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Transferable Hyperproduction of TEM-1 β-Lactamase in *Shigella flexneri* Due to a Point Mutation in the Pribnow Box

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TEM-1 hyperproduction in two ampicillin-sulbactam-resistant *Shigella flexneri* strains was studied. In both strains the bla_{TEM} gene was encoded as a single copy on a large conjugatively transferable plasmid. A single G→T transversion at position 1 of the −10 consensus sequence was identified to be the mechanism of TEM-1 hyperproduction.

Hyperproduction of β-lactamases was found to be an important cause of resistance to β-lactam–β-lactamase inhibitors among clinical isolates of the family *Enterobacteriaceae*. For example, hyperproduction of the plasmid-encoded β-lactamase TEM-1 was reported to be the predominant mechanism of resistance to amoxicillin-clavulanate in about 60% of *Escherichia coli* strains (13, 19). Three mechanisms may lead to hyperproduction. First, an increase in the gene dosage as a result of repetitions of β-lactamase genes; e.g., repetition of the wild-type alleles of the gene encoding AmpC (17), has been known to confer a high level of resistance to amoxicillin-clavulanate. Second, multiple copies of the bla_{TEM} gene due to the presence of multiple copies of plasmid per cell have been reported to cause hyperproduction of β-lactamase (21). Third, hyperproduction of enzyme due to various genetic events affecting the promoter region has been widely reported in various bacteria (2, 3, 10), although this mechanism was much less studied for β-lactamases.

We have isolated in Hong Kong two *Shigella flexneri* strains resistant to ampicillin-sulbactam due to the hyperproduction of TEM-1 β-lactamase, the gene of which was encoded on a large 120-kb plasmid and was transferable to *E. coli* by conjugation. The purpose of the present study was to find the molecular basis of TEM-1 hyperproduction by isolating and analyzing the plasmid DNA and sequencing the β-lactamase genes from the two strains. The results thus obtained were compared with those for a TEM-1-producing, ampicillin-sulbactam-susceptible *S. flexneri* strain in which the bla_{TEM} gene was also found to be encoded on a 120-kb conjugatively transferable plasmid.

The strains used in this study and their characteristics are listed in Table 1. The MICs of ampicillin, ampicillin-sulbactam, amoxicillin, and amoxicillin-clavulanate were determined by the agar dilution method (15). The ampicillin-sulbactam and amoxicillin-clavulanate combinations were tested at a fixed ratio of 2:1. Antibiotic powders were kindly provided by Pfizer Corporation, Hong Kong (ampicillin and sulbactam), and SmithKline Beecham, Hong Kong (amoxicillin and clavulanate). Susceptibility testing of sulfamethoxazole-trimethoprim, chloramphenicol, tetracycline, and rifampin were performed by the Kirby-Bauer disk diffusion method (16). A rifampin-resistant *E. coli* strain, strain JP-995 (kindly supplied by J. Pittard, Department of Microbiology, University of Melbourne, Melbourne, Australia), was used as the recipient in all conjugation experiments. TEM-1 was identified by isoelectric focusing of crude enzyme preparations (14) and by hybridization with a probe specific for bla_{TEM}, which consisted of a 998-bp fragment (1, 4). Plasmid DNA was prepared by alkaline extraction (11), and hybridization was performed on a nylon membrane (Amersham International, Buckinghamshire, United Kingdom) following Southern blotting. The β-lactamase activity in strains K-24 and K-25 was >10-fold higher than that detected in the ampicillin-sulbactam-susceptible CH-04 strain. This hyperproducing phenotype in strains K-24 and K-25 was found to be encoded on a 120-kb plasmid which was transferable to *E. coli* JP-995. The properties of transconjugants K-24, K-25, and CH-04 are presented in Table 1. In both the parental strains and transconjugants, TEM-1 was the only β-lactamase detected.

The possibility of β-lactamase hyperproduction due to an increase in gene dose was studied by restriction enzyme analysis of the 120-kb plasmid. The restriction enzymes *Eco*RI, *Hin*fI, and *Hin*cII (Pharmacia, Hong Kong) were used according to the manufacturer's instructions. The digested plasmid DNAs from the three transconjugants exhibited three different patterns by testing with each of the restriction enzymes used

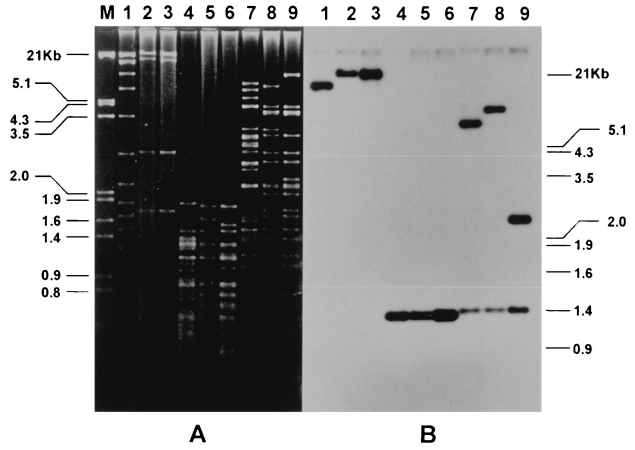

FIG. 1. (A) Restriction patterns of plasmids from the *E. coli* JP-995 transconjugants. Lanes 1 to 3, 4 to 6 and 7 to 9, digestions with *Eco*RI, *Hin*fI, and *Hin*cII, respectively. *E. coli* CH-04 (lanes 1, 4, and 7), K-24 (lanes 2, 5, and 8), and K-25 (lanes 3, 6, and 9) were tested. (B) Southern blot of the same gel shown in panel A after hybridization with a ^{32}P-labeled bla_{TEM} probe.

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TABLE 1. Properties of the strains studied

Strain[a]	Resistance phenotype[b]	MIC (μg/ml)[c]		β-Lactamase activity[d]	Hybridization with TEM probe[e]
		AMC	SAM		
Parental strains					
S. flexneri CH-04	Su, Tp, Cm, Tet	8	8	8	+
S. flexneri K-24	Su, Tp, Cm, Tet	32	≥128	640	+
S. flexneri K-25	Su, Tp, Cm, Tet	32	≥128	1,088	+
Transconjugants					
E. coli CH-04	Su, Tp, Cm, Tet, Rif	8	8	13	+
E. coli K-24	Su, Tp, Cm, Tet, Rif	32	≥128	233	+
E. coli K-25	Rif	32	≥128	157	+
Recipient, *E. coli* JP-995	Rif	4	4		−

[a] *E. coli* CH-04, K-24, and K-25 were transconjugants of *S. flexneri* CH-04, K-24, and K-25, respectively.
[b] Abbreviations: Su, sulfamethoxazole; Tp, trimethoprim; Cm, chloramphenicol; Tet, tetracycline; Rif, rifampin.
[c] AMC, amoxicillin-clavulanate; SAM, ampicillin-sulbactam. The combination of amoxicillin-clavulanate and ampicillin-sulbactam were tested at a fixed ratio of 2:1.
[d] Nanomoles of nitrocefin hydrolyzed per minute per milligram of protein.
[e] +, positive result; −, negative result.

(Fig. 1). Upon hybridization with the bla_{TEM} probe, a single band was detected with *Eco*RI and *Hin*fI digests and two bands were detected with the *Hin*cII digests. Since only *Hin*cII has a cutting site within the β-lactamase gene (20), the hybridization result indicated that in both the ampicillin-sulbactam-susceptible JP-04 strain and the ampicillin-sulbactam-resistant K-24 and K-25 strains, only a single copy of bla_{TEM} was present per plasmid. The presence of multiple copies of bla_{TEM} due to the presence of multiple copies of plasmid per cell was considered unlikely because of the large size of the plasmid. Plasmids reported to have multiple copies leading to β-lactamase hyperproduction were mostly 8 to 16 kb (18).

The bla_{TEM} genes from the three transconjugant strains were sequenced to look for mutations that might account for hyperproduction. For this purpose, purified plasmid DNA from the transconjugants was used as a template for PCR amplification by using deoxy oligonucleotide primers (12) 5′-ATA AAA TTC TTG AAG ACG ACG AAA (primer A) and 5′-GAC AGT TAC CAA TGC TTA ATC A (primer B), synthesized by GIBCO BRL, New York, N.Y.

The entire sequences of the 1,079-bp PCR products from *E. coli* transconjugants CH-04, K-24, and K-25 were determined. In all three strains, the bla_{TEM}-coding region was identical to that of TEM-1 reported in Tn*2* (8). The only difference observed between the genes cloned for the hyperproducing strains K-24 and K-25 and the nonhyperproducing strain CH-04 was a single substitution from G to T at position 1 of the −10 consensus sequence in the hyperproducing strains K-24 and K-25 (Fig. 2).

In an analysis of the promoter DNA sequences in 168 *E. coli* strains by Hawley and McClure (9), a general rule was identified: that the very presence of the two consensus promoter sequences, TTGAC for the −35 region and TATAAT for the −10 region (Pribnow Box), corresponds to maximal function. Mutations that increase the similarity of the sequences of the −10 and −35 regions to these consensus sequences have been reported to increase the expression of chromosomally encoded β-lactamases. In *Klebsiella oxytoca*, hyperproduction of chromosomal β-lactamase both in clinical isolates and in mutants selected in vitro has been reported to be a consequence of a point mutation in either the −10 or the −35 consensus sequence (5, 6). In a study of the β-lactamase gene promoters of 45 clinical isolates of *K. oxytoca* resistant to β-lactam and exhibiting β-lactamase hyperproduction, the most frequently identified mutations were either a transversion (G→T) at position 1 (27%) or a transition (G→A) at position 5 (67%) of the −10 consensus sequence (7). All of the −10 promoters in these strains were closer to the consensus sequence, TATAAT.

In this study, the mechanism of hyperproduction of TEM-1 in *S. flexneri* was found to be related to one point mutation (a change from G to T) at position 1 of the −10 consensus sequence. This G→T transversion increased its similarity to the optimal promoter sequence TATAAT, as has been reported for *E. coli* (9). As a result, more than a 10-fold increase in TEM-1 β-lactamase activity was observed. Previous reports of β-lactamase hyperproduction due to a mutation in the consensus sequence indicated that the mutations mostly occurred in chromosomally encoded genes. The promoter mutations reported here were carried on conjugatively transferable plasmids in naturally occurring strains isolated from patients. The

Source	Sequence		
	-35	-10	start codon
Tn*2*	TACA TTCAA ATATGTATCCGCTCATGA GACAAT	AACCCTGGTAAATGCTTCAATAATATTGAAAAAGGAAGAGT	ATG
CH-04	---- TTCAA ----------	GACAAT ----------	
K-24	---- TTCAA ----------	TACAAT ----------	
K-25	---- TTCAA ----------	TACAAT ----------	

FIG. 2. Comparison of promoter sequences of β-lactamase genes bla_{TEM} from Tn*2*, two hyperproducing *S. flexneri* mutants (K-24 and K-25), and one *S. flexneri* strain (CH-04) that produce low levels of TEM-1. The −35 and −10 regions are underlined. The start codon is indicated in boldface type.

possibility that resistance to β-lactam–β-lactamase inhibitors due to this mechanism might spread to other members of the family *Enterobacteriaceae* raises our concern and should be closely monitored.

Nucleotide sequence accession numbers. The nucleotide sequence data for strains K-24 and K-25 have been submitted to the GenBank nucleotide sequence data library under accession number U48775.

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