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Extended-Spectrum Beta-Lactamases among *Enterobacter* Isolates Obtained in Tel Aviv, Israel

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The extended-spectrum beta-lactamase (ESBL)-producing phenotype is frequent among *Enterobacter* isolates at the Tel Aviv Sourasky Medical Center, Tel Aviv, Israel. We examined the clonal relatedness and characterized the ESBLs of a collection of these strains. Clonal relatedness was determined by pulsed-field gel electrophoresis. Isoelectric focusing (IEF) and transconjugation experiments were performed. ESBL gene families were screened by colony hybridization and PCR for bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{IBC}, bla_{PER}, bla_{OXA}, bla_{VEB}, and bla_{SFO}; and the PCR products were sequenced. The 17 *Enterobacter* isolates studied comprised 15 distinct genotypes. All isolates showed at least one IEF band (range, one to five bands) whose appearance was suppressed by addition of clavulanate; pIs ranged from 5.4 to ≥8.2. Colony hybridization identified at least one family of beta-lactamase genes in 11 isolates: 10 harbored bla_{TEM} and 9 harbored bla_{SHV}. PCR screening and sequence analysis of the PCR products for bla_{TEM}, bla_{SHV}, and bla_{CTX-M} identified TEM-1 in 11 isolates, SHV-12 in 7 isolates, SHV-1 in 1 isolate, a CTX-M-2-like gene in 2 isolates, and CTX-M-26 in 1 isolate. In transconjugation experiments with four isolates harboring bla_{TEM-1} and bla_{SHV-12}, both genes were simultaneously transferred to the recipient strain *Escherichia coli* HB101. Plasmid mapping, PCR, and Southern analysis with TEM- and SHV-specific probes demonstrated that a single transferred plasmid carried both the TEM-1 and the SHV-12 genes. The widespread presence of ESBLs among *Enterobacter* isolates in Tel Aviv is likely due not to clonal spread but, rather, to plasmid-mediated transfer, at times simultaneously, of genes encoding several types of enzymes. The dominant ESBL identified was SHV-12.

Enterobacter spp. are leading nosocomial pathogens (26) that commonly cause pneumonia (25) and that are the most frequent gram-negative organisms causing bloodstream infections in intensive care units (14, 33). More than one-third of the *Enterobacter* sp. isolates in intensive care units reporting to the National Nosocomial Infection Surveillance System (26) are resistant to extended-spectrum cephalosporins. Moreover, treatment with extended-spectrum cephalosporins may lead to the emergence of resistance to these antimicrobial agents among susceptible strains of *Enterobacter* spp. (8, 19, 35). Emergence of resistance results in increased rates of mortality, longer hospital stays, and higher hospital charges (9, 10).

The resistance of *Enterobacter* spp. to beta-lactam antibiotics is most frequently mediated by hyperproduction of chromosomal AmpC beta-lactamase, caused either by induction or, more likely, by selection of derepressed mutants (1). In the last decade, the production of plasmid-mediated extended-spectrum beta-lactamases (ESBLs) has been recognized as an additional important emerging mechanism of resistance among members of the family *Enterobacteriaceae* (3, 17), including clinical isolates of *Enterobacter* spp. (31). The most common ESBLs found in clinical isolates of *Enterobacter* spp. belong to the TEM-, SHV-, and CTX-M-derived β-lactamases (2, 5, 36). However, other ESBLs have recently been reported in *Enterobacter* spp., including IBC-1, which was detected in an *Enterobacter cloacae* isolate in Greece (15, 18); VEB-1, which was found in clinical isolates of *E. cloacae* and *Enterobacter sakazakii* in Bangkok, Thailand (16); and SFO-1, which was detected in *E. cloacae* isolates in Japan (24). The prevalence of ESBLs among members of the family *Enterobacteriaceae* may vary significantly between geographical areas (40). For example, Schwaber et al. (34) recently reported that less than 2% of 152 U.S. isolates of *Enterobacter* spp. were confirmed to produce ESBLs. In sharp contrast, we recently investigated the occurrence of the ESBL-producing phenotype among members of the family *Enterobacteriaceae* at the Tel Aviv Sourasky Medical Center, Tel Aviv, Israel, and found that 22% of the *Enterobacter* clinical isolates in our institution had this phenotype (27). To understand the widespread occurrence of the ESBL phenotype in *Enterobacter* spp. at the Tel Aviv Sourasky Medical Center, we examined the genetic relatedness between these ESBL producers and characterized the ESBL enzyme families produced by these strains.

MATERIALS AND METHODS

Bacterial strains and susceptibility testing. Seventeen unique *Enterobacter* sp. patient isolates identified as ESBL producers (27) were studied. All the strains were isolated over a 6-month period, from June 2000 through December 2000, at the Tel Aviv Sourasky Medical Center, a 1,200-bed, tertiary-care, university-affiliated hospital. Bacterial identification to the species level and the MICs of the broad-spectrum cephalosporins cefotaxime, ceftazidime, and cefepime, the monobactam antibiotic aztreonam, and the ureidopenicillin piperacillin were performed with an automated identification and microdilution system (Microscan, Dade International, Inc., West Sacramento, Calif.) by using an over-

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TABLE 1. Oligonucleotides used for PCR amplification of ESBL genes

Primer type and gene family	Sequence[a]	Annealing temp (°C)	Reference or source	PCR product size (kb)
Screening primers				
16S	For: 5′-CCGCACAAGCGGTGGAGCA-3′ Rev: 5′-AGGCCCGGGAACGTATTCAC-3′	42	32	0.4
TEM	For: 5′-TCAACATTTCCGTGTCG-3′ Rev: 5′-CTGACAGTTACCAATGCTTA-3′	42	This study	0.86
SHV	For: 5′-ATGCGTTATATTCGCCTGTG-3′ Rev: 5′-AGATAAATCACCACAATGCGC-3′	47	This study	0.78
CTX-M	For: 5′-RGMAGYGYRMCGCTKYATGCSC-3′ Rev: 5′-ARTARGTSACCAGAAYVAGCGG-3′	55	This study	0.73
IBC	For: 5′-GGGCGTACAAAGATAATTTCC-3′ Rev: 5′-GAAGCAACGTCGGCTTGAACG-3′	47	This study	0.94
VEB	For: 5′-ACGGTAATTTAACCAGATAGG-3′ Rev: 5′-ACCCGCCATTGCCTATGAGCC-3′	46	This study	0.97
SFO	For: 5′-GTTAATCCATTTTATGTGAGG-3′ Rev: 5′-CAGATACGCGGTGCATATCCC-3′	44	This study	0.94
PER	For: 5′-ATGAATGTCATTATAAAAGC-3′ Rev: 5′-AATTTGGGCTTAGGGCAGAA-3′	42	39	0.93
OXA-4	For: 5′-ACACAATACATATCAACTTCGC-3′ Rev: 5′-AGTGTGTTTAGAATGGTGATC-3′	42	34	0.81
CTX-M	For: 5′-CGYTTTSCIATGTGCAG-3′ Rev: 5′-ACCGCRATATCRTTGGT-3′	54	This study	0.55
OXA-3	For: 5′-TTCAAGCCAAAGGCACGATAG-3′ Rev: 5′-TTCGAGTTGACTGCCGGGTTG-3′	42	34	0.7
Full gene amplification and sequencing primers				
TEM	For: 5′-KACAATAACCCTGRTAAATGC-3′ Rev: 5′-AGTATATATGAGTAAACTTGG-3′	42	This study	0.94
SHV	For: 5′-TTTATCGGCCYTCACTCAAGG-3′ Rev: 5′-GCTGCGGGCCGGATAACG-3′	50	This study	0.93

[a] For, forward primer; Rev, reverse primer. R, A or G; M, A or C; Y, C or T; K, G or T; S, G or C; I, Inosin.

night panel; and the results were recorded and interpreted according to NCCLS guidelines (28). *Escherichia coli* 4107 (bla_{TEM-26}; pI 5.6), *Klebsiella oxytoca* 4076 (K1; pI 6.5), *E. coli* 4075 (TEM-1; pI 5.4), *E. cloacae* 4080 (P99; pI 7.8), and *E. coli* 4133 (SHV-1; pI 7.6) were used as standards for isoelectric focusing (IEF). These strains, as well as *E. coli* J53(pMG267) ($bla_{CTX-M-14}$) and *E. coli* J53 R55 (bla_{OXA-3}), which were used as positive controls, and *E. coli* ATCC 25922, which was used as a negative control, were used in the PCR assays. A strain carrying bla_{PER-1} was used as a positive control in the PCR assay for the presence of this gene. *E. coli* HB101 (Str^r Amp^s; Promega) was used as the recipient strain in the conjugation experiments to study plasmid-transferable beta-lactamases. *E. coli* 4107 (bla_{TEM-26}), *E. coli* J53(pAFF2) (bla_{SHV-5}), and *E. coli* J53(pMG203) (bla_{OXA-4}) were used as positive controls and *Pseudomonas aeruginosa* PU21 was used as a negative control for colony hybridization.

Determination of ESBL-producing phenotype. The ESBL-producing phenotypes of the 17 isolates were determined by Kirby-Bauer disk diffusion test methodology. The zones of inhibition of each isolate were tested on Mueller-Hinton agar plates (Hy-Labs, Rehovot, Israel) with disks containing 30 μg of cefpodoxime, cefotaxime, and ceftazidime, alone and in combination with 10 μg of clavulanic acid (CA; Oxoid, Basingstoke, England). An organism was classified as having an ESBL-producing phenotype if the zone of inhibition produced by at least one combination disk was ≥5 mm larger than that produced by the corresponding lone antibiotic-impregnated disk (28). *E. coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 were used as negative and positive controls for ESBL production, respectively.

PFGE analysis. Pulsed-field gel electrophoresis (PFGE) was performed with all of the 17 ESBL-positive *Enterobacter* strains. Bacterial DNA was prepared and cleaved with 20 U of SpeI endonuclease (New England Biolabs, Boston, Mass.), as described previously (29). Plugs were loaded onto a 1% agarose gel (BMA Products, Rockland, Maine) prepared and run in 0.5× Tris-borate-EDTA buffer on a CHEF-DR III apparatus (Bio-Rad Laboratories, Inc., Hercules, Calif.). Electrophoresis was performed at 6 V/cm and 14°C. The run time was 23 h, with pulse times ranging from 5 to 40 s. Gels were stained with ethidium bromide, destained in distilled water, and photographed under UV light with a GelDoc camera (Bio-Rad). PFGE DNA macrorestriction patterns were visually compared and interpreted according to the criteria established by Tenover et al. (38).

Beta-lactamase characterization. *Enterobacter* sp. clinical isolates were cultured on tryptic soy broth (Biolife Italiana, Milan, Italy) and harvested, and cell lysates were prepared by sonication. The protein concentrations in the sonic extracts of the cells were determined with the Bradford reagent (Bio-Rad). Detection of beta-lactamases and determination of pIs were performed by IEF electrophoresis with Ampholine PAGplate pH gradient 3 to 9.5 gels in a Multiphor II electrophoresis system apparatus (Amersham Biosciences, Uppsala, Sweden). Beta-lactamases with known pIs (pIs 5.4, 5.6, 6.5, 7.6, and 7.8) were

TABLE 2. MICs of beta-lactams for *Enterobacter* clinical isolates

Strain	MIC (μg/ml)[a]						
	AMP	A/S	ATM	CTX	CAZ	CEP	IMP
E. aerogenes 1063	>32	>32	>64	>64	>64	>64	2
E. aerogenes 1220	>32	>32	2	8	>64	<1	<0.5
E. aerogenes 1620	>16	>32	>64	>64	>64	>64	<0.5
E. cloacae 1061	>32	>32	>64	16	>64	2	<0.5
E. cloacae 1434	>32	>32	>64	>64	>64	>64	<0.5
E. cloacae 1330	>32	>32	>64	>64	>64	2	<0.5
E. cloacae 1598	>32	>32	>64	>64	>64	4	<0.5
E. cloacae 1005	>32	>32	>64	>64	>64	<1	<0.5
E. cloacae 1018	>32	>32	>64	32	>64	8	<0.5
E. cloacae 1143	>32	>32	>64	>64	>64	2	<0.5
E. cloacae 1250	>32	>32	>64	8	>64	<1	<0.5
E. cloacae 1262	>32	16	16	2	16	<1	<0.5
E. cloacae 1274	>32	>32	2	8	16	2	<0.5
E. cloacae 1408	>32	>32	>64	>64	>64	16	<1
E. cloacae 1522	>32	>32	>64	8	>64	<1	<0.5
E. cloacae 1527	>32	>32	>64	8	>64	<1	<0.5
E. cloacae 1538	>32	>32	>64	>64	>64	2	<0.5

[a] AMP, ampicillin; A/S, ampicillin sulbactam; ATM, aztreonam; CTX, cefotaxime; CAZ, ceftazidime; CEP, cefepime; IMP, imipenem.

electrophoresed in parallel as controls. Beta-lactamase activity was revealed with nitrocefin (0.5 mg/ml; Calbiochem-Novabiochem Corp., San Diego, Calif.) (29) and with CA prior to testing with nitrocefin for determination of the CA inhibition effect.

Screening for ESBL gene families by colony hybridization. Clinical isolates of *Enterobacter* spp. were grown overnight on Mueller-Hinton agar plates containing 100 μg of ampicillin per ml. Single colonies were transferred to Hybond-N^+ membranes (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom) that were placed on Whatman papers sequentially saturated with a denaturation solution (0.5 M NaOH, 1.5 M NaCl) and a neutralization solution (0.5 M Tris-HCl, 1.5 M NaCl). The DNA was allowed to cross-link to the membrane for 2 min at 302 nm. The probes were prepared by labeling the DNA fragments with [α-^{32}P]dCTP with a random primer DNA labeling mixture (Biological Industries, Beit Haemek, Israel). The TEM-specific probe was prepared from the 467-bp PCR fragment of $bla_{\text{TEM-26}}$, the SHV-specific probe was prepared from the 480-bp PCR fragment of $bla_{\text{SHV-5}}$ (30), and the OXA-4-specific probe was prepared from the 813-bp PCR fragment of $bla_{\text{OXA-4}}$.

Detection of ESBL genes by PCR. Bacterial cell lysates were used as templates in the specific PCR amplifications for detection of the bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$, bla_{IBC}, bla_{PER}, bla_{OXA}, bla_{VEB}, and bla_{SFO} genes. Cell lysates were prepared from overnight cultures grown on solid medium (Luria-Bertani medium [LB] plus 12 μg of ceftazidime per ml) by inoculating a single colony into 100 μl of double-distilled water, boiling at 95°C for 10 min, and discarding the cellular debris by centrifugation (12,000 × *g*, 2 min, 4°C). PCRs were performed with Hot-StarTaq DNA polymerase (Qiagen, Hilden, Germany), according to the instructions of the manufacturer, in the presence of 1 μl of the template DNA preparation in a total volume of 50 μl. The PCR conditions were as follows: 15 min at 95°C and 35 cycles of 1 min at 94°C, 1 min at an annealing temperature designed for each primer set (Table 1), and 1 min at 72°C, followed by 10 min at 72°C. The primer pairs used (Metabion GmbH, Martinsried, Germany) and their respective annealing temperatures and expected product lengths are listed in Table 1. The resulting PCR products were analyzed and visualized in a 1.5% agarose gel with ethidium bromide staining and UV light. PCR analysis was performed with primers specific for the 16S rRNA gene (32). These primers were used as controls to determine whether the cell lysate could be amplified.

Sequence analyses. Sequences were analyzed with an ABI PRISM 3100 Genetic Analyzer (PE Biosystems) with DNA Sequencing Analysis Software and 3100 Data Collection Software (version 1.1).

The nucleotide and deduced protein sequences were analyzed and compared by use of the software available online at http://www.mbio.ncsu.edu/BioEdit/bioedit.html.

Transconjugation experiments. The *Enterobacter* sp. and *E. coli* HB101 isolates studied were grown overnight on LB plates containing either 12 μg of ceftazidime per ml or 200 μg of streptomycin per ml, cross-spread on Mueller-Hinton agar plates, and incubated overnight at 37°C. Colonies appearing in the cross area were pooled and spread on LB plates containing 12 μg of ceftazidime per ml and 200 μg of streptomycin per ml. Isolated colonies, obtained after overnight incubation at 37°C and referred to as transconjugant colonies, were further analyzed.

Plasmid isolation and Southern analysis. Plasmid DNA was isolated from two selected clinical *Enterobacter* strains and their *E. coli* transconjugants by using a Plasmid Midi kit (Qiagen). Plasmid DNA was digested with SmaI, XhoI, and BamHI endonucleases (MBI Fermentas); and the resulting restriction pattern was visualized in a 1% agarose gel by ethidium bromide staining. DNA was transferred from the agarose gel to a positively charged Hybond N^+ membrane (Amersham Biosciences, Little Chalfont, United Kingdom) and cross-linked with UV light. The TEM- and SHV-specific probes used in the Southern analysis were the same as those used in the colony hybridization described above.

RESULTS

Bacterial strains and antibiotic susceptibilities. Seventeen unique patient *Enterobacter* sp. isolates with an ESBL-producing phenotype were studied. All were identified to the species level: 14 as *E. cloacae* and 3 as *E. aerogenes*. The MICs for these isolates show that the MICs of at least one of the cephalosporins tested were within the susceptible range for 8 (47%) isolates and within the intermediately susceptible range for 2 (12%) isolates. Seven strains (41%) were resistant to all cephalosporins and aztreonam (Table 2).

PFGE analysis. The 17 clinical isolates possessed 15 distinct PFGE patterns; 13 clones included a single isolate each. Two clones, clone C and clone E (including subtype E′), included two isolates each (Fig. 1 and Table 3).

Screening for ESBL gene families. IEF followed by colony hybridization was carried out with all *Enterobacter* isolates to screen for the presence of ESBL gene families. All isolates showed at least one IEF band indicating hydrolysis of nitrocefin. The pIs of these beta-lactamases inhibited by CA ranged from 5.4 to ≥8.2 (Table 3). Thirteen isolates had a beta-lactamase with a pI of 5.4, and 11 strains had a CA-inhibited beta-lactamase with a pI of 8.2. All isolates were screened for the presence of the bla_{TEM} and bla_{SHV} genes by colony hybridization. Ten of them hybridized with the probe specific for the bla_{TEM} gene, and nine hybridized with the bla_{SHV} gene-specific probe (Table 3). Only the positive control strain hybridized with the probe specific for $bla_{\text{OXA-4}}$, and therefore, PCR screening for this gene was not performed.

Analysis of ESBL genes by PCR. The PCR results showed that one isolate (*Enterobacter* sp. strain 1063) contained three groups of ESBLs: bla_{TEM}, bla_{SHV}, and $bla_{\text{CTX-M}}$. Seven isolates harbored both bla_{TEM} and bla_{SHV}, two isolates harbored both bla_{TEM} and $bla_{\text{CTX-M}}$, and one isolate harbored only bla_{TEM}. These results were similar to the colony hybridization screening results, with a few exceptions: one isolate positive for bla_{TEM} and bla_{SHV} by colony hybridization did not yield either gene by PCR. On the other hand, two isolates whose colonies did not hybridize with a labeled bla_{TEM}-specific probe yielded a bla_{TEM} product by PCR (Table 3).

PCR analysis for the $bla_{\text{CTX-M}}$ family identified three isolates with a $bla_{\text{CTX-M}}$ product. All isolates were screened with primers specific for $bla_{\text{OXA-7}}$ and $bla_{\text{PER-1}}$, and all were negative. The results of the screening for bla_{IBC}, bla_{VEB}, and bla_{SFO} were negative as well, although these results must be interpreted with caution due to the absence of suitable positive control strains for these genes.

Sequence analysis of the entire open reading frame of bla_{TEM} revealed that all bla_{TEM} PCR products were TEM-1 (11

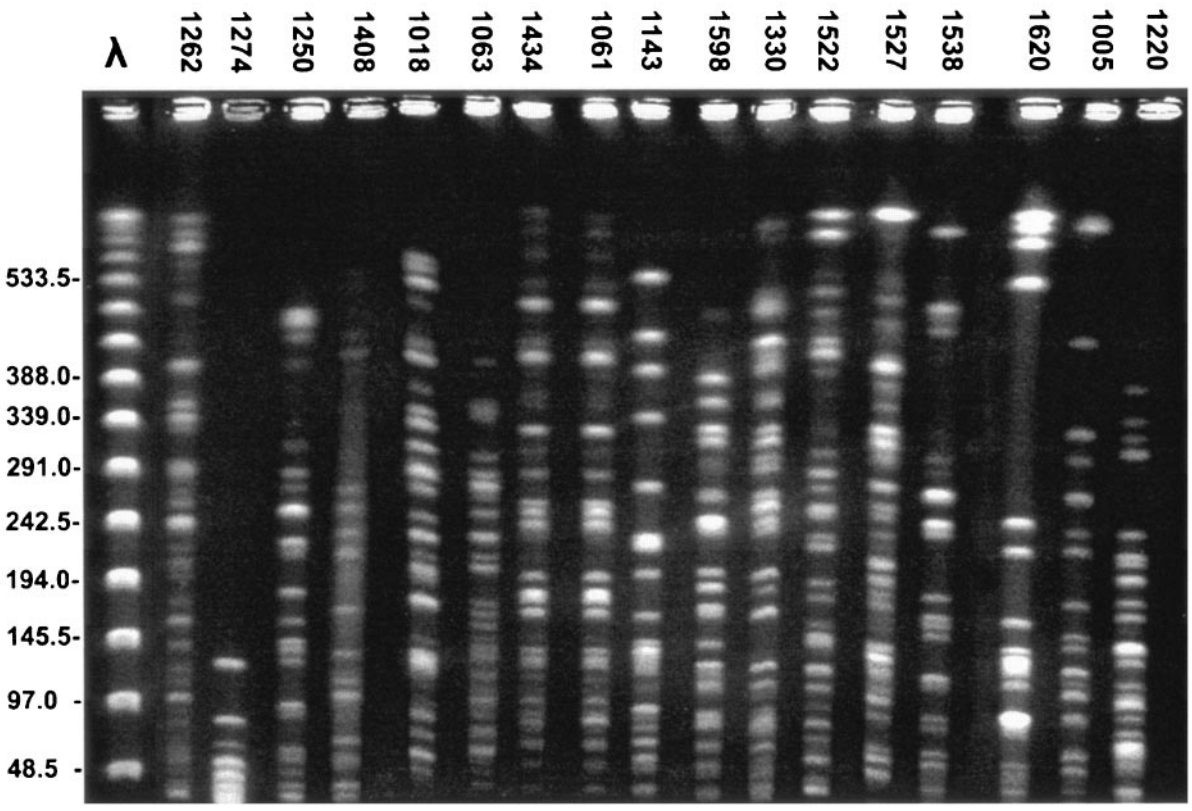

FIG. 1. PFGE of the *Enterobacter* sp. clinical isolates. Lane λ, bacteriophage lambda concatemers as molecular size markers; the remaining lanes contain different clinical isolates, as indicated above each lane.

isolates) and that all except one of the bla_{SHV} products were SHV-12 (7 isolates); one product was identified as bla_{SHV-1}. PCR detected bla_{CTX-M} in three of our *Enterobacter* isolates. Sequencing identified two of the three bla_{CTX-M} genes as being similar to $bla_{CTX-M-2}$ and the second as being similar to $bla_{CTX-M-26}$ (Table 3). The results of IEF and PCR suggested that the beta-lactamases with a pI of 5.4 were TEM-1 and that in several of the isolates the beta-lactamases with a pI of 8.2 were SHV-12. The band of pI 7.8 could be ascribed to isolates possessing $bla_{CTX-M-2}$.

SHV-12 was found to coexist with TEM-1 in both isolates

TABLE 3. IEF analysis and ESBL genes of ESBL-producing *Enterobacter* sp. isolates

Strain	PFGE type	pI(s)	Detection and characterization of ESBL genes				
			bla_{TEM}		bla_{SHV}		bla_{CTX-M} by PCR
			PCR	Colony hybridization	PCR	Colony hybridization	
E. aerogenes 1063	B	5.4, 5.8, 6.6, 7.6, 7.8	TEM-1	−	SHV-1	+	CTX-M-2 like
E. aerogenes 1220	K	8.2	−[a]	−	−	−	−
E. aerogenes 1620	I	5.4, 8.2	−	+	−	+	−
E. cloacae 1061	C	5.4, 6.8, 8.2	TEM-1	+	SHV-12	+	−
E. cloacae 1434	C	5.4, 5.6, 6.6, 8.2	TEM-1	+	SHV-12	+	−
E. cloacae 1330	E	5.4, 8.2	TEM-1	+	SHV-12	+	−
E. cloacae 1598	E′	5.4	TEM-1	−	−	−	−
E. cloacae 1005	J	5.4, 5.8, 6.8, 7.2, 7.6, 7.8	−	−	−	−	−
E. cloacae 1018	A	5.4, 5.8, 6.8	TEM-1	+	−	−	CTX-M-26 like
E. cloacae 1143	D	6.8, 7.8, 8.2, >8.2	−	−	−	−	−
E. cloacae 1250	N	5.4, 7.8, 8.2	TEM-1	+	SHV-12	+	−
E. cloacae 1262	L	5.4, 5.6, 8.2	TEM-1	+	SHV-12	+	−
E. cloacae 1274	M	5.4, 7.8, >8.2	TEM-1	+	−	−	CTX-M-2
E. cloacae 1408	O	6.8, 7, 7.6, 7.8	−	−	−	−	−
E. cloacae 1522	F	5.4, 8.2	TEM-1	+	SHV-12	+	−
E. cloacae 1527	G	5.4, 8.2	TEM-1	+	SHV-12	+	−
E. cloacae 1538	H	5.6, 6.5, 7.6, 7.8, 8.2	−	−	−	−	−

[a] −, tested, but yielded negative results.

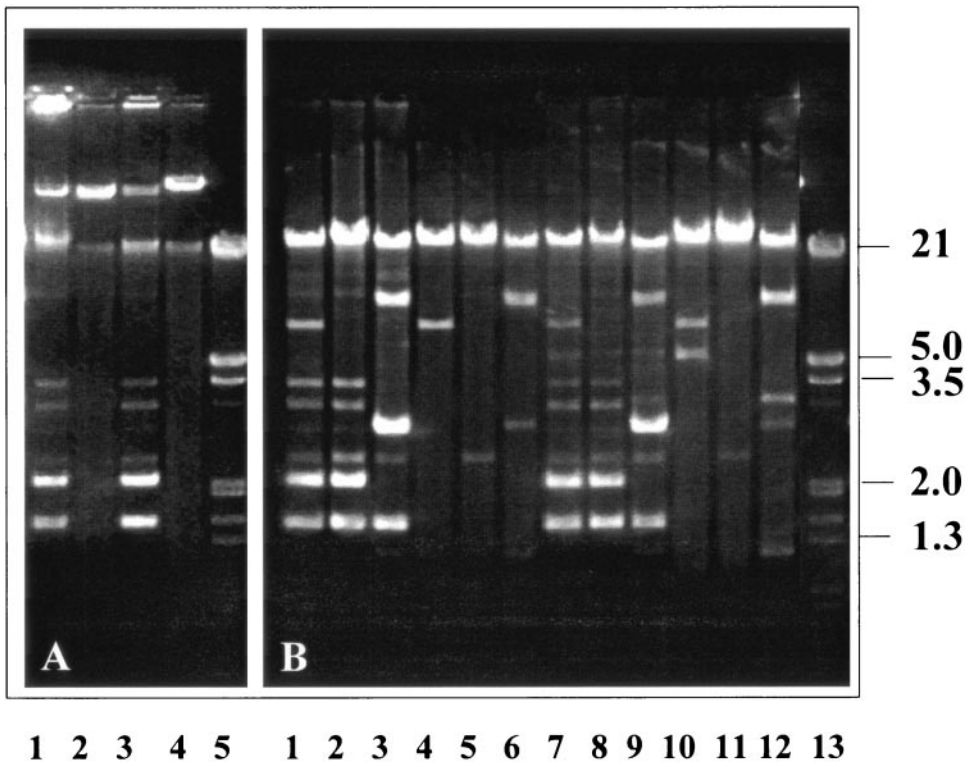

FIG. 2. Plasmid DNA (A) and restriction enzyme (B) profiles of two representative *Enterobacter* isolates and their respective transconjugants carrying both the TEM-1 and the SHV-12 genes. (A) Lane 1, *Enterobacter* sp. strain 1061; lane 2, transconjugant strain 1061; lane 3, *Enterobacter* sp. strain 1434; lane 4, transconjugant strain 1434; lane 5, EcoRI-HindIII-digested bacteriophage lambda DNA marker (in kilobases). Only one plasmid was transferred from both *Enterobacter* strains to the recipient strains. (B) The plasmids from both recipients had identical restriction profiles, suggesting that they are the same plasmid. The restriction patterns of plasmid DNAs digested with the SmaI, XhoI, and BamHI endonucleases are shown in each of the three sets of the following lanes, respectively: lanes 1 to 3, *Enterobacter* sp. strain 1061; lanes 4 to 6, transconjugant strain 1061; lanes 7 to 9, *Enterobacter* sp. strain 1434; and lanes 10 to 12, transconjugant strain 1434. Lane 13, DNA size marker.

belonging to clone C, whereas the other two genetically related *Enterobacter* strains belonging to clone E and E′ did not carry the same ESBL gene, SHV-12, by IEF and PCR.

Transconjugation experiments. Successful transconjugation was obtained with four of the isolates, *Enterobacter* sp. strains 1250, 1061, 1434, and 1262, all of which harbored TEM-1 and SHV-12. The other three *Enterobacter* strains carrying both TEM-1 and SHV-12 failed to conjugate. Both genes were transferred simultaneously to *E. coli* HB101 (as detected by PCR and colony hybridization). These transconjugant strains exhibited an ESBL phenotypic profile, based on the disk combination test, and their IEF profiles showed two distinct β-lactamases with pIs of 5.4 and 8.2, as did their parental *Enterobacter* strains. The transconjugant PCR analysis and sequencing data confirmed that the four transconjugants carried the same beta-lactamases as their parental strains, TEM-1 and SHV-12, proving a transferable ESBL phenotype.

Plasmids were isolated from two representative *Enterobacter* donor strains (strains 1061 and 1434) and from their respective transconjugants (Fig. 2A). The two donor strains contained various plasmids, one of which was transferred to the transconjugants, suggesting that it carried the ESBL genes.

Restriction analysis of plasmid DNA from donors and transconjugants revealed that each DNA fragment in the transconjugants corresponded to a similar band in the respective donor strain (Fig. 2B). DNA fragments of the same sizes in both *Enterobacter* donors and the respective transconjugants were hybridized with the TEM- and SHV-specific probes, proving the presence of both genes on the same plasmid (Fig. 3A and B). *Enterobacter* sp. strain 1434 and its transconjugant showed an additional DNA fragment that hybridized with the SHV-specific probe, suggesting the presence of an additional copy of the bla_{SHV} gene on the plasmid. Interestingly, Southern analysis showed an additional DNA fragment that hybridized with the TEM-specific probe in both the *Enterobacter* donor strains and the transconjugants (Fig. 3A).

DISCUSSION

In a recent study (27) we showed that the ESBL phenotype occurred in 22% of *Enterobacter* isolates at Tel Aviv Sourasky Medical Center. In the present study, we attempted to determine the resistance determinants that conferred this phenotype and to understand the molecular epidemiology of these organisms.

We characterized 17 unique patient isolates of *Enterobacter* spp. identified phenotypically as ESBL producers. We were able to prove by IEF and genetic sequencing the presence of at least one ESBL in 9 of the 17 isolates. We believe, however, on the basis of the IEF and colony hybridization results, that at least 13 isolates and, more likely, all 17 isolates carried an ESBL gene. For those isolates from which we failed to amplify

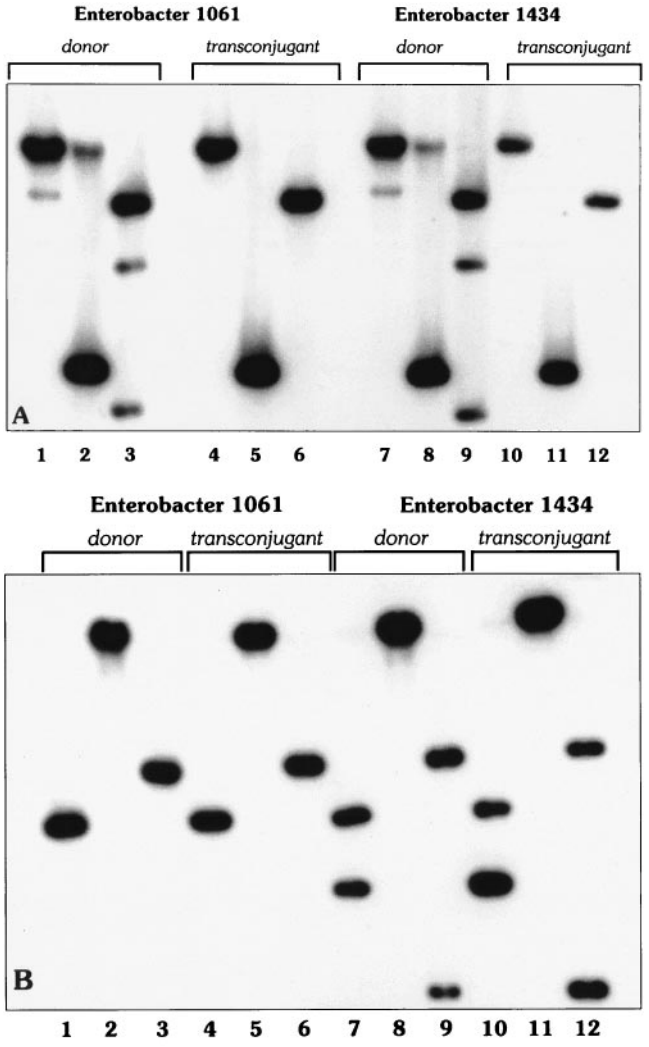

FIG. 3. Southern blot analysis of plasmid DNA from *Enterobacter* sp. strains 1061 and 1434 and their transconjugants. Plasmid DNA was digested with the SmaI, XhoI, and BamHI endonucleases in lanes 1 to 3, 4 to 6, 7 to 9, and 10 to 12, respectively, and hybridized with TEM-specific (A) and SHV-specific (B) probes. Hybridization with the TEM-specific and SHV-specific probes proves the existence of these genes on the same plasmid. *Enterobacter* sp. strain 1064 carried two copies of TEM, one of which was transferred together with SHV to the transconjugant. The plasmid of *Enterobacter* sp. strain 1434 had two copies of SHV, and both were transferred to the transconjugant, together with TEM.

and sequence an ESBL gene, we suspect the presence of other classes of ESBLs or technical problems with the PCR primers. There are two main mechanisms for the spread of ESBLs: the spread of an ESBL-producing clone or dissemination of an ESBL-encoding plasmid among various clones. In several studies, clonal spread was found to account for the dispersion of ESBL-producing *Enterobacter* spp. (12, 18, 23). In other cases, multiple clones were found, although usually only one was shown to be dominant (4, 11). In one of these cases, one specific enzyme was found among polyclonal isolates of *Enterobacter* (4). In one report from South Korea, SHV-12 was detected in several genetically unrelated *Enterobacter* isolates. This finding indicates that the ESBL phenotype had spread due not to an outbreak of a resistant strain but rather to the dissemination of the SHV-12 beta-lactamase (21). In France, where one *E. aerogenes* clone dominated, two populations of this clone were found: one carried the gene for TEM-24 and the other carried the gene for SHV-4 (23). Later, the TEM-24-carrying group spread further, causing a wide outbreak, while the SHV-carrying strains disappeared. In our study, PFGE typing revealed that *Enterobacter* isolates belonged to distinct clones, with only 2 of 17 cases explained by clonal spread. IEF, colony hybridization, and PCR results were consistent with the presence of various enzymes belonging to different gene families of ESBLs, SHV and CTX-M. Interestingly, TEM-1 was detected in all 11 isolates from which the TEM gene was sequenced. This finding does not explain the ESBL phenotype; moreover, one would not expect TEM-1 to confer any evolutionary advantage to AmpC-carrying organisms such as *Enterobacter* spp. The major ESBL gene, $bla_{\text{SHV-12}}$, was found to coexist with $bla_{\text{TEM-1}}$ in seven isolates. Moreover, conjugation experiments with four strains identified TEM-1 and SHV-12 as beta-lactamases transferred together. Indeed, plasmid isolation followed by Southern analysis proved the coexistence of these two genes on the same plasmid. We hypothesize that TEM-1-encoding plasmids, which have the ability to spread rapidly between *Enterobacter* isolates, acquired SHV-12. A similar linkage between a bla_{TEM} gene (TEM-1B) and a bla_{SHV} gene (SHV-12) was found in an *Enterobacter amnigenus* strain isolated in Thailand (6) and an *E. cloacae* strain isolated in Guangzhou, China (7) and in *E. cloacae* and *E. aerogenes* isolates in South Korea (20, 21). In all these cases, both genes cotransferred to a recipient *E. coli* strain in transconjugation experiments, as in our case. The coexistence of bla_{TEM} and bla_{SHV} in clinical isolates of *E. cloacae* was also reported in Italy, where the rate of coexistence was 50% (37), and in Philadelphia, Pa. (22). In France, on the other hand, one *E. cloacae* isolate was found to carry a TEM-1 gene together with CTX-M-1 on the same 55-kb plasmid, and another isolate was found to carry both TEM-1 and CTX-M-3 on the same 180-kb plasmid. Both plasmids were transferred to *E. coli* by transconjugation and, as revealed by IEF, expressed two beta-lactamases (one with a pI of 5.4 corresponding to TEM-1 and one with a pI of 8.4 corresponding to both CTX-M enzymes) (13).

In several isolates we found discrepancies between the results of IEF, colony hybridization, and PCR. Such discrepancies are not unusual and may be related to various degrees of expression of the ESBL genes, the existence of ESBL genes that belong to other families or that do not react with the specific primers used, and other factors. For example, the CTX-M family of enzymes is fairly diverse, with various $bla_{\text{CTX-M}}$ genes showing as little as 67% homology with one another.

Taken together, the high prevalence of ESBLs in *Enterobacter* spp. at the Tel Aviv Sourasky Medical Center is attributable not to clonal spread but, rather, to the dissemination of plasmids carrying different class A enzymes among genetically diverse isolates.

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