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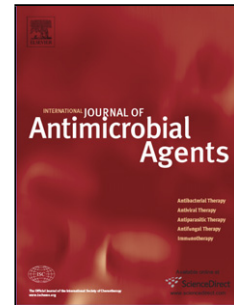
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Diversity of class 1 integron gene cassette Pc promoter variants in clinical *Escherichia coli* strains and description of a new P2 promoter variant

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ABSTRACT

Gene cassettes of class 1 integrons may be differently expressed depending on the Pc promoter variant as well as occasionally from a second promoter located downstream of Pc, named P2. So far, the distribution of the variants has only been described in an in silico study. In this study, the prevalence of these variants in vivo was analysed in a population of 85 *Escherichia coli* strains from a variety of phylogenetic groups isolated from healthy subjects and clinical samples in Spain and France from 2004 to 2007. The weakest variants (PcW and PcH1) prevailed (variants associated with the integrase having the most efficient excision activity), whilst the two strongest variants, PcW_{TGN-10} and PcS, were less frequent. Furthermore, a new variant of P2 associated with PcW was characterised in one integron (harbouring the gene cassette *bla*_{OXA-1}–*aadA1*) from a French strain of a healthy subject. This variant was hereafter named P2m3 and shows a G→A substitution in its –10 element (TACAGT to TACAAT), a mutation that doubled the strength of P2 and approached the level of expression of the strong PcW_{TGN-10} variant. When the correlation between the Pc variants and the origin of the strains was analysed, no significant difference ($P < 0.05$) was observed in the Pc variant distribution according to the geographic origin or clinical setting.

1. Introduction

Integrations are genetic elements strongly involved in the dissemination of antibiotic resistance among Gram-negative bacteria [1]. Integrations can acquire, exchange and express genes embedded within gene cassettes (GCs). The integrin functional platform is composed of an *intl* gene encoding an integrase, a recombination site *attI* and a promoter *Pc*. The integrase is the key element, able to catalyse through site-specific recombination either the insertion of a GC within the integrin or its excision. To date, more than 130 different GCs encoding resistance to almost all antibiotic families have been identified in integrins. Several classes of integrins have been characterised, with class 1 being the most prevalent. As GCs are usually promoterless, they are transcribed from the integrin promoter *Pc*, and the level of transcription is dependent upon their position within the integrin [2]. *Pc* variants with different strengths have been described in class 1 integrins based on the sequence of the –35 and –10 elements. Eight variants were initially described, with the four most prevalent being named *PcS* for ‘strong’, *PcW* for ‘weak’ (*PcS* being 30-fold stronger than *PcW*), *PcH1* for hybrid 1 and *PcH2* for hybrid 2, these two latter *Pc* variants containing the –35 and –10 hexamers of *PcW* and *PcS* in opposite combinations and having intermediate strengths [3–5]. In 10% of class 1 integrins a second promoter (*P2*) has been described 119 bp downstream of *Pc* [2,6]. We recently performed an in silico study and reported 13 *Pc* variants, including a new variant with a ‘TGN’ extended –10 motif, a motif known to increase the transcription efficiency of σ^{70} promoters in *Escherichia coli*. These 13 variants expressed different strengths and the most frequent ones were the weak variants *PcW* and *PcH1* and the

strong variants PcW_{TGN-10} and PcS [6]. However, such data might contain a bias because of the subjective selection of the deposited integrons.

Thus, class 1 integron-containing *E. coli* strains isolated in Spain and France from hospital patients and from healthy subjects were analysed to determine the Pc distribution in circulating strains.

2. Materials and methods

2.1. Bacterial isolates

In total, 85 class 1 integron-containing *E. coli* strains isolated from 2004 to 2007 were studied. The strains were geographically distinct (from Spain and France), from various origins (clinical strains from hospital patients and strains from faecal carriage of healthy subjects) and belonging to different phylogenetic groups (A, B1, B2 and D) (Table 1) [7].

2.2. Study of the integron and characterisation of the gene cassette promoter variants

The GC arrays of the Spanish strains ($n = 41$) had previously been characterised [8–10], whereas those of the French strains ($n = 44$) were characterised in this study by polymerase chain reaction (PCR) mapping as described elsewhere [10]. To characterise the Pc–P2 promoters, a 691-bp fragment obtained by PCR with primers int11-LC9 (5'-CCA AGC TCT CGG GTA ACA TC-3') and orf11-R (5'-GCG GGA TCC AGG TAA CTT TGT TTT AGG GCG ACT GCC CT-3') located, respectively, in *int11* and *attI* sequences was sequenced.

Statistical analysis was performed by means of Pearson's χ^2 test (with Yates' corrections) to check whether there were differences in the prevalence of promoter types among isolates of different origins or countries.

2.3. Estimation of the strength of promoters

The relative strength of a GC promoter was estimated in a transcriptional fusion in which both Pc and P2 were upstream of a promoterless *lacZ* gene in a reporter vector, as described previously [6]. The 'pPcW-P2m3' fusion was constructed by PCR assembly (see protocol in [6]) of the 'pPcW+P2' fusion with P2mut3L (5'-CTG TTT TTT TGG GGT ACA ATC TAT GCC TCG GGC ATC CAA G-3') and P2mut3R (5'-CCC GAG GCA TAG ATT GTA CCC CAA AAA AAC AGT CAT AAC-3') as internal primers. The efficiency of this promoter to drive *lacZ* gene expression was then estimated by the β -galactosidase assay [6].

3. Results and discussion

3.1. Integron detection and characterisation of gene cassette promoter variants

The 85 *E. coli* strains contained 88 class 1 integrons (3 strains had 2 integrons) with 22 different GC arrays (Table 2). Two integrons had no GC, and PCR failed to produce products for five strains. A new GC array (*dfrB1-aadA11*) was identified in a French clinical strain (GenBank accession no. GQ900749). All other GC arrays have been previously described in class 1 integrons and, as in other studies, the *dfr* and *aadA* GCs

associated with trimethoprim and streptomycin resistance, respectively, were the most prevalent [11,12].

Four different Pc variants were found in the 88 class 1 integrons: two weak variants (PcW and PcH1) predominated, whilst two strong variants (PcW_{TGN-10} and PcS) were less frequent (Table 3). These four variants were also the most prevalent in the in silico study, but strong variants have been more frequently found (16.8% for PcW_{TGN-10} and 23.7 % for PcS) [6]. Only a few studies have examined the prevalence of GC promoter variants in clinical strains. The PcW variant has been found to be the most prevalent in studies of clinical Enterobacteriaceae isolates [13,14]. However, the frequency of the PcW variant could have been overestimated in these studies because differentiation between PcW and PcW_{TGN-10} was not performed. Indeed, it has been recently shown that >40% of the PcW-containing integrons harboured the PcW_{TGN-10} variant, in which a -10 extended region increases the efficiency of PcW by 15-fold [6]. In this study, it was found that in 15 of 51 PcW-containing integrons, PcW was associated with the P2 form, a configuration known to be more efficient than PcW alone [6]. In the in silico study, P2 was also mostly found associated with PcW [6].

Among the 22 GC arrays identified, 8 were found in only one integron (Table 2). The 14 remaining GC arrays were found in at least two integrons, in strains from Spain and/or France belonging to different phylogenetic groups. Among these 14 GC arrays, 9 were always associated with the same Pc–P2 combination, whilst the other 5 could be transcribed from two or three different Pc–P2 combinations. In the previous in silico

study, we found that there was no link between a specific Pc variant and specific GC arrays (unpublished data). Here, the most frequent GC array (*dfrA1–aadA1*) was associated with three Pc–P2 combinations, suggesting also that Pc variants are not associated with specific GC arrays. All of the integrons containing *dfrA17* as the first GC bore the PcH1 promoter, which is in agreement with findings for *Salmonella enterica* serovar Typhimurium [14]. After statistical analysis, no significant difference ($P < 0.05$) was observed in the Pc variant distribution according to the geographic origin or clinical setting (Table 3).

3.2. Estimation of the strength of promoters

One integron (harbouring the GC *bla_{OXA-1}–aadA1*) from a French strain of a healthy subject presented a new P2 variant associated with PcW (Tables 2 and 3). This variant shows a G→A substitution in its –10 element (TACAGT to TACAAT), which is closer to the consensus sequence of the *E. coli* promoter, and it was named P2m3. When the strength of this promoter variant was determined by β-galactosidase assay, it was found that this mutation doubled the strength of P2 and approached the level of expression of the strong PcW_{TGN-10} variant (data not shown).

This work showed interesting data, with a predominance of the two weak variants PcW and PcH1 in the circulating *E. coli* strains studied and a low prevalence of the strong Pc variants (PcW_{TGN-10} and PcS), unlike in the in silico study. The population of strains studied was considered to be representative of the *E. coli* diversity as they came from different geographical and contextual origins. Moreover, they belonged to different

phylogenetic groups, which are known to have a good correlation to multilocus sequence type (MLST)-based phylogroups [15]. However, further studies with more isolates are needed to determine whether this higher prevalence of weaker GC promoter variants is widespread.

Interestingly, as the Pc sequence lies within the *intl1* gene, different IntI1 integrases correspond to different Pc variants, with PcW and PcH1 being associated with the integrase having the most efficient excision activity [6]. Thus, a better ability to excise cassettes might compensate for weak expression of antibiotic resistance factors and could theoretically enhance the capacity of the integron to adapt to antibiotic pressure and thus represent a survival advantage. If so, this may be one reason for the high prevalence of the weakest Pc variants observed here. It remains to be shown whether environmental factors (antibiotic pressure, stress, etc.) influence the selection of weak variants during bacterial evolution.

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Competing interests

None declared.

Ethical approval

Not required.

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Table 1

Distribution of 85 *Escherichia coli* strains included in the study according to origin and phylogenetic group

Phylogenetic group	Total (N= 85) [n (%)]	Spain (n = 41)		France (n = 44)	
		Healthy subjects (n = 7)	Clinical samples (n = 34)	Healthy subjects (n = 20)	Clinical samples (n = 24)
A	31 (36.5)	2	10	6	13
B1	10 (11.8)	1	5	1	3
B2	23 (27.1)	2	9	5	7
D	21 (24.7)	2	10	8	1

Table 2

Gene cassette arrays in 88 class 1 integrons from 85 *Escherichia coli* strains and their relationship with the promoter variant

Gene cassette array	No. of integrons (Spain/France)	Pc variant				
		PcW	PcH1	PcW+P2	PcW _{TGN} - ¹⁰	PcS
<i>aadA1</i>	8 (3/5)			8		
<i>aadA2</i>	1 (1/0)			1		
<i>aadB-aadA1-cmlA1</i>	2 (1/1)		2			
<i>dfrA1</i>	3 (2/1)	3				
<i>dfrA1-aadA1</i>	20 (4/16)	15	3	2		
<i>dfrA12-orfF-aadA2</i>	3 (1/2)				3	
<i>dfrA12-orfF-aadA2-</i> <i>cmlA1-aadA1-qacH-</i> <i>IS10-IS440-sul3</i>	1 (1/0)				1	
<i>dfrA12-orfF-aadA2-</i> <i>cmlA1-aadA1-qacH-</i> <i>IS440-sul3</i>	2 (2/0)				1	1
<i>dfrA12-orfF-aadA2-</i> <i>cmlA1-aadA1-IS440-</i> <i>sul3</i>	1 (1/0)				1	
<i>dfrA14</i>	1 (0/1)		1			
<i>dfrA15-aadA1</i>	1 (1/0)		1			
<i>dfrA16-aadA2-aadA2</i>	3 (2/1)	1	2			
<i>dfrA17</i>	4 (3/1)		4			
<i>dfrA17-aadA5</i>	10 (4/6)		10			
<i>dfrA25</i>	1 (0/1)		1			
<i>dfrA5</i>	9 (4/5)	9				
<i>dfrA5-ereA2</i>	2 (2/0)	1	1			
<i>dfrA7</i>	2 (2/0)	2				

<i>dfrB1</i> – <i>aadA11</i> ^a	1 (0/1)					1
<i>bla</i> _{OXA-1} – <i>aadA1</i>	3 (1/2)			3 ^b		
<i>estX</i> – <i>psp</i> – <i>aadA2</i> – <i>cmlA1</i> – <i>aadA1</i> – <i>qacH</i> – IS440– <i>sul3</i>	2 (2/0)	1	1			
<i>estX</i> – <i>psp</i> – <i>aadA2</i> – <i>cmlA1</i> ::IS1294– <i>aadA1</i> – <i>qacH</i> –IS440– <i>sul3</i>	1 (1/0)		1			
No gene cassettes	2 (1/1)	1	1			
Unknown ^c	5 (4/1)	3	1	1		
Total	88 (43/45)	36	29	15	6	2

^a Included in GenBank under accession no. GQ900749.

^b One of these integrons presented the new P2 variant named P2m3.

^c Class 1 integrons for which polymerase chain reaction (PCR) failed to amplify the gene cassette array.

Table 3

Pc and P2 promoter variants detected among 88 class 1 integrons detected in 85

Escherichia coli strains from Spain and France

Pc–P2 combinations ^a	Total no.	Healthy	Clinical	Spain (<i>n</i> = 43)	France (<i>n</i> = 45)
		subjects (<i>n</i> = 27)	samples (<i>n</i> = 61)		
		<i>n</i> (%)	<i>n</i> (%)		
PcW	36	14 (51.9)	22 (36.1)	18	18
PcH1	29	8 (29.6)	21 (34.4)	14	15
PcW+P2	14	3 (11.1)	11 (18.0)	6	8
PcW+P2m3	1	1 (3.7)	0 (0.0)	0	1
PcW _{TGN-10}	6	1 (3.7)	5 (8.2)	4	2
PcS	2	0 (0.0)	2 (3.3)	1	1

^a P2 is indicated when present.