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Promoters *P3*, *Pa*/*Pb*, *P4*, and *P5* Upstream from bla_{TEM} Genes and Their Relationship to β-Lactam Resistance

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Using an isogenic system, we have determined the impact that the four promoters known to control bla_{TEM} gene expression have on β-lactamase activity. For both TEM-1 and TEM-30, this activity gradually increased in relation to the presence of promoters *P3*, *Pa*/*Pb*, and *P4* upstream of the corresponding gene. Promoter *P5*, only found upstream of the bla_{TEM-1B} gene, was related to the highest expression of this gene.

The extensive nucleotide sequence analysis of plasmid-mediated bla_{TEM} genes performed over the last 15 years because of their role in resistance to extended-spectrum cephalosporins and to amoxicillin-clavulanate has allowed us to observe that the expression of these genes is controlled by four different promoters (3, 6). The first promoter, called *P3* (Fig. 1,) corresponds to the promoter of the bla_{TEM-1A} gene which is located on a Tn*3* transposon and plasmid pBR322 (10). The second promoter, called *Pa*/*Pb* and found upstream of the bla_{TEM-2} gene, is composed, in fact, by two overlapping promoters resulting from the mutation C→T at position 32 according to the Sutcliffe numbering system (10). The −35 and −10 regions of promoter *Pa* are TTGAAG and TACGCT, respectively, whereas the −35 and −10 regions of promoter *Pb* are GTGATA and TAATGT, respectively, the first 2 nucleotides of the −10 region of *Pa* corresponding to the last 2 nucleotides of the −35 region of *Pb* (Fig. 1). Chen et al. (2), studying the separately cloned promoters *P3* and *Pa*/*Pb*, showed that the *Pb* sequence which was present in the fragment containing the promoter *P3* did not work as a promoter and that the overlapping promoter *P3* were stronger than promoter *P3*, with the increase in strength resulting from both the strength of *Pa* and the cooperative activity of *Pa* and *Pb*. The third promoter, called *P4* by Goussard et al. (3), differs from promoter *P3* by the substitution G162T (Sutcliffe numbering system) corresponding to the first nucleotide of the −10 region of the promoter (TACAAT instead of GACAAT). This substitution makes the sequence of the −10 region of promoter *P4* closer to the consensus sequence (TATAAT) (Fig. 1). We suggest that the fourth promoter, which we have recently published, be called *P5* (7). This promoter has the same sequence as promoter *P3* at the −10 region but has a sequence at the −35 region (TTGAAA) closer to the consensus sequence (TTGACA) than that of promoter *P3* (TTCAAA) (Fig. 1). To assess and compare the respective impact of the four promoters on β-lactam resistance, we established an isogenic system in which the only difference consisted of the type of promoter upstream from bla_{TEM} genes coding for either a β-lactamase susceptible (TEM-1) or resistant (TEM-30 or IRT-2) to class A β-lactamase inhibitors.

By using primers 5′-ATA AAA TTC TTG AAG AC-3′ and 5′-TTA CCA ATG CTT AAT CA-3′, four bla_{TEM-1B} genes, each preceded by one of the four promoters, were amplified and cloned from previously published *Escherichia coli* isolates (6). The bla_{TEM-30} gene coding for the inhibitor-resistant TEM-30 or IRT-2 enzyme was also amplified and cloned from previously published *E. coli* isolates but only with promoter *P3*, *Pa*/*Pb*, or *P4* upstream from it, as a bla_{TEM-30} gene controlled by promoter *P5* has not been discovered thus far (6). Plasmid pPCR Script (Cam^r) (Stratagene, La Jolla, Calif.) and *E. coli* strain Epicurian Coli XL10-Gold (Stratagene) were used in the first stage of the cloning experiments. After the sequences of inserted fragments (promoter and coding region) were checked (automated cycle sequencing system on a Perkin-Elmer R377 sequencer), the fragments were then cloned into the *Bam*HI-restricted and dephosphorylated plasmid pACYC184 (Tet^r and Cam^r) and expressed in *E. coli* strain NM554 (8). The size of the inserts and their insertion in the opposite orientation of the promoter of the pACYC184 tetracycline resistance-encoding gene were assessed by restriction analyses with *Bam*HI and *Ase*I enzymes.

The *E. coli* NM554 transformants containing the different recombinant plasmids were then tested in three independent experiments for β-lactam susceptibility by using the agar dilution method on Mueller-Hinton agar with a Steers multiple inoculator and 10^4 CFU per spot. The β-lactamase activity of each transformant was also tested from the crude extracts of three independent cultures, as previously described (5). Briefly, β-lactamase was extracted from 100 ml of an exponential-growth-phase culture at 37°C in Trypticase soy broth containing amoxicillin (100 μg/ml) combined with chloramphenicol (30 μg/ml). Bacterial suspensions were disrupted by sonication, and the β-lactamase activity was measured from the crude extracts with a Pharmacia UV2000 spectrophotometer, using benzylpenicillin (100 μM) as the substrate. One unit

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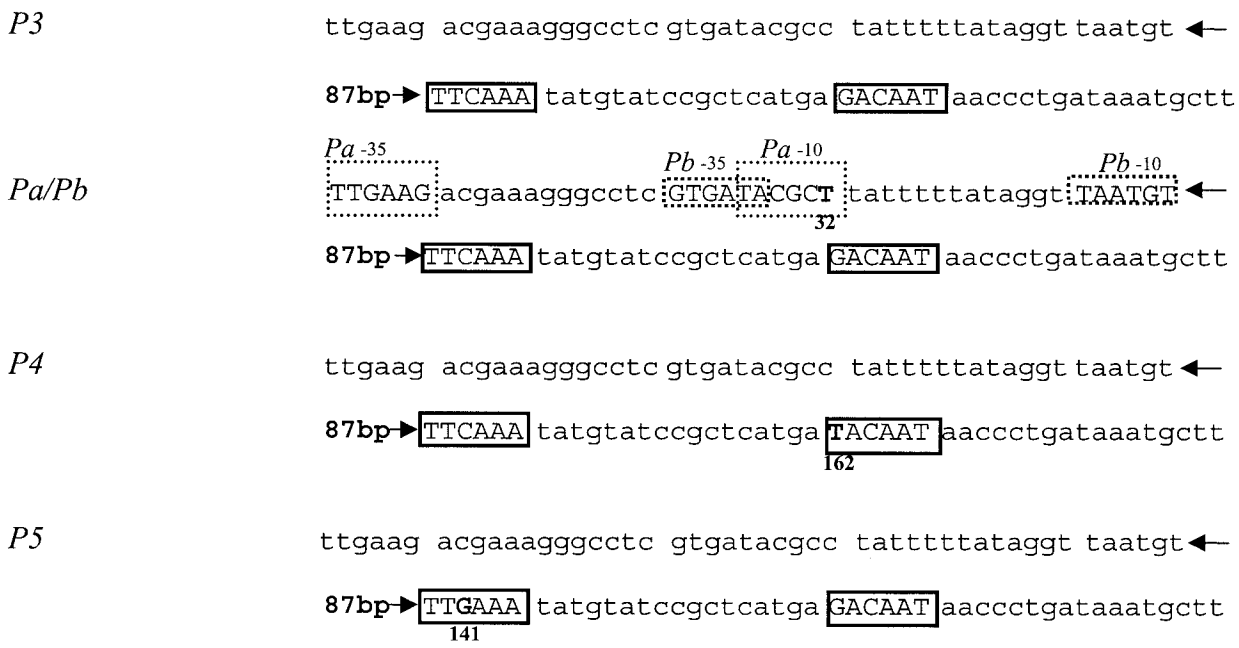

FIG. 1. Nucleotide sequences of promoters *P3*, *Pa*/*Pb*, *P4*, and *P5* of bla_{TEM} genes. The sequences of the −35 and −10 regions of promoters *P3*, *P4*, and *P5* are boxed by solid lines, whereas these of the overlapping promoters *Pa*/*Pb* are boxed by broken lines. Boldface letters indicate the mutations which led to the creation of promoters *Pa*/*Pb*, *P4*, and *P5*. The numbers under sequences indicate Sutcliffe numbering positions.

of β-lactamase activity was defined as the amount of enzyme which hydrolyzed 1 μM benzylpenicillin per min, and the specific activity was defined as the β-lactamase activity per milligram of protein.

As indicated in Table 1, β-lactamase activity as well as MICs of amoxicillin-clavulanate, ticarcillin-clavulanate, piperacillin, and cephalothin gradually increased from the *E. coli* transformant expressing the bla_{TEM-1B} gene controlled by the *P3* promoter to that expressing the bla_{TEM-1B} gene controlled by the *P5* promoter, with higher values for the two types of parameters when bla_{TEM-1B} was controlled by the *P4* promoter than when it was controlled by the *Pa*/*Pb* promoters. Concerning ticarcillin, the concentrations used in this study did not allow us to observe a gradual increase in MICs in relation to the promoter type. For piperacillin-tazobactam, we observed different levels of MICs: low MICs, from 1 to 2 μg/ml for the transformants whose bla_{TEM-1B} gene was controlled by promoters *P3* and *Pa*/*Pb*, respectively, and high MICs, from 128 to 512 μg/ml for those whose bla_{TEM-1B} gene was under the control of promoters *P4* and *P5*, respectively. The first two transformants were categorized as susceptible to piperacillin-tazobactam, whereas the last two were categorized as resistant following the recommendations of the French Antibiogram Committee (9). These results indicate that the C→G substitution, which oc-

TABLE 1. β-Lactam susceptibility and β-lactamase activity of TEM-1- and TEM-30 (IRT-2)-producing *E. coli* transformants according to the type of promoter upstream from the bla_{TEM-1B} and $bla_{TEM-30B}$ genes

Strain and bla_{TEM} gene (promoter)	MIC (μg/ml)[a]						β-Lactamase activity (U/mg) (mean ± SD)
	AMC (≤4–>16)	TIC (≤16–>64)	TCC (≤16–>64)	PIP (≤8–>64)	TZB (≤8–>64)	CEP (≤8–>32)	
E. coli							
ATCC 25 922	4	4	4	2	1	8	ND[b]
NM554	2	2	2	1	1	2	ND
Transformant NM554							
bla_{TEM-1B} (*P3*)	32	8,192	32	128	1	8	6.8 ± 3.3
bla_{TEM-1B} (*Pa*/*Pb*)	128	>8,192	256	512	2	32	13.8 ± 2.1
bla_{TEM-1B} (*P4*)	512	>8,192	1,024	1,024	128	64	38 ± 9
bla_{TEM-1B} (*P5*)	1,024	>8,192	2,048	>1,024	512	128	87.4 ± 8
$bla_{TEM-30B}$ (*P3*)	256	128	32	8	1	8	14 ± 4.4
$bla_{TEM-30B}$ (*Pa*/*Pb*)	2,048	512	128	64	2	8	32.6 ± 5.6
$bla_{TEM-30B}$ (*P4*)	4,096	2,048	512	64	32	8	120 ± 7.9

[a] Drug abbreviations: AMC, amoxicillin with 2 μg of clavulanate per ml; TIC, ticarcillin; TCC, ticarcillin with 2 μg of clavulanate per ml; PIP, piperacillin; TZB, piperacillin with 4 μg of tazobactam per ml; CEP, cephalothin. The MIC breakpoints (in micrograms per milliliter) are shown in parentheses after the drug abbreviations.

[b] ND, not determined.

curred in the −35 region of promoter *P5*, is more efficient in terms of promoter strength than the G→T substitution, which occurred in the −10 region of promoter *P4*. In fact, promoter *P5* possesses the trimer TTG in the −35 region that was considered by Kobayashi et al. (4) to be the most essential sequence for determining promoter strength. However, although the overlapping promoters *Pa*/*Pb* possess the trimer TTG in the −35 region of promoter *Pa*, they were related to a weaker β-lactamase activity than that related to promoter *P5*. This difference suggests that other nucleotide motifs than TTG in the promoter region might be involved in gene expression.

Concerning the three transformants containing the $bla_{\text{TEM-30}}$ gene, they differed from each other with regard to the β-lactamase activity and the β-lactam MICs with the exception of cephalothin. The lowest MICs, regardless of the penicillins tested, were found in the transformant harboring the $bla_{\text{TEM-30B}}$ gene preceded by promoter *P3*, whereas the highest MICs were found in the transformant harboring the $bla_{\text{TEM-30B}}$ gene controlled by promoter *P4*; intermediate MICs were found in the transformant harboring the $bla_{\text{TEM-30}}$ gene preceded by promoters *Pa*/*Pb* (Table 1). For cephalothin, all of the transformants were susceptible. For piperacillin-tazobactam, only those transformants with promoters *P3* and *Pa*/*Pb* upstream from the $bla_{\text{TEM-30B}}$ gene were susceptible. For piperacillin, only the transformant with promoter *P3* was susceptible. For the transformants with $bla_{\text{TEM-30B}}$, the lowest β-lactamase activity was observed in the transformant harboring the $bla_{\text{TEM-30B}}$ gene with promoter *P3*, whereas the highest activity was observed in the transformant harboring the $bla_{\text{TEM-30B}}$ gene with promoter *P4*.

Overall, the increase in β-lactamase activity observed in relation to the presence of promoters *P3*, *Pa*/*Pb*, and *P4* was demonstrated by using either the $bla_{\text{TEM-1B}}$ gene coding for TEM-1 or the $bla_{\text{TEM-30B}}$ gene coding for TEM-30 or IRT-2. Thus, we were able to compare not only the expression of a given bla_{TEM} gene in relation to different promoters but also the expression of different bla_{TEM} genes in relation to a given promoter. In this study, it has been unambiguously demonstrated that, for any one promoter, the β-lactamase activity measured by using benzylpenicillin as the substrate was higher for TEM-30 or IRT-2 than for TEM-1 from which the IRT enzyme is derived (1). However, despite this higher β-lactamase activity, the MICs of the penicillins tested were lower, with the exception of amoxicillin-clavulanate, for the transformants producing TEM-30 or IRT-2 than for those producing TEM-1 (Table 1). This result emphasizes that MICs are linked to both the type of promoters upstream of the TEM-encoding gene and to the biochemical properties of the TEM enzyme.

In conclusion, although our isogenic system has probably slightly overestimated MICs and β-lactamase activity for all of the transformants, as the vector used (pACYC184) is a low-number but not a single-copy plasmid, it has allowed us to compare the roles that the four promoters that have been described thus far and that are upstream from the bla_{TEM} genes have on β-lactam resistance according to the type of TEM enzyme involved.

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