ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 1996, p. 460–463 Vol. 40, No. 2
0066-4804/96/$04.00+0

NOTES

β-Lactamase Gene Promoters of 71 Clinical Strains of *Klebsiella oxytoca*

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Received 3 July 1995/Returned for modification 2 October 1995/Accepted 11 December 1995

β-Lactamase gene promoters of 45 clinical *Klebsiella oxytoca* isolates resistant to β-lactams and exhibiting β-lactamase hyperproduction differed from those in 26 susceptible strains. Direct sequencing revealed one mutation in either the −10 or −35 conserved sequences: a G-to-A transition of the fifth base (67%) or a G-to-T transversion of the first base of the −10 sequence (27%) or a T-to-A transversion in the fourth base in the −35 sequence (4%). One strain carried both the −10 transition and the −35 transversion.

Klebsiella oxytoca produces small amounts of a chromosomally encoded β-lactamase. Overproduction of this β-lactamase causes resistance to penicillins, narrow-spectrum cephalosporins, ceftriaxone, and aztreonam (5); about 8 to 10% of *K. oxytoca* strains overproduce this β-lactamase (3, 11, 14–16). Overproduction can result from a mutation in the promoter of the β-lactamase gene. In aztreonam-resistant mutants obtained in vitro, one of two different mutations was observed in the −10 consensus sequence of the promoter (6). Recently, a second type of β-lactamase gene (bla_{OXY-2}) with a high degree of nucleotide identity (87.3%) was described for *K. oxytoca* (7). Several strains carrying the bla_{OXY-2} gene also present the characteristic pattern of resistance (to broad-spectrum cephalosporins and monobactams) observed for *K. oxytoca* strains carrying bla_{OXY-1}.

In this study, we investigated whether aztreonam-resistant mutants selected in vitro from strains harboring bla_{OXY-2} carried promoter mutations. In this work, we also report the characterization of 71 clinical strains susceptible and resistant to β-lactams harboring one or the other of the bla_{OXY} genes. β-Lactam susceptibility, β-lactamase activity, and promoter sequences were determined for these strains. Our study demonstrates that mutations were present in the promoters of the resistant strains.

In vitro selection of resistant strains. Mutants were selected in vitro to verify that the β-lactamase OXY-2 could be overproduced following mutations in the promoter. *K. oxytoca* SL902 and SL911 were isolated from St.-Louis Hospital (Paris, France) in 1990 and 1991 respectively. *K. oxytoca* mutants resistant to aztreonam were selected from these two susceptible strains on Mueller-Hinton agar containing 1 μg of aztreonam per ml as described previously (5). One mutant (SL9111) was obtained from strain SL911 (frequency, 10^{-10} per viable cell), and two mutants (SL9021 and SL9022) were obtained from strain SL902 (frequency, 2×10^{-10} per viable cell). The mutation frequencies were similar to that observed for the bla_{OXY-1} mutant (6). Mutants and parental strains were analyzed by the API 20E and 50CHE systems (bioMérieux). Isoelectric points of in vitro mutants were identical to that of their parental strain, i.e., 5.2 for strain SL911 and its mutant and 6.4 for strain SL902 and its mutants.

Susceptibility testing. *K. oxytoca* strains were recovered from hospitals in the following countries: France (n = 27), Germany (n = 10), Spain (n = 6), Switzerland (n = 14), United Kingdom (n = 6), and the United States (n = 8). They were identified with the API 20E system (bioMérieux) and by two carbon substrate assimilation tests (histamine and ethanolamine) as described previously (12). The type of the β-lactamase gene (bla_{OXY-1} or bla_{OXY-2}) in each of these isolates was previously determined by colony hybridization (7). The susceptibilities to β-lactams of the 71 *K. oxytoca* strains were tested by a disk diffusion method with Mueller-Hinton agar (4). The susceptibility patterns clearly fell into two groups

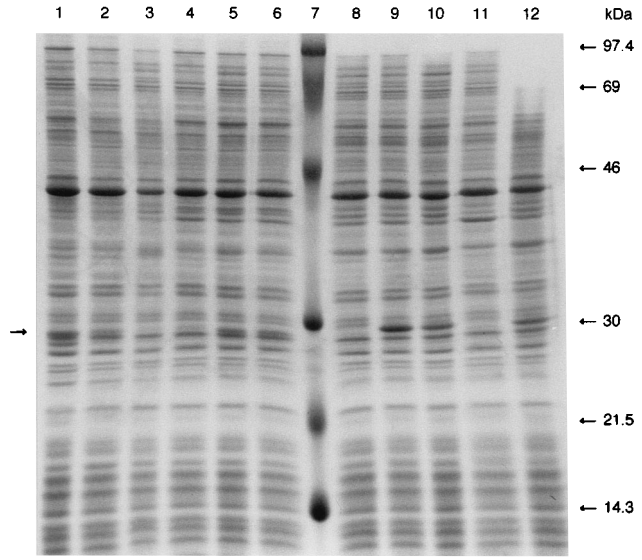

FIG. 1. SDS-PAGE of proteins released by osmotic shock. Volumes of shock fluid equivalent to 40 μg of protein were loaded. Lanes: 1, SL7811; 2, SL7812; 3, SL781; 4, SL901; 5, SL9011; 6, SL9012; 7, standard protein size markers; 8, SL902; 9, SL9021; 10, SL9022; 11, SL911; 12, SL9111. The β-lactamase protein is indicated by the arrow on the right.

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TABLE 1. Susceptibility patterns, according to level of resistance against β-lactams, of the 71 *K. oxytoca* strains[a]

Strain resistance phenotype (no.)	Diameter (mean ± SD [mm]) of inhibition zone with[b]:								
	Amoxicillin (25 μg)	Amoxicillin (20 μg) + clavulanate (10 μg)	Ticarcillin (75 μg)	Cephalothin (30 μg)	Cefuroxime (30 μg)	Cefoxitin (30 μg)	Cefotaxime (30 μg)	Ceftazidime (30 μg)	Aztreonam (30 μg)
Susceptible (26)	11.2 ± 3.2	28.5 ± 2.1	16.8 ± 3.8	24.9 ± 3.3	28.1 ± 2.7	29.3 ± 1.8	35.7 ± 1.5	33.7 ± 1.4	35.0 ± 2.6
Resistant (45)	6.6 ± 0.9	14.9 ± 2.3	6.5 ± 0.9	7.7 ± 2.0	9.1 ± 3.9	27.4 ± 2.7	28.6 ± 3.3	30.6 ± 2.4	15.8 ± 5.7

[a] Resistance determined by a disk diffusion method.
[b] The disk contents are specified in parentheses.

(Table 1): the first included the 26 strains resistant to penicillins (amoxicillin and ticarcillin) and susceptible to the cephalosporins examined; the second included 45 strains resistant to penicillins, narrow-spectrum cephalosporins (cephalothin), cefuroxime, and aztreonam. The susceptibility patterns of in vitro mutants and the resistant clinical strains harboring either bla_{OXY-1} or bla_{OXY-2} were indistinguishable.

β-Lactamase assays. The strains resistant to β-lactams, including cefuroxime and aztreonam, were tested for overproduction of their β-lactamase by determination of β-lactamase activity by the iodometric method and estimation, on sodium

TABLE 2. Summary of promoter consensus sequences of 71 clinical aztreonam-susceptible and -resistant *K. oxytoca* strains

bla gene	Promoter sequence[a]		Strain		β-Lactamase activity[c] according to origin	Ave. β-lactamase activity[c] for each promoter	Susceptibility[d]
	−35	−10	No.	Origin[b] (no.)			
bla_{OXY-1}	TTGTCA	GATAGT	14	France (5)	17.2 (5.3)	19.0 (6.6)	36.3 (1.8)
				Germany (1)	7.1		
				Spain (2)	24.7 (8.4)		
				Switzerland (1)	18.5		
				U.K. (2)	17.2 (4.4)		
				U.S.A. (3)	23.5 (6.3)		
	TTGTCA	GATA**A**T	12	France (8)	1,618 (1,275)	1,569 (1,023)	20.6 (5.8)
				Spain (1)	1,687		
				U.K. (2)	1,368 (13)		
				U.S.A. (1)	1,464		
	TTGTCA	**T**ATAGT	7	France (2)	2,727 (245)	2,539 (2,083)	16.3 (2.1)
				Spain (1)	6,341		
				Switzerland (4)	1,565 (1,483)		
	TTG**A**CA	GATAGT	1	France (1)	2,546		25
bla_{OXY-2}	TTGTCA	GATAGT	12	France (2)	24.3 (9.0)	30.2 (11.4)	34.7 (1.8)
				Germany (1)	28.1		
				Spain (2)	48.0 (6.5)		
				Switzerland (3)	28.4 (5.0)		
				U.K. (1)	44.9		
				U.S.A. (3)	19.9 (3.0)		
	TTGTCA	GATA**A**T	18	France (6)	3,850 (1,135)	4,360 (1,429)	12.9 (1.8)
				Germany (6)	4,940 (1,822)		
				Switzerland (4)	4,514 (1,244)		
				U.K. (1)	5,029		
				U.S.A. (1)	2,628		
	TTGTCA	**T**ATAGT	5	France (2)	6,433 (2,613)	6,501 (1,884)	12.6 (4.4)
				Germany (2)	6,535 (2,714)		
				Switzerland (1)	6,567		
	TTG**A**CA	GATAGT	1	France (1)	2,481		22
	TTG**A**CA	GATA**A**T	1	Switzerland (1)	5,142		9

[a] Consensus sequences at −35 and −10, separated by 17 bp. Mutations are indicated in boldface type.
[b] U.K., United Kingdom; U.S.A., United States of America.
[c] β-Lactamase activity in microunits of β-lactamase per milligram of protein. Values in parentheses are standard deviations.
[d] Susceptibility to aztreonam expressed as diameters of inhibition zones (in millimeters). Values in parentheses are standard deviations.

TABLE 3. Comparison of promoter sequences of β-lactamase gene bla_{OXY-2} from in vitro *K. oxytoca* mutants and that of the SL781 β-lactamase gene (bla_{OXY-1})

Sequence[a]	Strain
EcoRI -35 -10 +1	
GAATTCCATCATCAATAAATTGCTTGTCAAAATAGCGGGAGTCGCAGATAGTCCGCTGCGACTTATCACTCTCAAGGAGTCAGAAATG	SL911
----------A----------	SL9111
----------A---------	SL902
----------T----------A---------	SL9021
----------A----------A---------	SL9022
----------G-C-T-G----------GC----------G---------GGCA- --C------A----------T--G---	SL781

[a] The −35 and −10 regions and the start codon are singly underlined. The transcription start sites are shown in boldface type and labeled +1. The ribosome-binding site is indicated by double underlining. The *Eco*RI site is underlined with a dotted line. The nucleotide sequence data reported in this paper will appear in the EMBL-GenBank-DDBJ nucleotide sequence data libraries under accession no. Z49084 for SL911 and its mutant and accession no. Z49083 for SL902 and its mutants.

dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), of the amount of β-lactamase produced by osmotic shock.

An overnight culture was diluted 100-fold in 50 ml of Luria-Bertani broth. After 3 h of incubation at 37°C, 20 ml of the culture was centrifuged and the pellet was sonicated as previously described (5) to obtain crude extract. β-Lactamase activity in this crude extract was measured by a quantitative iodometric method with benzylpenicillin as the substrate (13). The remaining 30 ml of culture was used for osmotic shock by a procedure previously described (1). After protein denaturation, shock fluid containing 40 μg of protein was loaded onto a polyacrylamide gel (12.5%) in the presence of SDS. β-Lactamase amounts were estimated after staining with Coomassie blue.

The level of β-lactamase activity estimated by the iodometric method was higher in the 45 resistant strains (3,539 ± 2,132 mU of β-lactamase per mg of protein) than that in the 26 susceptible strains (24.2 ± 10.6) (Comparison of the average values of the two groups by the Student-Fisher t test, $P < 0.001$) (Table 2).

β-Lactamase enzyme was obtained by osmotic shock of all the susceptible and resistant strains and migrated with a molecular mass of 29.0 ± 0.5 kDa as observed by Arakawa et al. (2) (Fig. 1). The susceptible strains SL781 and SL901 and their respective overproducing mutants SL7811, SL7812, SL9011, and SL9012 carry the bla_{OXY-1} gene (6). The previously described susceptible strains SL911 and SL902 (7) and their respective mutants carry the bla_{OXY-2} gene. The susceptible strains produced small quantities of β-lactamase. The mutants obtained in vitro produced large amounts of β-lactamase (Fig. 1). Similarly, the 26 susceptible clinical strains of *K. oxytoca* produced small amounts of β-lactamase whereas the 45 resistant strains produced large amounts (data not shown).

Promoter sequencing. Primers used to amplify the β-lactamase promoter were primer Q, 5′-d[TTC ACA AAG CGC TCG GCA AT]-3′ and primer R, 5′-d[CTT TAC TGG TGC TGC ACA TG]-3′. The sequences of PCR products were determined directly with the T7 sequencing kit (Pharmacia).

One of two different point mutations was identified in the in vitro mutant promoters (Table 3): a transversion (G→T) of the first base of the −10 consensus sequence (SL9021) and a transition (G→A) of the fifth base (in the other two mutants, SL9111 and SL9022). The three mutants carried the same mutations as those previously reported for bla_{OXY-1} (6).

The promoter sequences of β-lactamase genes from the 71 clinical strains were sequenced (Table 2). As expected, all the 26 susceptible *K. oxytoca* strains had the same −10 and −35 consensus sequences (GATAGT and TTGTCA, respectively). All the resistant strains were mutated in one of the two consensus sequences, more often in the −10 consensus sequence of the promoter (95% of the resistant strains). The most frequent mutation was the transition (G→A) of the fifth base (60% of the bla_{OXY-1} and 72% of the bla_{OXY-2} overproducing strains). The same mutation was similarly frequent among the in vitro mutants (75% of the bla_{OXY-1} mutants [6] and 66% of the bla_{OXY-2} mutants). The transversion (G→T) of the first base was less common: 35% for the bla_{OXY-1} and 20% for the bla_{OXY-2} clinical resistant strains.

Only two clinical strains (one with bla_{OXY-1} and one with bla_{OXY-2}) presented a mutation in the −35 consensus sequence: a transversion (T→A) in the fourth base. The same mutation has been described for the *ampC* β-lactamase gene in *Escherichia coli*. It causes a 22-fold increase in transcription from the promoter (9). This mutation was rare in our series (occurring in 5% of the bla_{OXY-1} and 4% of the bla_{OXY-2} resistant strains). Finally both this mutation and the transition (G→A) in the −10 consensus sequence were found in one strain.

Changes in the −35 and −10 regions have strong influences on the promoter strength (10). In *E. coli*, the promoter is stronger the closer the sequence is to the consensus (−35 consensus sequence, TTGACA; −10 consensus sequence, TATAAT) (8). All of the mutations reported in this work resulted in greater similarity of the promoter sequence with the consensus *E. coli* promoter sequence.

Studies of β-lactamase activity and aztreonam resistance suggest that the transition in the −10 consensus sequence seems to give a weaker promoter than does the transversion. We are now testing the strengths of these various promoters with a reporter gene on a promoter-probe plasmid.

We thank P. C. Appelbaum (Hershey Medical Center, Hershey, Pa.), F. Baquero (Hospital Ramon y Cajal, Madrid, Spain), A. Bauernfeind (Max von Pettenkofer-Institut, Munich, Germany), F. Goldstein (Hôpital Saint-Joseph, Paris, France), V. Jarlier (Hôpital de la Pitié, Paris, France), D. M. Livermore (London Hospital Medical College, London, United Kingdom), F. C. Tenover (National Center for Infectious Diseases, CDC, Atlanta, Ga.), and R. L. Then (Hoffmann-La Roche, Basel, Switzerland) for the gifts of *K. oxytoca* strains. We also thank P. H. Roy (Université Laval, Québec, Canada) for critical reading of the manuscript.

B.F. was supported by a grant from the Ministère de la Recherche et de l'Espace.

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