RNA was extracted, treated with DNase I and purified from 10 mL of the culture using the Bacterial RNA kit (Omega, USA), according to the manufacturer's instructions. The quality and quantity of RNA were measured according to the values of OD_{260} and OD_{280} obtained from the RNA sample. Total RNA was reversed-transcribed using random primers with Transcriptor First Strand cDNA Synthesis kit (Roche, USA) in a final volume of 100 μL. The cDNAs were stored at -70°C until used. Other cDNA samples from the strains JM/pAC-Int-aadA2-144, JM/pAC-Int-aadA2-157 and JM/pAC-Int-aadA2-196 were prepared in the same manner as JM/pAC-Int-aadA2-145.

Quantitative real-time PCR to determine the relative transcription level of aadA2 and integrase genes: Using the cDNA (diluted one hundred times) from JM/pAC-Int-aadA2-145 stains as templates, quantitative real-time PCR was performed to measure the transcription of the aadA2, integrase and tetracycline genes using the corresponding primers (Table 1). Each PCR was performed in 50 μL reactions containing 1×SYBR Preminx Ex Taq (TaKaRa, Japan), 4 μL cDNA and 0.3 μM each of the sense primer and reverse primer. The thermal cycling conditions were shown in Table 1. A positive control (DNAs) and a negative control (distilled water) were included in each run. For each cDNA sample, the Cycle Threshold (CT) values of each gene transcription were obtained in triplicate and the mean of these values was used for further analysis.

As the same way, the CT values of aadA2, integrase and tetracycline genes were measured using the cDNA from JM/pAC-Int-aadA2-144, JM/pAC-Int-aadA2-157 and JM/pAC-Int-aadA2-196 as templates, respectively.

The relative quantification of aadA2 and integrase genes at the transcriptional level in each clone was

Table 1: Primers for quantitative real-time PCR assay

Primers	Primers sequences (5'-3')	Corresponding gene	Length of amplified fragments (bp)	Amplification procedure
aadA2-F	CATTGAGCGCCATCTGGAATC	aadA2	216	94°C for 4 min followed by 40 cycles of 94°C for 15 sec, 60°C for 30 sec and 72°C for 15 sec
aadA2-R	ACAAGGGTGACTTCTATAGCG			
INT-F	AGTTGCAAACCCTCACTGATC	Integrase	185	
INT-R	AAGGTGCTGTGCACGGATCTG			
Tet-F	ATAGGCTTGGTTATGCCGGTA	Tetracycline	183	
Tet-R	AAGTAGCGAAGCGAGCAGGA			

expressed by $2^{-\Delta CT}$ corresponding to the differences in CT values between the corresponding gene and the internal reference tetracycline gene.

The relative expression level of aadA2 and integrase genes: The 1 mL cultures of JM/pAC-Int-aadA2-144, JM/pAC-Int-aaA2-145, JM/pAC-Int-aadA2-157 and JM/pAC-Int-aadA2-196 strains were centrifuged, respectively and the cell pellets were resuspended in 100 μL PBS followed by the addition of 100 μL of 2×loading buffer. The samples boiled for 10 min were seperated by 15% SDS-PAGE eletrophoresis and transfered to PVDF membrane. Subsequentlly, Western blot assay was performed. The anti-aadA2 serum, anti-integrase serum and anti-tetracycline serum were combined to the corresponding proteins, respectively. Then, conjugated-HRP goat-anti-rabbit antibodies were combined to the positive sera. At last, aadA2, integrase and tetracycline proteins were detected by ECL Method. The expression levels of aadA2 and integrase gene relative to the internal control tetracycline gene were detected by optical density analysis.

Statistical analysis: The data of relative transcription levels of aadA2 and integrase genes based on quantitative real-time PCR assay were analyzed using SPSS Software (Version 15.0). The t-test was used to compare the differences among the strains.

RESULTS

Design and synthesis of integron-gene cassette carrying different attI sites: According to the sequences of the five integron-gene cassettes, Int-aadA2 and Int-aadA5 had the same attI sites of 145 bp in size and the attI sites of Int-dfrA12-orfF-aadA2, Int-dfrA17-aadA5 and Int-aacA4-aadA1 were 144, 157 and 196 bp, respectively. Based on the four attI sites, four DNA fragments (Int-aadA2-144, Int-aadA2-145, Int-aadA2-157 and Int-aadA2-196) carrying the same integrase gene, PcS promoter, *aadA2* gene cassette and attC site were determined except for the substitutions of the attI sites. The same components of the DNA fragments were naturally carried by Int-aadA2. Int-aadA2-145 fragments, namely Int-aadA2, naturally carried the attI site of 145 bp. Then, the sequences of 145 bp were replaced with the attI site sequences of 144, 157 and 196 bp carried by Int-dfrA12-orfF-aadA2, Int-dfrA17-aadA5 and Int-aacA4-aadA1 to produce Int-aadA2-144, Int-aadA2-157 and Int-aadA2-196 fragments, respectively. The four fragments were cloned into pUC-57 vector (sample) and sequenced. The results indicated that the DNA sequences were correct as expected.

Construction of JM/pAC-Int-aadA2-144, JM/pAC-Int-aadA2-145, JM/pAC-Int-aadA2-157 and JM/pAC-Int-aadA2-196 stains: By PCR amplification and sequencing, three DNA fragments were successfully cloned into pACYC184 vector with NcoI and ScaI restriction enzymes. The positive strains of JM/pAC-Int-aadA2-144, JM/pAC-Int-aadA2-145, JM/pAC-Int-aadA2-157 and JM/pAC-Int-aadA2-196 carrying the corresponding positive plasmids were successfully constructed.

RNA extraction and reverse transcription: The values of OD_{260}/OD_{280} obtained from the RNA samples were between 1.82 and 2.02 indicating that the quality of RNA samples was high. High quality cDNA samples were successfully obtained from the strains JM/pAC-Int-aadA2-144, JM/pAC-Int-aadA2-157 and JM/pAC-Int-aadA2-196, respectively.

Quantitative real-time PCR to determine the relative transcription level of aadA2 and integrase genes: The amplification efficiencies of aadA2, integrase and tetracycline genes were very close. Therefore, the relative transcription levels of aadA2 and integrase genes, relative to the internal control tetracycline gene could be calculated by $2^{-\Delta CT}$ in each corresponding clone of the JM/pAC-Int-aaaA2-144, JM/pAC-Int-aaaA2-145, JM/pAC-Int-aadA2-157 and JM/pAC-Int-aadA2-196 strains. The relative transcription levels of the aadA2 and integrase genes had no differences in the four strains. The results were shown in Fig. 2.

The relative expression level of aadA2 and integrase genes: Western blot and optical density analysis showed

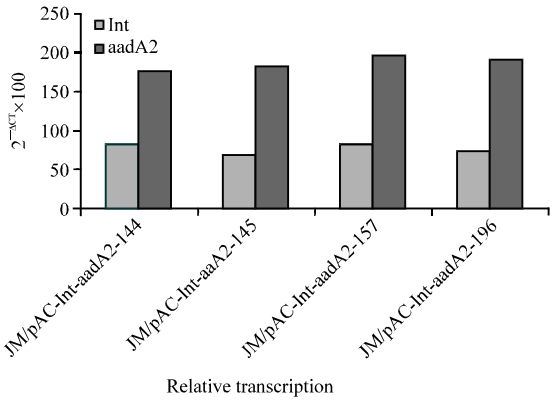

Fig. 2: The relative transcription levels of the aadA2 and integrase genes in the JM/pAC-Int-aaaA2-144, JM/pAC-Int-aaaA2-145, JM/pAC-Int-aadA2-157 and JM/pAC-Int-aadA2-196 strains. Int and aadA2 represented the integrase and *aadA2* genes, respectively

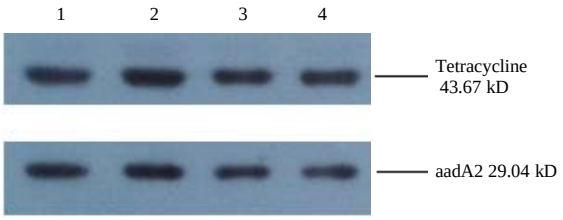

Fig. 3: The expression levels of aadA2 and tetracycline genes in the four strains. Lanes 1, 2, 3 and 4 represented the samples derived from the JM/pAC-Int-aaaA2-144, JM/pAC-Int-aaaA2-145, JM/pAC-Int-aadA2-157 and JM/pAC-Int-aadA2-196 strains, respectively

that the expression levels of *aadA2* gene relative to the internal control tetracycline gene had no difference in JM/pAC-Int-aaaA2-144, JM/pAC-Int-aaaA2-145, JM/pAC-Int-aadA2-157 and JM/pAC-Int-aadA2-196 strains as shown in Fig. 3. The band corresponding to integrase was not detected.

Statistical analysis: The relative transcription and expression levels of the aadA2 and integrase genes did not display significant differences ($p>0.05$) in JM/pAC-Int-aaaA2-144, JM/pAC-Int-aadA2-145, JM/pAC-Int-aadA2-157 and JM/pAC-Int-aadA2-196 strains.

DISCUSSION

Integrons play a major role in mediation of the horizontal transfer of resistance genes, particularly among Gram-negative bacteria (Boucher *et al*., 2007; Cambray *et al*., 2010). Integrons have three essential core features of intI, attI and promoter Pc. The expression of integron-associated gene cassettes rely on Pc promoter because they often only contain an open reading frame and an attC recombination site (Collis and Hall, 1995). So, Pc promoter has significant influence on the transcription and expression levels of cassette genes, the stronger the Pc, the higher the transcription and expression levels of the cassette genes (Guerin *et al*., 2011; Wei *et al*., 2011). However, internal promoters can allow the transcription and expression of some cassette genes such as the cmlA chloramphenicol resistance gene and the quinolone resistance genes of the qnrVC family (Stokes and Hall, 1989; Da Fonseca and Vicente, 2012).

Other factors including attC site, Shine Dalgarno (SD) sequences, intrinsic short ORF (called ORF-11) present in the 5CS, the copies of the cassette genes and so on have effects on the transcription and expression levels of cassette genes (Hanau-Bercot *et al*., 2002; Szczepanowski *et al*., 2004; Jacquier *et al*., 2009).

It is worth mentioning that the distance between Pc and gene cassettes can also affect the expression of genes. The expression level of the cassette gene is highest when the gene is closest to Pc promoter. The strength of expression drops off as cassettes become more distal to the promoter (Collis and Hall, 1992). The attI site is between Pc promoter and the first cassette. Thus, the distances between a Pc promoter and the first gene cassette are different if the attI sites have various lengths. The effects of the attI sites on the transcription and expression levels of the integron-associated gene cassette are not been reported until now.

Three attI sites with different nucleotides in size were found in our previous research. In this study, the effects of the three attI sites on the transcription and expression levels of the first gene cassette and the integrase gene were researched. The artificially constructed integrons were individually cloned into a pACYC184 vector, respectively. A chloramphenicol gene carried by the pACYC184 plasmid was destroyed and could not be expressed because of insertions of the constructed integrons. Hence, the tetracycline gene was used as the internal control in all assays. The recombinant plasmids were transferred into *E. coli* JM109 and the transcription and expression of the first gene cassette of aadA2 and the integrase gene in each corresponding strain were detected. The transcription levels of aadA2 and integrase genes had no significant differences by quantitative real-time PCR assay. Similarly, the expression levels of *aadA2* gene did not display differences. However, the integrase expression was not detected by Western blot assay which was consistent with previous reports (Wei, 2010). It is likely that integron can cause very little integrase expression.

CONCLUSION

This research reported the effects of the attI sites on the transcription and expression levels of the cassette and integrase genes carried by the artificially constructed integrons. The negative results indicated that the attI sites could not regulate transcription and expression of the gene cassettes and integrase genes.

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