Different promoters of SHV-2 and SHV-2a β -lactamase lead to diverse levels of cefotaxime resistance in their bacterial producers

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Clinical *Klebsiella pneumoniae* isolates as well as *Escherichia coli* transformants producing the β -lactamases SHV-2 or SHV-2a demonstrate MIC values for cefotaxime of 4 mg l⁻¹ or 64 to >128 mg l⁻¹, respectively. The β -lactamases differ by one possibly insignificant amino acid exchange at position number 10 of the mature protein; their kinetic parameters are rather similar. The 5' untranslated regions of both corresponding genes show no homology starting 74 nucleotides upstream to the start codon. Hybridization of intragenically annealing oligonucleotides to dot-blotted serial dilutions of total cellular RNA from *E. coli* transformants harbouring these genes cloned into the same vector plasmid gave a positive signal down to 1.2 μ g (SHV-2) and 0.32 to 0.16 μ g (SHV-2a), indicating a four to eight times higher amount of specific transcript in the case of SHV-2a. By primer extension analysis and S1 nuclease digestion the starting point of transcription was located 100 nucleotides (SHV-2) and 50 nucleotides (SHV-2a) in front of the start codon. No other transcripts of different length could be detected after prolonged exposure. Northern blot analysis demonstrated the length of the β -lactamase mRNA to be about 1.6 kb in both cases, thus comprising a potential open reading frame downstream of the two enzymes' genes. Selective PCR amplification of both promoter regions and of the structural gene of SHV-2 and subsequent combined cloning of each of the promoters and the SHV-2 gene into pBGS19 using a *Bam*HI restriction site introduced by three point mutations into the cloned sequences was employed to transform *E. coli* DH5 α . The MIC value for cefotaxime of transformants harbouring the SHV-2 promoter and SHV-2 structural gene was 8 mg l⁻¹, but was 64 mg l⁻¹ in the case of the SHV-2a promoter and SHV-2 structural gene combination, indicating that the quantitative change of resistance to cefotaxime of SHV-2a-producing bacteria is caused solely by a significant promoter mutation.

Introduction

In the last few years an increasing number of plasmid-coded β -lactamases from members of the *Enterobacteriaceae* such as *Escherichia coli* and *Klebsiella* spp. have been described. Initially the investigators' attention was drawn to these bacteria because of the increased resistance of a given isolate to a newer β -lactam, e.g. a methoxyiminocephalosporin. Analysis of the mechanism of resistance most often yielded an altered β -lactamase, mainly from the groups of TEM or SHV enzymes. At present, at least 19 TEM-group and eight SHV-group β -lactamases (references summarized in: Mabilat & Courvalin, 1990; Podbielski *et al.*, 1991) have been characterized, many of them at the nucleotide sequence level. In all cases the genes of these enzymes

differed in a few point mutations, leading to even fewer amino acid exchanges, with one or two of these in the pouch of the active site of the enzyme, thus in some cases affecting the enzyme's affinity for a given β -lactam compound and by this means the rate and effectivity of its hydrolysis (Labia *et al.*, 1988; Collatz *et al.*, 1989). Besides the possibility of reaching an increased level of resistance to recently developed β -lactams by introducing appropriate point mutations within the structural gene of the β -lactamase, there exists another mechanism of adaptation for the bacteria, namely by augmenting the rate of expression of the enzyme, a point that only a few investigators have considered. Obvious differences between β -lactamase promoters of Gram-negative bacteria are well characterized only in the case of transposon- and plasmid-encoded TEM enzymes (Chen & Clowes, 1984, 1987; Sougakoff *et al.*, 1988; Mabilat *et al.*, 1990). Recently, we described a variant of the SHV-2 β -

Abbreviation: PCR, polymerase chain reaction.

lactamase, SHV-2a, whose kinetic parameters with respect to different methoxyiminocephalosporins did not differ significantly from the original SHV-2 enzyme; but when SHV-2a was expressed by the natural *Klebsiella pneumoniae* host, the bacteria showed an increased minimum inhibitory concentration (MIC) for cefotaxime of at least 128 mg l^{-1} (Podbielski *et al.*, 1991). Comparison of the genes and neighbouring nucleotide sequences revealed three point mutations within the gene leading to one amino acid exchange of questionable importance. Gene dosage effects could be excluded, since SHV-2 and SHV-2a appear as single-copy genes in their original hosts, as judged by hybridization experiments using intragenic probes and restriction-enzyme-digested genomic and plasmid host cell DNA. In contrast, the 5' untranslated regions of both genes were totally different starting 74 nucleotides upstream of the start codon, which is within the putative promoter region. Since only one research group (Arakawa *et al.*, 1986) had tried to establish the SHV promoter sequence for the chromosomally encoded SHV-type β -lactamase LEN-1 (presumably by homology identification), we decided to characterize the SHV-2 and SHV-2a promoters experimentally and subsequently to analyse the actual strength of each promoter in order to explain the different MICs for cefotaxime of *Klebsiella* isolates producing these enzymes.

Methods

Bacterial strains, plasmids and media. *E. coli* DH5 α (BRL) was used as a host strain for recombinant plasmids. High-copy-number plasmid pBGS19 (Spratt *et al.*, 1986) served as a vector plasmid. Recombinant plasmids pBP60-1-2 and pZMP1-1-1, harbouring a 2.3 kb insert including the gene for SHV-2 and a 2.2 kb insert including the SHV-2a gene, respectively, each cloned into pBGS18, have been described elsewhere (Kliebe *et al.*, 1985; Podbielski *et al.*, 1991).

DST agar (Unipath Oxoid) was used for routine culturing of bacteria. When required, it was supplemented with ampicillin (50 mg l^{-1}) or cefotaxime (2 mg l^{-1}). Recombinant clones were screened on H agar (protocol by Boehringer Mannheim) supplemented with kanamycin (30 mg l^{-1}), X-Gal and IPTG.

MICs were assessed by the MicroScan system (Baxter) in ready-to-use microtitre plates, following the protocol of the manufacturer.

Preparation of nucleic acids. Small-scale preparation of plasmid DNA was done by the method outlined by Zhou *et al.* (1990). In the case of DNA sequencing this method was scaled up, followed by phenol extraction and ethidium bromide/CsCl density gradient centrifugation in a Beckman TLV 100 K rotor at 100 000 r.p.m. for 3 h. Bands containing covalently closed circular plasmid DNA were punctured and drawn off with a hypodermic syringe and ethidium bromide was extracted from these by phenol treatment. Two micrograms of this DNA suspended in TE buffer (10 mM-Tris pH 7.5, 1 mM-EDTA) was used for an individual sequencing reaction. When appropriate, single DNA fragments generated by polymerase chain reaction (PCR) amplification or restriction digests were electroeluted from agarose gels

and purified by affinity chromatography on NACS-52 (BRL) according to the manufacturer's instructions. Total cellular RNA was isolated according to the protocol of Reddy *et al.* (1990). Concentration of RNA solutions was measured spectrophotometrically at 260 nm.

DNA techniques. Restriction enzyme digests, DNA ligation, 1% (w/v) agarose gel electrophoresis and Southern blotting by vacuum onto Biotodyne B nylon membranes (Pall Bio Support) were done by conventional methods (Sambrook *et al.*, 1989). The enzymes necessary for these procedures were purchased from Boehringer Mannheim. Transformation of *E. coli* recipients was performed as outlined by Hanahan (1983); colony blots were done according to Maas (1983). Hybridization with non-radioactively labelled oligonucleotide probes and visualization with the Photogene system (BRL) was described earlier (Podbielski *et al.*, 1990); stringent hybridization temperatures were selected 5 to 10 °C below the calculated melting temperature (T_D), using the OLIGO program (National Bioscience Inc.) for calculation. Oligonucleotides for probing, sequencing and primer extension experiments (Fig. 1) were synthesized on a Beckman 200A DNA synthesizer, eluted, deprotected and desalted following the instructions of the manufacturer. Oligonucleotides for RNA dot-blot and Northern blot hybridization were 5' labelled using [γ - ^{32}P]ATP (Amersham) according to Sambrook *et al.* (1989).

DNA sequencing was performed by the dideoxy chain-termination method (Sanger *et al.*, 1977) using the T₇ sequencing kit (Pharmacia), pUC standard forward and reverse primers and [α - ^{35}S]dATP (Amersham) as exemplified in the instructions of the manufacturers. Sequences were analysed with the aid of the PC GENE program (IntelliGenetics) on a IBM PS-2 personal computer.

PCR was run in a Techne PHC 1 thermal cycler. The reaction volume of 100 μl contained (final concentrations): 1 μg template DNA, 200 μM -dNTPs, 1 μM of each primer, 50 mM KCl, 10 mM-Tris pH 8.3, 1.5 mM-MgCl₂ and 0.01% (w/v) gelatin; it was overlaid with 100 μl mineral oil, heated to 100 °C for 5 min, 2 U of *Taq* polymerase (BRL) was added and the assay was placed into the cycler where the cycling programme was immediately started. The programme consisted of 27 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2.5 min, followed by a final segment of 72 °C for 7.7 min. The mineral oil overlay was extracted with 100 μl chloroform, and, if appropriate, the amplification products were analysed or prepared for further experiments by electrophoresis on a 1% agarose minigel using Tris/phosphate/EDTA as running buffer.

RNA techniques. Northern and dot-blotting of total cellular RNA onto Biotodyne B nylon membrane was done as outlined by Davis *et al.* (1986). Hybridization of dot-blotted and Northern-blotted RNA with 5' radioactively labelled oligonucleotides was performed under the same conditions as hybridization with non-radioactively labelled probes (see above).

For primer extension analysis as modified from Inoue & Cech (1985), 1 pmol oligonucleotide primer was dissolved in 5 μl hybridization buffer I (60 mM-NaCl/50 mM-Tris pH 8.3/10 mM-DTT), and 20 μg total cellular RNA dissolved in 5 μl hybridization buffer I was added. The mixture was heated to 90 °C, gradually cooled to 30 °C over a period of 45 min, then 1 μl 60 mM-MgCl₂ was added. Five microlitres of this mixture were transferred to a new tube containing 3 μl dCTP/dGTP/dTTP dissolved in hybridization buffer I/6 mM-MgCl₂ at a final concentration of 200 μM of each nucleotide, 1.5 μl [α - ^{35}S]dATP and 2 μl (400 U) MMTV reverse transcriptase (BRL). The assay was incubated at 37 °C for 5 min, then 1 μl 2 mM-dATP was added and the incubation continued for a further 30 min at 37 °C. After this the reaction was stopped by adding 5 μl loading buffer (90%, v/v, formamide/20 mM-EDTA/bromophenol blue and xylene cyanol 1 mg ml⁻¹ each). Before loading onto a 8% (w/v) polyacrylamide sequencing gel with dideoxy termination sequencing reaction mixtures

in parallel as nucleotide length markers, the primer extension reaction mixture was denatured by the conventional procedure (5 min at 95 °C, 5 min on ice, spin in a centrifuge, 2 min at 95 °C, 2 min on ice, spin again).

For S1 nuclease digestion mapping as modified from Aiba *et al.* (1981), a 1672 bp *Bam*HI–*Not*I DNA fragment and a 1076 bp *Pst*II–*Not*I DNA fragment from the cloned SHV-2a and SHV-2 genes, respectively, both containing 146 intragenic bp and the adjacent 5' untranslated regions, were separated on 1% agarose gels, electroeluted, extracted with phenol/chloroform and 5' labelled with [γ -³²P]ATP as outlined for oligonucleotides (see above). Samples (0.2 µg) of each of these labelled fragments were mixed with 150 µg total cellular RNA, precipitated with ethanol and dissolved in 30 µl hybridization buffer II (80% formamide/40 mM-PIPES pH 7.4/1 mM-EDTA/0.4 M-NaCl) at 60 °C.

The DNA–RNA hybridization assay was heated to 85 °C for 10 min, and then kept at the hybridization temperature overnight. The hybridization temperature to be used depends on the GC content of the annealing region (SHV-2a, 51.2%; SHV-2, 46.7%) and was deduced from Sambrook *et al.* (1989). Thus, 59 °C and 52 °C for SHV-2, and 64 °C and 55 °C for SHV-2a were chosen. At the end of hybridization, 300 µl ice-cold S1 nuclease mapping buffer (0.3 M-NaCl/50 mM-sodium acetate/5 mM-ZnSO₄/20 µg ml⁻¹ fresh denatured calf thymus DNA/750 U S1 nuclease per ml final volume) was directly added, mixed quickly and transferred to 37 °C for 2 h. The reaction was stopped by immediate cooling on ice and addition of 80 µl stop mixture (4 M-ammonium acetate/50 mM-EDTA/50 µg ml⁻¹ yeast tRNA), extracted once with phenol/chloroform and precipitated with ethanol. The pellet was dissolved in 4 µl TE buffer, 6 µl loading buffer was added and after conventional denaturation loaded onto a sequencing gel in the same way as for the primer extension analysis.

Results

Northern blot hybridization

For hybridization to Northern blots loaded with identical amounts of total cellular RNA of *E. coli* DH5 α transformed with the SHV-2 and SHV-2a genes cloned into pBGS18 (pBP60-1-2 and pZMP1-1-1, respectively) a mixture of three intragenically annealing oligonucleotides (Fig. 1a) was selected as a probe. Comparing the major signal on the blot of the λ DNA/*Hind*III size standard, the length of the specific transcript was estimated as approximately 1.6 kb. Densitometric evaluation of the hybridization bands indicated an approximately four to five times stronger signal from the SHV-2a RNA.

RNA dot-dot hybridization

Total cellular RNA from *E. coli* transformants harbouring the recombinant plasmids pBP60-1-2 (SHV-2) and pZMP1-1-1 (SHV-2a) was serially diluted in twofold steps and dot-blotted onto the nylon membrane. The highest amount of blotted RNA was 40 µg in both cases. The mixture of intragenically annealing oligonucleotides (Fig. 1a) was again used as detection probe. The results are shown in Fig. 2. A signal clearly above background

could be detected with 1.25 µg blotted total cellular RNA in the case of SHV-2, compared to 0.32 to 0.16 µg in the case of SHV-2a. Thus, in the preparation of total cellular RNA containing the SHV-2a mRNA there was about four to eight times more of the specific transcript compared to that of SHV-2.

Promoter mapping of the SHV-2 and SHV-2a genes

Promoter mapping was achieved by the use of two different methods, primer extension analysis and S1 nuclease digestion. The three different primers used for the primer extension analysis are shown in Fig. 1(b).

As a control for irregularities in the sequencing gels, each individual assay as well as each sequencing reaction as a size standard was loaded onto the gel twice in one run, at different times.

Reverse transcription of the mRNA templates led to identical results irrespective of the primer chosen. S1 nuclease digestion was only effective in the case of the lower hybridization temperatures. The transcription start points defined by primer extension analysis and by S1 nuclease mapping were in excellent agreement.

As examples, representative regions of the sequencing gels loaded with reaction mixtures from primer extension and S1 nuclease assays are shown in Figs 3 and 4, respectively. The transcription start point (position +1) for the SHV-2 gene was mapped 100 nucleotides in front of the translation start codon ATG; that of SHV-2a was mapped 50 nucleotides in front of ATG. The alignment of the SHV-2 and SHV-2a sequences upstream of the start codons and the deduced sequences of the corresponding –35 and –10 boxes are shown in Fig. 5.

Selected PCR-mediated amplification and cloning of the SHV-2 and SHV-2a promoters and SHV-2 structural gene; determination of MICs of resulting recombinant clones

Two DNA fragments of 510 nucleotides, including the SHV-2 and SHV-2a gene promoters, respectively, were successfully amplified by PCR using the primers shown in Fig. 1(c) and both were cloned separately into pBGS19 via restriction sites for *Eco*RI (5' end) and *Bam*HI (3' end) added to the 5' ends of the PCR primers. The *Bam*HI site lies between the Shine–Dalgarno sequence and the start codon of the gene and is created by introducing three point mutations in this stretch of sequence. Positive recombinant clones were initially spotted by white/blue selection and subsequently after colony blotting by hybridization with oligonucleotide no. I in Fig. 1(b). Effective cloning was confirmed and unwanted mutations introduced by the PCR reaction were excluded by sequencing these recombinant clones

(a) Oligonucleotides used as hybridization probes annealing to sequences within the SHV-2 structural gene

- I 5' GCG CCG CAG AGC ACT ACT TT 3'
 nt. pos. no. 1154 - 1135 in SHV-2
- II 5' AAT CCT GCG GGG CCG CCG AC 3'
 nt. pos. no. 1361 - 1342 in SHV-2
- III 5' TTG CTG ATT TCG CTC GGC CA 3'
 nt. pos. no. 1746 - 1727 in SHV-2

(b) Oligonucleotides used in primer extension analysis

- I 5' AGA AGC GAC AAA TAA GAA TA 3'
 nt. pos. no. 887 - 868 in SHV-2
- II 5' TAC ACA GGC GAA TAT AAC GC 3'
 nt. pos. no. 952 - 933 in SHV-2
- III 5' AAT TTG CTC AAG CGG CTG CG 3'
 nt. pos. no. 1017 - 998 in SHV-2

(c) Oligonucleotides used as primers for selective PCR amplification of SHV-2 and SHV-2a promoters and SHV-2 structural gene

- FOR 2 5' ^{Clamp} CCC CCC ^{EcoRI} GAA TTC GAC CCG CGT TCA CTT ATC GC 3'
 nt. pos. no. 409 - 428 in SHV-2
- FOR 2a 5' CCC CCC GAA TTC ACT TCT TCA CGT TGT TGA GG 3'
 nt. pos. no. 1006 - 1025 in SHV-2a
- REV 2 5' CCC CCC ^{BamHI} GGA TCC ^{SD} ATC CTT GAG TGA GGG CCG AT 3'
 nt. pos. no. 919 - 900 in SHV-2
- REV 2a 5' CCC CCC GGA TCC ATC CTT GAG TGA AGG CCG AT 3'
 nt. pos. no. 1515 - 1496 in SHV-2a
- FOR 2/2a 5' CCC CCC GGA TCC TGG ^{Start} TTA TGC GTT ATA TTC GC 3'
 nt. pos. no. 926 - 945 in SHV-2
- REV 2/2a 5' GTC ATA ^{HindIII} AAG CTT GGC ACG CCT GCG AGG TGC TG 3'
 nt. pos. no. 1843 - 1824 in SHV-2

Fig. 1. Sequences of oligonucleotides used in this study for hybridization and primer extension experiments. The purpose of additional nucleotides added to the 5' end of a specific oligonucleotide or special features within a given oligonucleotide are shown above the region of interest (SD, Shine-Dalgarno sequence; Start, translation start codon). Nucleotide position numbers (nt. pos. no.) refer to the complete SHV-2 and SHV-2a nucleotide sequences. For comparison: the start codons of the SHV-2 and SHV-2a genes comprise nucleotide positions no. 931-933 and 1527-1529, respectively, in the published total sequences (Podbielski & Melzer, 1990; Podbielski *et al.*, 1991).

Fig. 2. Hybridization of intragenically annealing oligonucleotides to dot-blotted serial dilutions of SHV-2 and SHV-2a mRNA. The amounts (μ g) of dot-blotted total cellular RNA are shown above the photograph. For hybridization a mixture of radioactively labelled oligonucleotides (Fig. 1a) was used.

on one strand prior to further recombination experiments. Cloning of the SHV-2 structural gene followed the procedures outlined above, using artificial *Bam*HI (5' end) and *Hind*III (3' end) restriction sites for cloning, and the mixture of oligonucleotides of Fig. 1(a) for screening in colony blot hybridization thereafter.

The recombinant plasmids containing the SHV-2 or SHV-2a promoters or the SHV-2 structural gene were designated pZMP2-P, pZMP2a-P and pZMP2-S, respectively. The SHV-2 gene was cut out of pZMP2-S by *Bam*HI and *Hind*III digestion and after separation and cleaning of the fragment was cloned into the corresponding sites of pZMP2-P and pZMP2a-P. *E. coli* transformants harbouring the new recombinant plasmid were selected on DST agar supplemented with ampicillin or cefotaxime. Plasmid DNA of cefotaxime-resistant clones was analysed by triple digestion with *Bam*HI, *Eco*RI and *Hind*III and confirmed to contain the desired compound genes by DNA sequencing.

Five clones each harbouring the recombinant plasmids designated pZMP2-P/2-S (SHV-2 promoter and structural gene) and pZMP2a-P/2-S (SHV-2a promoter and SHV-2 structural gene) were subjected to MIC determination for a panel of β -lactams. The mean values are presented in Table 1. Recipient *E. coli* DH5 α devoid of any plasmid was tested in parallel and proved to exhibit MIC values as already published (Podbielski *et al.*, 1991; data not shown).

Discussion

The first description of a promoter of a chromosomally encoded SHV type β -lactamase, LEN-1, was by Arakawa *et al.* (1986). However, these authors did not

Fig. 3. Example of a primer extension analysis for the assessment of the transcription start point of SHV-2a mRNA. Lane 1, sequencing assay as size reference; lanes 3, 5 and 7, extension of primers I, III and II (Fig. 1b), respectively, using SHV-2a mRNA as a template; lanes 2, 4, 6 and 8, each reaction mixture loaded onto the gel for a second time 1.5 h after starting the first run.

Fig. 4. Example of an S1 nuclease digestion assay for the assessment of the transcription start point of SHV-2 and SHV-2a mRNA. Lane 1, sequencing assay as size reference; lanes 2 and 3, SHV-2 and SHV-2a mRNA-derived products, respectively. In both assays an SHV-2a-derived DNA fragment was used for annealing. Thus, the hybrid with SHV-2 mRNA is only 22 nucleotides longer than the SHV-2a mRNA-derived fragment, reflecting the dissimilarity of the two sequences beyond this point.

elaborate in detail how they came to the conclusion that the described nucleotide sequences belong to the promoter. All other groups who later published new nucleotide sequences of plasmid-coded SHV β -lactamases cited the study of Arakawa *et al.* (1986) when mentioning a putative promoter site. Since almost all nucleotide sequences adjacent to the 5' end of a given

SHV gene are highly homologous, differences in MIC values for newer β -lactams have largely been ascribed to amino acid exchanges found in the structural gene. Only the 5' untranslated region of SHV-2a and another very similar SHV-2 variant enzyme (Garbarg-Chenon *et al.*, 1990) differ from the rest of the sequences in so far showing homology of less than 50% starting 74 nucleotides 5' to the start codon in the upstream direction. Because the much higher MIC values for cefotaxime of the original *Klebsiella pneumoniae* producers of SHV-2a compared to those harbouring SHV-2 did not correspond with the similar kinetic values of the SHV-2 and SHV-2a enzymes, we decided to analyse the promoters involved.

In this paper we present the different promoters of the β -lactamases SHV-2 and SHV-2a, both assessed for the first time by experimental means. We determined the starting point of transcription by two different assays to circumvent the specific problems inherent in each method, which might lead to wrong or ambiguous results when using only one of them. In addition we used three different primers for the primer extension analysis since the reverse transcriptase tends to leave the template stand prematurely when it encounters secondary structures often found in single-stranded RNA. The results by every assay of both methods are in excellent agreement.

The starting points of the specific transcripts for the two β -lactamases differ by 50 nucleotides, being 100 nucleotides in front of the start codon ATG in the case of SHV-2 and 50 nucleotides in the case of SHV-2a.

Having a guanosine as the first nucleotide of the SHV-2 transcript is in accordance with the majority of transcripts presenting a purine nucleotide at this position (Horwitz & Loeb, 1990); in contrast, the transcript of SHV-2a starts with a cytidine. Knowing these starting points we deduced the -10 and -35 boxes of the corresponding promoters by simply counting the nucleotides in the 5' direction and applying rules like the special conservation of the TTG motif in the -35 box, which is said to lead to heteronomous DNA configuration and therefore to facilitate strand selection for the RNA polymerase (Horwitz & Loeb, 1990), and the conservation of spacer length between the -35 and -10 box necessary for a coordinate contact of the RNA polymerase to the template strand prior to the beginning of transcription (Beutel & Record, 1990). Comparison of the nucleotide sequences of the deduced -10 and -35 boxes of the SHV promoters with the consensus sequences given by Hawley & McClure (1983) shows that the promoter of SHV-2, and especially its -10 box, deviates more from the consensus sequence than that of SHV-2a (Fig. 5). This could be a reason for the lower strength of the SHV-2 promoter, since promoters which lack homology with the consensus sequence are generally less efficient at initiating transcription than promoters

SHV-2	702-	CAGC-GACGCCCGACAGAGTGC GGTA-TT-TAGTACTTTCTTATAGTTCA
SHV-2a	1298-	CAGCTCACG-GTAACTGA-TGCCGTATTTGCAGTACCAGCGTACGGCCCA
SHV-2	748-	-TCACGGCCTTGAGTCAAAAAATAGCGTGCTTAGGCAGGGCT-AGATATT
SHV-2a	1346-	CAGAATGATGTCACGCTGAAAAATGCCGGCCTTTGAATGGGTTTCATGTGCA
SHV-2	797-	GAT-TATTCGAAATAAAAGATGAAAAATGATGAAGGAAAAAGAGGAATT
SHV-2a	1396-	GCTCCATCAGCAAAAGGGGATG-ATAA-G-TTTATCACCACCGA-CTATT
SHV-2	846-	GTGAATCAG-CAAAACGCCGGGTTATTCTTATTGTGCTTCTTTACTCG
SHV-2a	1441-	-TGCAACAGTGCCAACGCCGGGTTATTCTTATTGTGCTTCTTTACTCG
SHV-2	895-	CCTTTATCGGCCCTCACTCAAGGATGTATTGTGGTT
SHV-2a	1491-	CCTTTATCGGCCTTCACTCAAGGATGTATTGTGGTT

Fig. 5. Alignment of the nucleotide sequences immediately upstream of the start codons of the SHV-2 and SHV-2a β -lactamase genes. Nucleotide position numbers for the first nucleotide of each line are according to the published sequences of the SHV-2 and SHV-2a genes (Podbielski & Melzer, 1990; Podbielski *et al.*, 1991), respectively. Lines and numbers above or below a sequence mark the -35 and -10 box of the promoter and the transcription start point of each gene.

Table 1. Mean MIC values for selected β -lactams of five *E. coli* transformants each harbouring pZMP2-P/2-S and pZMP2a-P/2-S

pZMP2-P/2-S and pZMP2a-P/2-S are the recombinant plasmids containing the SHV-2 structural gene and the SHV-2 or SHV-2a promoter, respectively. For comparison, the corresponding MIC values of *E. coli* transformants harbouring pZMP1-1-1 (SHV-2a; Podbielski *et al.*, 1991) and pBP60-1-2 (SHV-2; Kliebe *et al.*, 1985) are shown. ND, Not determined.

β -Lactam	MIC value (mg l ⁻¹)			
	pZMP2a-P/2-S	pZMP2-P/2-S	pZMP1-1-1	pBP60-1-2
Ampicillin	> 128	> 128	> 128	> 128
Mezlocillin	> 128	> 128	> 128	> 128
Amoxycillin/ clavulanic acid	8/4	2/1	8/4	ND
Cefazolin	> 32	> 32	> 32	> 32
Cefotaxime	64	8	64	4
Ceftriaxone	32	4	16	4
Ceftazidime	4	≤ 1	8	2
Imipenem	1	≤ 0.5	≤ 0.5	1
Aztreonam	4	≤ 1	2	≤ 1

with a high degree of homology (Rosenberg & Court, 1979; Hawley & McClure, 1983; Kobayashi *et al.*, 1990). The lower homology of the SHV-2 promoter to the consensus sequence and the simultaneous presence of potential promoter sequences at the position corresponding to the SHV-2a promoter exhibiting a higher degree of homology explains why a computer search for potential promoter sites on the SHV-2 DNA may fail. Even if present, this site is not used for the start of transcription of SHV-2 mRNA, because there is no evidence for a second transcript of different length, in the case of either SHV-2 mRNA or SHV-2a mRNA, as judged from the results of primer extension and S1 nuclease digestion assays and the Northern blots even after prolonged exposure. The inactivity of the site given by Arakawa *et al.* (1986) as a promoter may be caused by the aberrant length of the spacer region between the potential -35

and -10 boxes: it is one nucleotide longer than the consensus length of 18 nucleotides.

Both promoters and the intervening regions downstream of the start codons show no significant similarity to those of the TEM β -lactamases (Russell & Bennett, 1981), to inducible promoters of chromosomally encoded β -lactamases (Lindberg & Normark, 1986; Forsman *et al.*, 1989) or to regulatory sequences sensitive to heat-labile protein, which may couple the β -lactamase expression to the SOS response (Kuriki, 1989).

Starting 178 bp downstream of the stop codon of the SHV genes, there is a 318 bp open reading frame of unknown function perfectly conserved in all SHV nucleotide sequences published so far. Considering the starting points of transcription and the length of the specific transcripts, SHV mRNAs may be polycistronic, because they include this open reading frame.

The PCR-mediated cloning of the different SHV promoters and of the structural gene of SHV-2 and the subsequent recombinant cloning of both promoters in front of the β -lactamase gene could only be achieved by creating a restriction site, here between the Shine-Dalgarno sequence and the start codon, via the introduction of three point mutations. The resulting potential hairpin structure may influence the efficiency of transcription or translation by an attenuation process (Lindberg & Normark, 1986; Lovett, 1990; de Smit & van Duin, 1990). But we think we can exclude any influences of the manipulations we made with these sensitive parts of the genes, because: (a) the clones containing the SHV-2 promoter/SHV-2 structural gene combination exhibit nearly the same MIC values for β -lactams as those harbouring the complete natural gene cloned into the same vector plasmid; and (b) the numerical difference of the amount of specific mRNA produced by bacteria transformed with recombinant plasmids containing the complete SHV-2 and SHV-2a genes, respectively, is in the same range as the difference of the MIC values, especially for cefotaxime, of *E. coli* transformants harbouring the PCR-derived substructures of the genes.

On the other hand we cannot exclude the influence of upstream regulatory sequences like those described by Urabe *et al.* (1990) because the amplified promoter-containing DNA fragments are long enough to comprise such regions and the sequences 500 to 300 bp upstream of the start codon are diverse, so they may exert different influences on the downstream promoters. This influence would have to be caused by *cis*-acting elements, because we did not amplify the open reading frame situated upstream of the SHV-2a β -lactamase gene (1135 to 899 nucleotides in front of the start codon; there is none in front of the SHV-2 gene). The method of selective PCR amplification of potential regions of interest on the DNA and their successive combined cloning to functional structures that we employed in this study may serve to answer such questions in future.

In conclusion, we have demonstrated by *in vitro* and *in vivo* experiments an alternative means of quantitative adaptation of a plasmid-borne β -lactamase to confer upon its *Klebsiella* host a higher level of resistance to a third-generation cephalosporin. The different promoter of SHV-2a alone is sufficient to generate the increased level of resistance to cefotaxime found in bacteria producing SHV-2a. This was confirmed by testing the *in vivo* effect of the different promoters in front of the same structural gene in otherwise identical host bacteria. This study documents that when looking for the reason for increased *in vivo* activity of a bacterial isolate against a given substrate, in some cases it may be insufficient to concentrate solely on small mutations of the structural

gene of the enzyme, thereby overlooking changes in the regulatory domains.

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