Relative Strengths of the Class 1 Integron Promoter Hybrid 2 and the Combinations of Strong and Hybrid 1 with an Active P2 Promoter[∇]

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The relative strengths of the uncommon promoters hybrid 2, hybrid 1 with an active P2 promoter (hybrid 1+P2), and strong+P2, which drive transcription of resistance genes in class 1 integrons, were evaluated using *bla*_{GES-1} as a reporter gene cassette. Hybrid 2 was stronger than hybrid 1. Coupling P2 with the strong promoter and with hybrid 1 caused a measurable increase in GES-1 expression.

Expression of antibiotic resistance genes in the variable region of class 1 integrons is driven by a promoter (Pc) that is 214 bases from the inner boundary of the 5' conserved segment and located within the coding sequence of *intI1*. There are four versions of Pc, designated "weak," "strong," "hybrid 1," and "hybrid 2," exhibiting differences in the –35 and/or –10 sequences, separated by 17 bases. Additionally, a second promoter, P2, complements the Pc weak promoter in various integrons. P2 occurs 119 bases downstream from Pc and is formed by an insertion of three G bases optimizing the spacing (17 bases) between the –35 and –10 hexamers (P2 active) (5). The relative strengths of the weak, strong, and hybrid 1 versions of the Pc promoter, as well as that of the weak promoter complemented by P2 (weak+P2), have been determined previously (5, 9), while that of hybrid 2, which occurs in a relatively small number of class 1 integrons (Table 1) (3, 4, 6, 8, 10, 14, 16), has not been studied. The increasing number of characterized integrons revealed the existence of additional promoter sequences, such as the combinations of the strong promoter and hybrid 1 with P2 (Table 1) (7, 12). In this study, we evaluated the relative strengths of hybrid 2 and the latter promoter combinations introduced into a GES-1 extended-spectrum-β-lactamase-encoding integron. Comparative assessments included amounts of *bla*_{GES-1} transcripts, levels of hydrolytic activity of GES-1, and MICs of β-lactams.

Cloning of a GES-1-encoding integron and construction of the Pc-P2 sequence variants. A GES-1-producing isolate of *Pseudomonas aeruginosa* (Pa-G1) was used as a source of In58-G1, a typical class 1 integron with a weak promoter containing a single gene cassette, *bla*_{GES-1} (GenBank accession no. EU598463). A 1,641-bp fragment of In58-G1, including the *bla*_{GES-1} cassette and the promoter sequence, was amplified by PCR and cloned into the Topo TA vector (Invitrogen, Carlsbad, CA), yielding plasmid pTOPO-G1. An EcoRI fragment of 1,690 bp from pTOPO-G1 was treated with a DNA polymerase Klenow fragment and introduced into the single HincII site of plasmid pACYC177, thereby disrupting the resident *bla*_{TEM-1}.

A recombinant plasmid of 5,607 bp (pHP327) carrying the 1,641-bp segment of In58-G1 in an orientation opposite that of the 246-bp 5' remnant of *bla*_{TEM-1} was selected for the construction of the various Pc-P2 sequence combinations. Site-specific mutagenesis was performed using 10 mutagenic primers (Table 2) and a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Seven variants of plasmid pHP327 carrying the weak, strong, hybrid 1, hybrid 2, weak+P2, strong+P2, and hybrid 1+P2 promoter sequences were studied. Additionally, a pHP327 variant with a promoterless integron was constructed by reverting the 29-bp Pc sequence and also deleting 5 of the 17 bp between the –35 and –10 hexamers. Entire plasmid sequences were verified on both strands by use of an API377 sequencer (Applied Biosystems, Foster City, CA). *Escherichia coli* MC4100, a *ΔampC* mutant, was used as a host. Isoelectric focusing and staining with nitrocefin showed that GES-1, with a pI of 5.8, was the sole β-lactamase produced by the respective clones.

Characterization of *bla*_{GES-1} transcripts by Northern hybridization and real-time reverse transcription-PCR (RT-PCR). Total RNA was prepared using RNA protect and RNeasy mini kits (Qiagen, Hilden, Germany). RNA samples (10 μg from each strain) were electrophoresed through formaldehyde-containing agarose, transferred to Hybond-N⁺ membranes (Amersham Biosciences, United Kingdom), and hybridized with a digoxigenin-labeled *bla*_{GES-1}-specific probe (618 bp, nucleotides [nt] 744 to 1361; GenBank accession no. EU598463) following standard techniques (15). These experiments confirmed production of similarly sized *bla*_{GES-1} transcripts (approximately 1.2 kb) in all seven strains and also indicated higher transcription levels in the strains carrying the Pc strong sequence (data not shown).

RNA samples were treated with RNase-free DNase I using a Turbo DNA-free kit (Ambion Inc., Foster City, CA). Real-time RT-PCR was performed using a Quantifast Sybr green RT-PCR kit (Qiagen). Three reactions per sample were performed, using 10, 20, and 50 ng of total RNA. Each reaction mixture also contained Quantifast SYBR green RT-PCR master mix (12.5 μl), Quantifast RT mix (0.25 μl), and 500 nM of each oligonucleotide primer (Ven-8P and Ven-12 [Table 2]) in a final volume of 25 μl. The pACYC177 resident gene *aphA1* was used as a reference. The respective reactions were carried

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TABLE 1. Characteristics of class 1 integrons with the uncommon promoter sequence Pc hybrid 2 and the combinations of P2 with Pc hybrid 1 and Pc strong

Promoter	Integron designation	Gene cassette(s) (5'-3')	Species	Location	GenBank accession no.	Reference or source
Pc hybrid 2	In-t4	<i>aadB</i> , <i>orfE</i> , <i>orf416</i> , <i>cmlA7</i>	<i>Klebsiella pneumoniae</i>	Plasmid pCC416 (IncA/C ₂)	AJ704863	4
		<i>aadB</i> , <i>int</i> , <i>cmlA1</i>	<i>K. pneumoniae</i>	Plasmid pKPN5 (IncF; 88.6 kb)	CP000650	McClelland et al., unpublished
	In7	<i>aacA1</i> , <i>orfG</i> ^a	<i>E. coli</i>	Plasmid pCMXR1	AB061794	6
		<i>bla</i> _{VIM-2} , <i>aacA4</i>	<i>Pseudomonas aeruginosa</i>	Chromosome	AM180753	8
	In70	<i>aacA7</i> , <i>bla</i> _{VIM-2} , <i>dfrB5</i> , <i>aacC-A5</i>	<i>P. aeruginosa</i>	Unknown	DQ522233	Shevchenko and Edelstein, unpublished
		<i>bla</i> _{VIM-1} , <i>aacA4</i> , <i>aphA15</i> , <i>aadA1</i>	<i>Achromobacter xylosoxidans</i>	Plasmid pAX22 (30 kb)	AJ278514	14
	Tn402-like	<i>oxa2</i> , <i>aacA4</i> , <i>aadA1</i>	Uncultured bacterium	Plasmid pTB11 (IncP; 68.9 kb)	AJ744860	16
		<i>aacA7</i> , <i>catB3</i> , <i>aadB</i> , <i>oxa2</i> , <i>orfD</i>	<i>Enterobacter aerogenes</i>	Plasmid pBWH301	U13880	3
		<i>aacA4</i> ^b	<i>Salmonella enterica</i> serovar Typhimurium	Plasmid pST2	S45954	10
Pc hybrid 1+P2		<i>aadA2</i>	<i>E. coli</i>	Plasmid pMUR050 (IncN; 56.6 kb)	AY522431	7
Pc strong+P2	In-h12	<i>aacA7</i> , <i>bla</i> _{VIM-12} , <i>aacA7</i>	<i>K. pneumoniae</i>	Plasmid p2873 (70 kb)	DQ143913	12

^a *aacA1* and *orfG* occur in the same cassette.^b Only the first gene cassette has been characterized.

out as described above with primers Aph-F and Aph-R (Table 2). RT was carried out for 10 min at 50°C and terminated by heating at 95°C for 5 min. A two-step cycling PCR was then performed for 40 cycles, with a denaturation step at 95°C for 10 s and an annealing-extension step at 60°C for 30 s. All reactions were carried out in triplicate using a Corbett Rotor-Gene 6000 (Corbett Life Science, Sydney, Australia). Rotor-Gene 6000 software (version 1.7) was used for data analysis. Quantities of *bla*_{GES-1} and *aphA1* in the same sample were expressed in threshold cycle values. The target-reference ratios were calculated, and the normalized values were used to compare differential *bla*_{GES-1} expression levels. The relative amounts of *bla*_{GES-1} transcripts under the control of the seven promoter sequences are presented in Table 3. The lowest value, observed for the weak promoter, was set to 1. Differences in strength among the weak, strong, hybrid 1, and weak+P2 promoters were generally in line with those reported previously (9). The strong promoter appeared to be 17 times more efficient than the weak promoter. The respective increase was lower for the weak+P2 sequence and marginal for hybrid 1 (4.7- and 1.9-fold, respectively). Association of P2 with hybrid 1 caused a 3.5-fold increase in the amount of *bla*_{GES-1} transcript, while the respective increase in the strength of the strong sequence was minor. The efficiency of hybrid 2 was comparable to that of the weak+P2 combination and approximately 2.5-fold higher than that of hybrid 1. *bla*_{GES-1} transcripts from the promoterless integron sequence were not detected (Table 3).

β-Lactamase activities and resistance levels to β-lactams.

β-Lactamase-containing extracts were derived by ultrasonic treatment of bacterial cell suspensions and clarified by ultracentrifugation (4). The protein content was measured with a protein assay kit (Bio-Rad Laboratories, Hercules, CA).

β-Lactamase activity was quantified by spectrophotometry using nitrocefin as a reporter substrate ($\Delta\epsilon_{M}^{482} = 17,400 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [17]). Results were expressed as units of activity. One unit was the amount of enzyme hydrolyzing 1 nmol of substrate/min/mg of protein at 30°C and pH 7.0. Hydrolytic activities of the extracts from strains carrying the Pc strong sequences were higher than those of the strains with the hybrid promoters. The lowest activity was observed in the strain carrying the weak version of Pc (at least 26- and 3-fold lower than those of the strains with strong and hybrid promoters, respectively). The β-lactamase activity of the strain with hybrid 2 was approximately twofold higher than that of the hybrid 1-possessing strain. The presence of a P2 promoter along with the weak and hybrid 1 sequences was associated with an increase in β-lactamase activity (approximately 6.2- and 3.4-fold, respectively). Also, the hydrolytic activity of the strain with the strong+P2 combination was slightly higher than that of the strain carrying the strong promoter (Table 3).

Susceptibilities to ampicillin, piperacillin, cefaclor, cefuroxime, ceftazidime, aztreonam, and cefepime were determined by Etest (AB Biodisk, Solna, Sweden). MIC differences were in line with those of the respective β-lactamase activities. As expected, differences in resistance levels were more pronounced with the narrow-spectrum cephalosporins and ceftazidime, which are GES-1 substrates preferable to piperacillin, cefepime, and aztreonam (11). The higher MICs were seen with the two strains carrying the strong promoter and the lower MICs with the strains with the weak and hybrid 1 promoters. MICs for *E. coli* strains harboring the plasmids with the hybrid 2, weak+P2, and hybrid 1+P2 sequences did not differ significantly. Resistance levels of the strains carrying strong and strong+P2 sequences were also similar. Most β-lactams tested

TABLE 2. Oligonucleotide primers

Primer function and name	Sequence (5'–3')	Usage	GenBank accession no. (nt) or source
Cloning and real-time PCR			
Int-F	CGTTCATACAGAAGCTG	Cloning of the In58-G1 integron	EU598463 (1–18)
3-CS	AAGCAGACTTGACCTGA	Cloning of the In58-G1 integron	EU598463 (1625–1641)
Ven-8P	GTAATCTCTCTCCTGGGCT	RT-PCR for <i>bla</i> _{GES-1}	EU598463 (1343–1361)
Ven-12	CCCCAAGGAGAGATCGT	RT-PCR for <i>bla</i> _{GES-1}	EU598463 (744–762)
Aph-F	GGAAACGTCTTGCTCGAGG	RT-PCR for <i>aphA1</i>	X06402 (1940–1958)
Aph-R	ACTGAATCCGGTGAGAATGG	RT-PCR for <i>aphA1</i>	X06402 (2480–2500)
Site-directed mutagenesis			
P1st-F ^a	AACCCAGTTGACATAAGCCTGTT CGGTTCTGTAAGTGAATG	Construction of the strong promoter from the weak promoter	This study
P1st-R ^a	CATTACAGTTTACGAACCGAACA GGCTTATGTCAACTGGGT	Construction of the strong promoter from the weak promoter	This study
P1h1-F ^b	GCACGAACCCAGTGGACATAAG CCTGTTCG	Construction of hybrid 1 from the weak promoter (–35 box)	This study
P1h1-R ^b	CGAACAGGCTTATGTCCACTGGG TTCGTGC	Construction of hybrid 1 from the strong promoter (–35 box)	This study
P1h2-F ^b	GCACGAACCCAGTTGACATAAG CCTGTTCG	Construction of hybrid 2 from the weak promoter (–35 box)	This study
P1h2-R ^b	CGAACAGGCTTATGTCAACTGGG TTCGTGC	Construction of hybrid 2 from the weak promoter (–35 box)	This study
P2ac-F ^c	TGACTGTTTTTTTGGGTACAGT CTATGCCTCGGGA	Construction of the P2 promoter	This study
P2ac-R ^c	TGCCCGAGGCATAGACTGTACCC CAAAAAACAGTCA	Construction of the P2 promoter	This study
PC-F ^d	GGATGAAGGCACGAACCCAGAG CTTACGAACCGAACAGTGTCCAG TAATGCAAGTAGCGTATGC	Construction of a promoterless sequence upstream of the <i>bla</i> _{GES-1} cassette	This study
PC-R ^d	GCATACGCTACTTGCTATTACTGG ACACTGTTCCGGTTCGTAAGCTCT GGGTTCTGTCCTTCATCC	Construction of a promoterless sequence upstream of the <i>bla</i> _{GES-1} cassette	This study

^a Underlined bases correspond to the –35 and –10 hexamers of the Pc strong promoter.

^b Underlined bases correspond to the –35 hexamer of the hybrid Pc promoters.

^c Underlined bases correspond to the insertion of three G bases in the P2 promoter sequence.

^d Bases in boldface type correspond to the reversed Pc weak promoter sequence. The –10 and –35 hexamers are underlined.

were more active against the strain with hybrid 1 than against the strain possessing the hybrid 1+P2 combination (Table 3).

Conclusions. Determination of enzymatic activity and susceptibility testing have been used for the characterization of the common promoters of the class 1 integrons (5, 9). We used

here a similar methodology to study hybrid 2, strong+P2, and hybrid 1+P2. Additionally, we employed a real-time RT-PCR assay for the relative quantification of the *bla*_{GES-1} transcripts. Results of the latter method were in agreement with the quantitative assessment of hydrolytic activities as well as resistance

TABLE 3. Effect of integron promoter sequences on the relative amounts of *bla*_{GES-1} transcripts, β-lactamase activities, and β-lactam resistance levels in *E. coli* MC4100 strains carrying a plasmid-borne *bla*_{GES-1} gene cassette

Promoter	Sequence at hexamer		Relative amt of <i>bla</i> _{GES-1} transcript ^b	Hydrolysis of nitrocefin (U) ^c	Etest MIC (μg/ml) of β-lactam ^a						
	–35	–10			Amx	Pip	Cec	Cxm	Caz	Atm	Fep
Pc weak	TGGACA	TAAGCT	1	32	>256	2	6	8	0.75	0.047	0.032
Pc strong	TTGACA	TAAACT	17.0	838	>256	24	256	>256	12	0.19	0.19
Pc hybrid 1	TGGACA	TAAACT	1.9	107	>256	4	8	32	1.5	0.047	0.064
Pc hybrid 2	TTGACA	TAAACT	4.6	225	>256	12	24	128	4	0.094	0.125
Pc weak+P2	TTGTTA	TACAGT	4.7	198	>256	12	24	96	4	0.094	0.094
Pc strong+P2			20.5	1,030	>256	24	256	>256	16	0.25	0.19
Pc hybrid 1+P2			6.7	365	>256	16	24	256	6	0.125	0.125
Control ^d			Not detectable	<1	4	1	1.5	2	0.064	0.032	0.032

^a Amx, amoxicillin; Pip, piperacillin; Cec, cefaclor; Cxm, cefuroxime; Caz, ceftazidime; Atm, aztreonam; Fep, cefepime.

^b Values are the means of three measurements differing by <25% and expressed as relative to that of the Pc weak promoter, set to 1.

^c Values are the means of three measurements differing by <10%.

^d Values are from *E. coli* MC4100 containing a promoterless *bla*_{GES-1}-carrying integron sequence.

levels conferred by the indicator β -lactamase. Additionally, classification of the weak, strong, hybrid 1, and weak+P2 promoters according to strength was as published by Lévesque et al. (9), although the different expression systems and assays do not allow for a direct comparison. Nevertheless, the results clearly indicate that the hybrid 2 version of Pc is an effective promoter similar in strength to weak+P2 and stronger than hybrid 1. It is also shown that the active configuration of P2 is functional when coupled with the strong and hybrid 1 promoters, resulting in a measurable increase in expression of an immediately downstream gene cassette. The levels of this increase were comparable in all three Pc+P2 combinations studied here, suggesting that the operation of P2 is probably not affected by the type of the preceding Pc sequence.

Promoter strength is a key factor in determining transcription of resistance gene cassettes in class 1 integrons. Therefore, the evaluation of the strengths of hybrid 2, hybrid 1+P2, and strong+P2 may be useful in explaining resistance phenotypes in strains containing these sequences. Cassette position as well as the sequence of the respective *attC* site also influences expression in multicassette arrays (5, 13). The potential of a resistance gene cassette to confer a wide range of resistance levels may facilitate establishment of these determinants under various conditions. Also, it may have implications in the phenotypic recognition of microorganisms carrying antibiotic resistance integrons.

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