

## Promoters *P3*, *Pa/Pb*, *P4*, and *P5* Upstream from *bla*<sub>TEM</sub> Genes and Their Relationship to $\beta$ -Lactam Resistance

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**Using an isogenic system, we have determined the impact that the four promoters known to control *bla*<sub>TEM</sub> gene expression have on  $\beta$ -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*<sub>TEM-1B</sub> gene, was related to the highest expression of this gene.**

The extensive nucleotide sequence analysis of plasmid-mediated *bla*<sub>TEM</sub> 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*<sub>TEM-1A</sub> gene which is located on a Tn3 transposon and plasmid pBR322 (10). The second promoter, called *Pa/Pb* and found upstream of the *bla*<sub>TEM-2</sub> 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* (TTCAA) (Fig. 1). To assess and compare the respective impact of the four promoters on  $\beta$ -lactam resis-

tance, we established an isogenic system in which the only difference consisted of the type of promoter upstream from *bla*<sub>TEM</sub> genes coding for either a  $\beta$ -lactamase susceptible (TEM-1) or resistant (TEM-30 or IRT-2) to class A  $\beta$ -lactamase inhibitors.

By using primers 5'-ATA AAA TTC TTG AAG AC-3' and 5'-TTA CCA ATG CTT AAT CA-3', four *bla*<sub>TEM-1B</sub> genes, each preceded by one of the four promoters, were amplified and cloned from previously published *Escherichia coli* isolates (6). The *bla*<sub>TEM-30</sub> 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*<sub>TEM-30</sub> gene controlled by promoter *P5* has not been discovered thus far (6). Plasmid pPCR Script (Cam<sup>r</sup>) (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<sup>r</sup> and Cam<sup>r</sup>) 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  $\beta$ -lactam susceptibility by using the agar dilution method on Mueller-Hinton agar with a Steers multiple inoculator and 10<sup>4</sup> CFU per spot. The  $\beta$ -lactamase activity of each transformant was also tested from the crude extracts of three independent cultures, as previously described (5). Briefly,  $\beta$ -lactamase was extracted from 100 ml of an exponential-growth-phase culture at 37°C in Trypticase soy broth containing amoxicillin (100  $\mu$ g/ml) combined with chloramphenicol (30  $\mu$ g/ml). Bacterial suspensions were disrupted by sonication, and the  $\beta$ -lactamase activity was measured from the crude extracts with a Pharmacia UV2000 spectrophotometer, using benzylpenicillin (100  $\mu$ M) as the substrate. One unit

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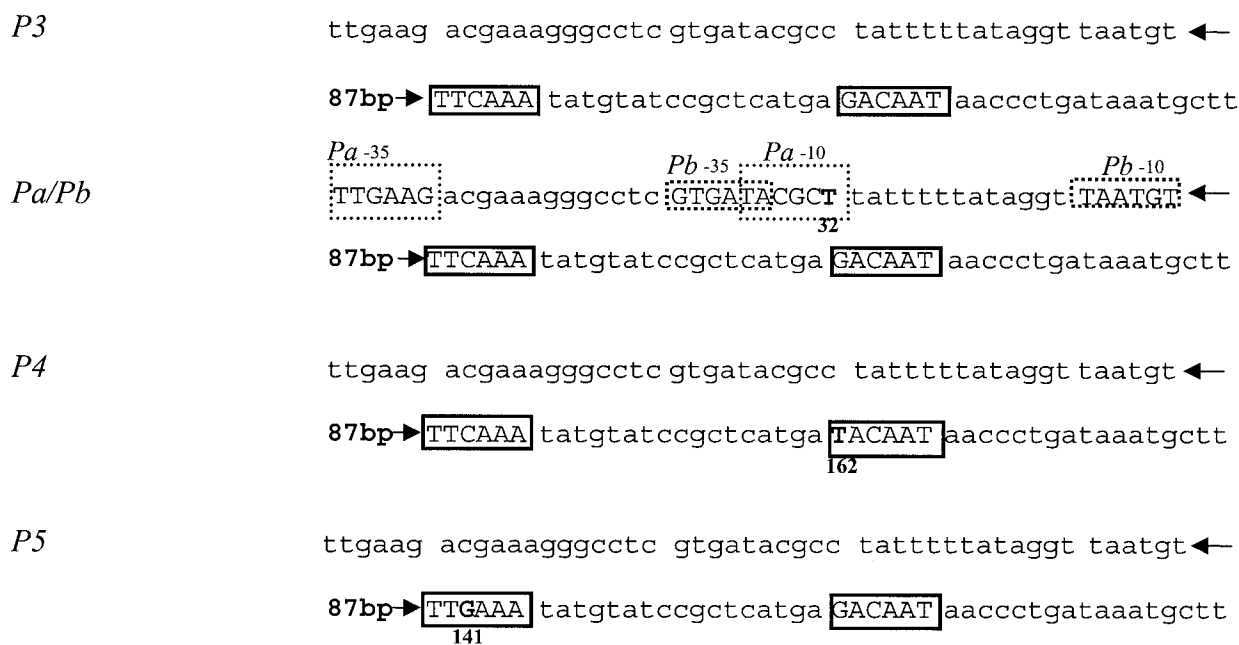


FIG. 1. Nucleotide sequences of promoters *P3*, *Pa/Pb*, *P4*, and *P5* of *bla*<sub>TEM</sub> genes. The sequences of the  $-35$  and  $-10$  regions of promoters *P3*, *P4*, and *P5* are boxed by solid lines, whereas these of the overlapping promoters *Pa/Pb* are boxed by broken lines. Boldface letters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

of  $\beta$ -lactamase activity was defined as the amount of enzyme which hydrolyzed 1  $\mu$ M benzylpenicillin per min, and the specific activity was defined as the  $\beta$ -lactamase activity per milligram of protein.

As indicated in Table 1,  $\beta$ -lactamase activity as well as MICs of amoxicillin-clavulanate, ticarcillin-clavulanate, piperacillin, and cephalothin gradually increased from the *E. coli* transformant expressing the *bla*<sub>TEM-1B</sub> gene controlled by the *P3* promoter to that expressing the *bla*<sub>TEM-1B</sub> gene controlled by the *P5* promoter, with higher values for the two types of parameters when *bla*<sub>TEM-1B</sub> 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  $\mu$ g/ml for the transformants whose *bla*<sub>TEM-1B</sub> gene was controlled by promoters *P3* and *Pa/Pb*, respectively, and high MICs, from 128 to 512  $\mu$ g/ml for those whose *bla*<sub>TEM-1B</sub> 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 $\rightarrow$ G substitution, which oc-

TABLE 1.  $\beta$ -Lactam susceptibility and  $\beta$ -lactamase activity of TEM-1- and TEM-30 (IRT-2)-producing *E. coli* transformants according to the type of promoter upstream from the *bla*<sub>TEM-1B</sub> and *bla*<sub>TEM-30B</sub> genes

Strain and <i>bla</i> <sub>TEM</sub> gene (promoter)	MIC ( $\mu$ g/ml) <sup>a</sup>						$\beta$ -Lactamase activity (U/mg) (mean $\pm$ SD)
	AMC ( $\leq 4$ - $>16$ )	TIC ( $\leq 16$ - $>64$ )	TCC ( $\leq 16$ - $>64$ )	PIP ( $\leq 8$ - $>64$ )	TZB ( $\leq 8$ - $>64$ )	CEP ( $\leq 8$ - $>32$ )	
<i>E. coli</i>							
ATCC 25 922	4	4	4	2	1	8	ND <sup>b</sup>
NM554	2	2	2	1	1	2	ND
Transformant NM554							
<i>bla</i> <sub>TEM-1B</sub> ( <i>P3</i> )	32	8,192	32	128	1	8	6.8 $\pm$ 3.3
<i>bla</i> <sub>TEM-1B</sub> ( <i>Pa/Pb</i> )	128	>8,192	256	512	2	32	13.8 $\pm$ 2.1
<i>bla</i> <sub>TEM-1B</sub> ( <i>P4</i> )	512	>8,192	1,024	1,024	128	64	38 $\pm$ 9
<i>bla</i> <sub>TEM-1B</sub> ( <i>P5</i> )	1,024	>8,192	2,048	>1,024	512	128	87.4 $\pm$ 8
<i>bla</i> <sub>TEM-30B</sub> ( <i>P3</i> )	256	128	32	8	1	8	14 $\pm$ 4.4
<i>bla</i> <sub>TEM-30B</sub> ( <i>Pa/Pb</i> )	2,048	512	128	64	2	8	32.6 $\pm$ 5.6
<i>bla</i> <sub>TEM-30B</sub> ( <i>P4</i> )	4,096	2,048	512	64	32	8	120 $\pm$ 7.9

<sup>a</sup> Drug abbreviations: AMC, amoxicillin with 2  $\mu$ g of clavulanate per ml; TIC, ticarcillin; TCC, ticarcillin with 2  $\mu$ g of clavulanate per ml; PIP, piperacillin; TZB, piperacillin with 4  $\mu$ g of tazobactam per ml; CEP, cephalothin. The MIC breakpoints (in micrograms per milliliter) are shown in parentheses after the drug abbreviations.

<sup>b</sup> 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  $\beta$ -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*<sub>TEM-30</sub> gene, they differed from each other with regard to the  $\beta$ -lactamase activity and the  $\beta$ -lactam MICs with the exception of cephalothin. The lowest MICs, regardless of the penicillins tested, were found in the transformant harboring the *bla*<sub>TEM-30B</sub> gene preceded by promoter *P3*, whereas the highest MICs were found in the transformant harboring the *bla*<sub>TEM-30B</sub> gene controlled by promoter *P4*; intermediate MICs were found in the transformant harboring the *bla*<sub>TEM-30</sub> 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*<sub>TEM-30B</sub> gene were susceptible. For piperacillin, only the transformant with promoter *P3* was susceptible. For the transformants with *bla*<sub>TEM-30B</sub>, the lowest  $\beta$ -lactamase activity was observed in the transformant harboring the *bla*<sub>TEM-30B</sub> gene with promoter *P3*, whereas the highest activity was observed in the transformant harboring the *bla*<sub>TEM-30B</sub> gene with promoter *P4*.

Overall, the increase in  $\beta$ -lactamase activity observed in relation to the presence of promoters *P3*, *Pa/Pb*, and *P4* was demonstrated by using either the *bla*<sub>TEM-1B</sub> gene coding for TEM-1 or the *bla*<sub>TEM-30B</sub> gene coding for TEM-30 or IRT-2. Thus, we were able to compare not only the expression of a given *bla*<sub>TEM</sub> gene in relation to different promoters but also the expression of different *bla*<sub>TEM</sub> genes in relation to a given promoter. In this study, it has been unambiguously demonstrated that, for any one promoter, the  $\beta$ -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  $\beta$ -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  $\beta$ -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*<sub>TEM</sub> genes have on  $\beta$ -lactam resistance according to the type of TEM enzyme involved.

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