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Direct Involvement of IS*26* in an Antibiotic Resistance Operon

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The plasmid pBWH77, originally found in an isolate of *Klebsiella pneumoniae*, harbors a new antibiotic resistance operon containing two resistance genes transcribed from an IS*26*-hybrid promoter, as shown by nucleotide sequencing, mRNA mapping, and the effect of inserting a transcription terminator within the promoter-proximal gene. The nucleotide sequence of this region revealed that the operon (IAB) is made up of three sections that are closely related to previously described genetic elements. The −35 region of the promoter, together with the adjacent sequence, is identical to sequences of the IS*26* element. One of the resistance genes, *aphA7*, which is located next to the hybrid promoter, confers assistance to neomycin and structurally related aminoglycosides. This *aphA7* gene is highly homologous to *aphA1* of Tn*903*, with five nucleotide differences. The second gene, *blaS2A*, encodes an evolved SHV-type β-lactamase with a pI of 7.6 that confers resistance to the broad-spectrum cephalosporins cefotaxime and ceftizoxime. The deduced amino acid sequence of SHV-2A shows that amino acid 238 is a serine, a residue reported to confer resistance to cefotaxime. We discuss how the operon may have evolved by a combination of insertion sequence-mediated genetic rearrangements and acquisitive evolution. Using phylogenetic parsimony, we show that *aphA7* in the IAB operon evolved from an ancestral form similar to *aphA1* in Tn*903* and that *blaS2A* evolved from an ancestral form similar to *blaS1*.

Insertion sequences (ISs) are widely distributed on bacterial chromosomes and plasmids even though they only encode the gene products needed for their own transposition. As these ISs are frequently associated with unstable genetic traits, it has been suggested that they are sustained by direct selection acting on genetic versatility (9, 22, 30), either through enhancing mobility or through inducing beneficial regulatory mutations. This selectionist hypothesis (35) is an alternative to the hypothesis that ISs are parasitic or selfish (10, 27). The best evidence in favor of the selectionist hypothesis has been the close linkage between insertion sequences and plasmid-borne antibiotic resistance genes, often in the form of complex transposons. This linkage provides correlative evidence that ISs have been involved in either the evolution or the dissemination of these antibiotic resistance genes.

In this report, we present evidence that IS*26* has recently been involved in the assembly of a new antibiotic resistance operon (IAB) and show that this insertion element provides part of the promoter of this operon. IS*26* has been associated with several antibiotic resistance genes, including *aphA1* in Tn*2680* and other transposons (20) and a *blaT-aacC5* operon in the plasmid pUZ3644 (17), and has been assumed from sequencing data to provide part of a hybrid promoter for *aacC* genes in the plasmids pWP7b and pWP14a (7). In addition, Prentki et al. (29) suggested that IS*26* is a portable −35 promoter site.

The IAB operon contains the *aphA7* gene encoding an aminoglycoside-3′,5″-phosphotransferase-I (K.-Y. Lee, J. D. Hopkins, and M. Syvanen,submitted for publication). This gene and promoter-proximal sequences are almost identical to sequences from the transposon Tn*903* (Lee et al., submitted). The second gene of the IAB operon is *blaS2A*, which encodes a β-lactamase (SHV-2A) that hydrolyzes the new broad-spectrum cephalosporin cefotaxime, whereas the ancestral *blaS1* gene encodes a β-lactamase (SHV-1) that is unable to hydrolyze this β-lactam antibiotic. Resistance to cefotaxime can occur as a result of modification of SHV-1 (3, 11, 15, 28) and TEM (28, 33) β-lactamases. Thus, cefotaxime use has placed selective pressure on SHV-1 to acquire the ability to hydrolyze cefotaxime.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are described in Table 1. The 150-kilobase (kb) plasmid pBWH77 (13) was transferred from *Klebsiella pneumoniae* 5214773 into an *Escherichia coli* K-12 strain (SY203) by conjugation (19). The chimeric plasmid pJH194 was constructed by subcloning a *Bam*HI fragment of pBWH77 into the vector pACYC184 to give pJH194, a plasmid with a 12.5-kb fragment from pBWH77 that conferred resistance to neomycin and cefotaxime. Deletion of a *Sal*I fragment in pJH194 reduced the size of the pBWH77 DNA to 3.5 kb, to give the plasmid pJH196 (Fig. 1). This plasmid also conferred resistance to cefotaxime and neomycin.

Chemicals and reagents. Cefotaxime was a gift from Hoechst-Roussel Pharmaceuticals Inc., Somerville, N.J. Other antibiotics were purchased from Sigma Chemical Co., St. Louis, Mo. Restriction endonucleases, nuclease *Bal* 31, T4 DNA ligase, and T7 DNA polymerase were purchased from New England BioLabs, Inc., Beverly, Mass. DNase I was purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind. The DNA-sequencing kit was purchased from U.S. Biochemical Corp., Cleveland, Ohio, and [^{32}P]dATP was from Amersham Corp., Arlington Heights, Ill. All enzymes were used according to the specifications of the manufacturers.

Construction and preparation of plasmid subclones. Endonuclease digestion, cloning, and transformation procedures were done by the methods of Maniatis et al. (16). Plasmid DNA was prepared by the method of Birnboim and Doly (5).

Isoelectric focusing. Isoelectric focusing analysis of SHV-2A was kindly performed by A. Mederios, using the technique of Matthew et al. (18).

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant markers	Reference or source
Bacteria		
Klebsiella pneumoniae 5214773	*aphA7 blaS2A*(pBHW77)	13
Escherichia coli		
SY203	Δ(*lac-pro*)XIII *argE*(Am) *araD gyrA rpoB thi*	Laboratory collection
JM101	Δ(*lac-pro*) *supE thi*(F′ *traD36 lacI*^{q} *lacZ*M15 *proA*$^+$*B*$^+$)	24
Plasmids		
pBWH77	*aphA7 blaS2A*	13
pJH194	pACYC184::IS*26 aphA7 blaS2A* (12.5 kb)	This study
pJH196	pACYC184::IS*26 aphA7 blaS2A* (3.5 kb)	This study
p453	*blaS1*	31
pACYC184	*cat tet*	8
pET112	T1 and T2 of *rrnB*	R. Doi
pKL250	pJH196::rRNA operon terminator	This study
pKL217	M13mp19 Δ(*aphA7 blaS2A*)	This study

M13 subcloning and nucleotide sequencing. Deletion derivatives of pJH196 were constructed by *Bal* 31 deletion from the *Sal*I and *Bam*HI sites, followed by ligation with *Sal*I and *Bam*HI linkers. The resulting short segments of pBWH77-derived DNA were subcloned into the polylinker of the replicative forms of bacteriophage M13 derivatives mp18 and mp19 (38). *E. coli* JM101 (24) was transfected with the recombinant phage. Additional deletions were obtained by digestion of M13 subclones with DNase I-Mn^{2+}. Also, five 17-base oligonucleotides were synthesized to provide an overlapping set of sequencing primers. Nucleotide sequences were determined by the dideoxynucleotide-chain termination method (32) with [α-^{32}P]dATP. The nucleotide-sequencing strategy is shown in Fig. 1. DNA fragments were resolved by electrophoresis on vertical 6% polyacrylamide gels (80 cm by 21 cm by 0.4 mm) containing 8 M urea. After drying, the gels were autoradiographed with Kodak X-OMAT film. The complete DNA sequence was arranged by using the software package of Hitachi America Ltd.

S1 protection assay. Total RNA was extracted from *E. coli* SY203(pJH196) by the method of von Gabain et al. (36). Total cellular RNAs (100 μg) were allowed to hybridize with ^{32}P-labeled DNA probes (200 ng) of *aphA7* and *blaS2A* in 20 μl of 80% formamide–0.4 M NaCl–0.04 M PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.4)–1 mM EDTA (50°C, 16 h). Hybrids were digested with S1 single-stranded endonuclease by the method of Berk and Sharp (4). Digestions were performed at 45°C in 0.25 M NaCl–0.03 M sodium acetate (pH 4.6)–1 mM $ZnSO_4$–20 μg of thermally denatured calf thymus DNA per ml. The reactions were quenched by the addition of EDTA (4 mM) and extraction with phenol-chloroform. After precipitation with ethanol, the samples were denatured at 90°C in 95% formamide–20 mM EDTA–0.05% bromophenol blue–0.05% xylene cyanol FF. The protected DNA fragments were separated on 6% polyacrylamide–8 M urea gradient gels and visualized by direct autoradiography. An mRNA size standard was run on each gel and visualized by ethidium bromide staining.

Insertion of transcription terminator into *aphA7*. The 600-base-pair (bp) *Xho*I-*Sma*I fragment from pET112 that contains the T1 and T2 rRNA transcription terminator sequence of *rrnB* (6) was inserted into the *Xho*I-*Sma*I site in *aphA7* by digesting the two plasmids with *Xho*I and *Sma*I and then ligating the mix of fragments. *E. coli* SY203 was transformed with this recombinant DNA, selecting for chloramphenicol resistance. Resistant colonies were screened for kanamycin sensitivity and their cefotaxime phenotype.

RESULTS

Genes encoding neomycin and cefotaxime resistance are closely linked in pBWH77. The 150-kb plasmid pBWH77, isolated in a cefotaxime-resistant strain of *K. pneumoniae* (13), encodes resistance to ampicillin, cefotaxime, neomycin, gentamicin, streptomycin, amikacin, tobramycin, chloramphenicol, trimethoprim, and sulfonamide. Earlier work (15) suggested that plasmid-borne resistance to the broad-spectrum cephalosporin cefotaxime is due to the presence of an evolved β-lactamase. Because of our interest in understanding mechanisms of acquisitive evolution, we cloned the cefotaxime resistance gene from pBWH77 (see Materials and Methods) and in doing so found that the cefotaxime and neomycin determinants were closely linked.

The neomycin resistance gene located on pJH196 is *aphA7*, which encodes an aminoglycoside-3′,5″-phosphotransferase-I. This enzyme inactivates neomycin, kanamycin, paramomycin, lividomycin, and G418 (Lee et al., submitted). The cefotaxime resistance gene is *blaS2A*, which encodes the SHV-2A β-lactamase. This β-lactamase is designated an SHV enzyme on the basis of its isoelectric point of 7.6 and its sequence (see below). Antibiotic susceptibility testing showed that SHV-2A also conferred resistance to ceftizoxime and ceftriazone but not to ceftazidime or the monobactam moxalactam (data not shown).

Nucleotide sequence of IS*26-aphA7-blaS2A* region. The complete nucleotide sequence of the IAB operon was determined (Fig. 2). The first 343 nucleotides from the *Sal*I site are nearly identical to sequences from IS*26*, including the 14-bp terminal inverted repeat sequence (IRS) (21). Sequences homologous to Tn*903* begin immediately past the outside base of the IRS. Although we did not determine the sequence to the left of the *Sal*I site, a complete IS*26* appears to be present in pJH194 based on the presence of characteristic restriction sites. The coding sequence of *aphA7* begins at nucleotide 410 and extends to 1222. This sequence is closely related to *aphA1* of Tn*903* (K.-Y. Lee et al., unpublished data). The *blaS2A* sequence extends from base 1281 to base 2138 and differs by 11 bases from the sequence of *blaS1A* while being identical to a previously described *blaS2* allele (R. C. Levesque, personal communication). In this report, we are using the amino acid sequences deduced from the nucleotide sequences. Barthelemy et al. (2, 3) have reported the amino acid sequences for SHV-1 and SHV-2. Because these differ from the sequences being discussed

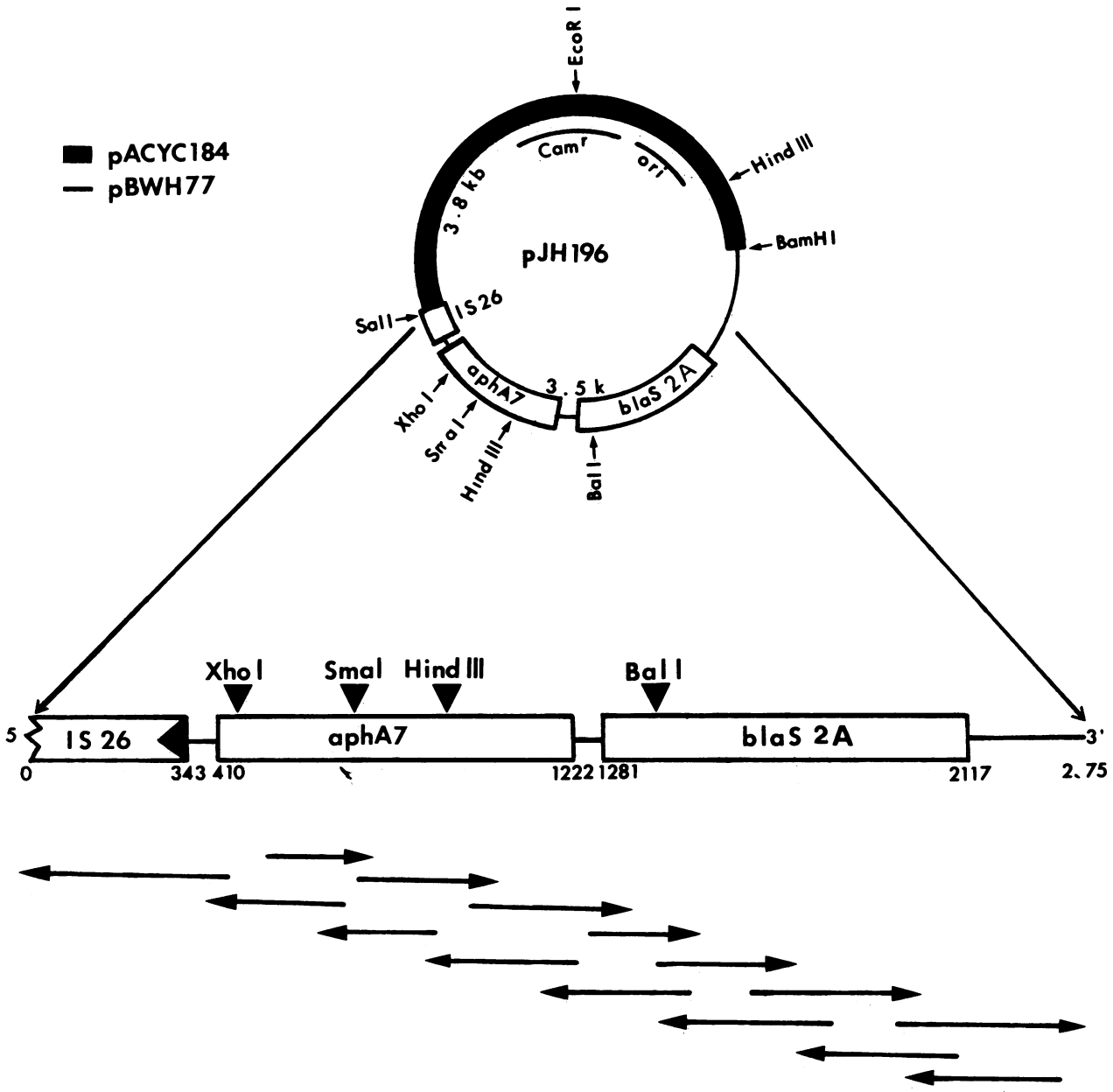
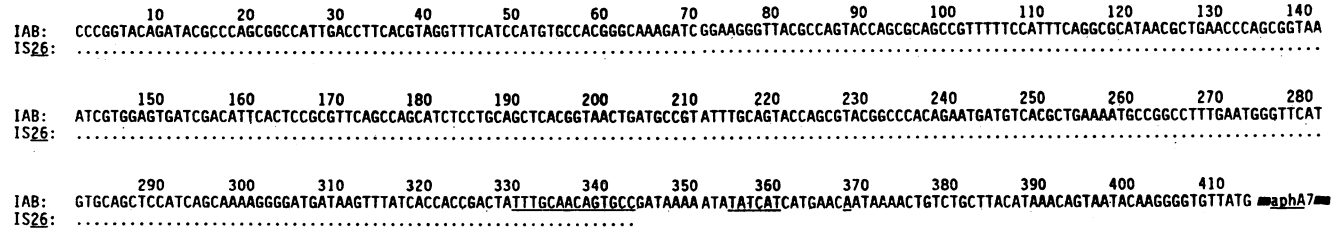


FIG. 1. IS*26*-*aphA7*-*blaS2A* operon (IAB): organization and DNA sequencing strategy. The sequenced portion of pJH196 is expanded, with the numbers indicating nucleotides from the *Sal*I site. The coding regions for *aphA7* and *blaS2A* are shown by the rectangles, and the intragenic region is shown by the solid line. The sideways triangle at the end of IS*26* indicates the 14-bp IRS. M13mp18 and M13mp19 subclones were constructed and subjected to dideoxy-chain termination sequencing. The direction and extent of the sequence determinations are shown by the horizontal arrows below the map.

here, we designate the Levesque sequences as SHV-1A and *blaS1A*. The 58 bases between the translation termination codon of *aphA7* and the translation start codon of *blaS2A*, as well as bases 2139 to 2375, have a high degree of homology with the sequences flanking the *K. pneumoniae* chromosomal β-lactamase gene *len-1* (1), whose product is known to be closely related to the SHV-1 β-lactamase (2), as well as those flanking *blaS1A*. The open reading frame gives a deduced amino acid sequence that is 99% identical with the deduced amino acid sequence of SHV-1A and 89% homologous with that of LEN-1 (1). The first 21 N-terminal amino acids are the putative signal sequence.

***aphA7* and *blaS2A* form an operon.** Simple inspection of the nucleotide sequence in Fig. 2 suggests that *aphA7* and *blaS2A* form an operon. A reasonably good σ^{70} promoter is apparent at the 5′ end of the *aphA7* gene. This promoter straddles the IS*26*-Tn*903* sequence junction, with the −35 region in the IS*26* IRS and the −10 region in the Tn*903*-like sequence. DNA sequences suggestive of hybrid promoters, with the −35 region provided by an IS element, have been previously noted with IS*26* (7), IS*1* (29), and IS*2* (14). In IS*26*, the −35 region IRSs are perfect palindromes (TTGCAA) and provide the −35 region of hybrid promoters, as well as the IS*26* transposase promoter at one end of the insertion element. A promoterlike sequence specific for *blaS2A* is not seen; indeed, *blaS2A* is missing the short

A. Nucleotide sequences of the 5'-end of the IAB and IS26.



B. Sequences of the flanking and coding regions of IAB, SHV-1 and LEN-1

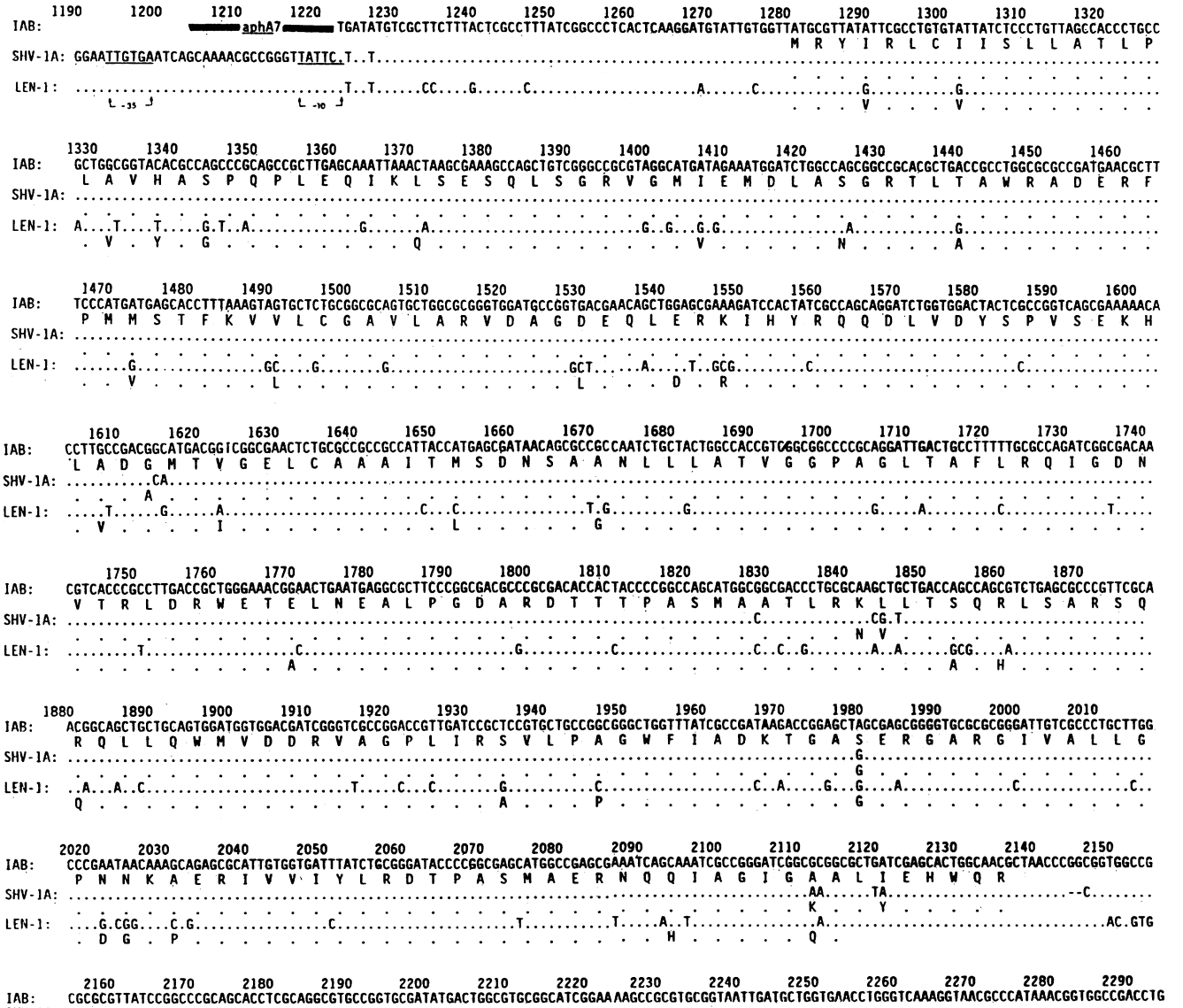


FIG. 2. Nucleotide sequences of promoter region and the *blaS2A* region of the IAB operon. (A) Sequence of the end of IS*26* and the region between IS*26* and *aphA7*. The published sequence for IS*26* (21) is indicated below, with a single dot indicating identity. The underlined sequences are, from left to right, the 14-bp IRS of IS*26*, the −10 promoter sequence, and the transcription start site. (B) Sequences of the flanking and coding region of *blaS2A*. The open reading frame for *blaS2A* was assigned by alignment with the *len* and *blaS1* genes. The −35 and −10 regions of the IAB promoter are underlined and indicated. Additional lines show deduced amino acid sequence for SHV-2A; nucleotide sequence for *blaS1A* (Levesque, personal communication); deduced amino acid sequence for SHV-1A (Levesque, personal communication); nucleotide sequence for *len-1* (1); deduced amino acid sequence for LEN-1 (1). A single dot designates an identity with *blaS2A* or SHV-2A.

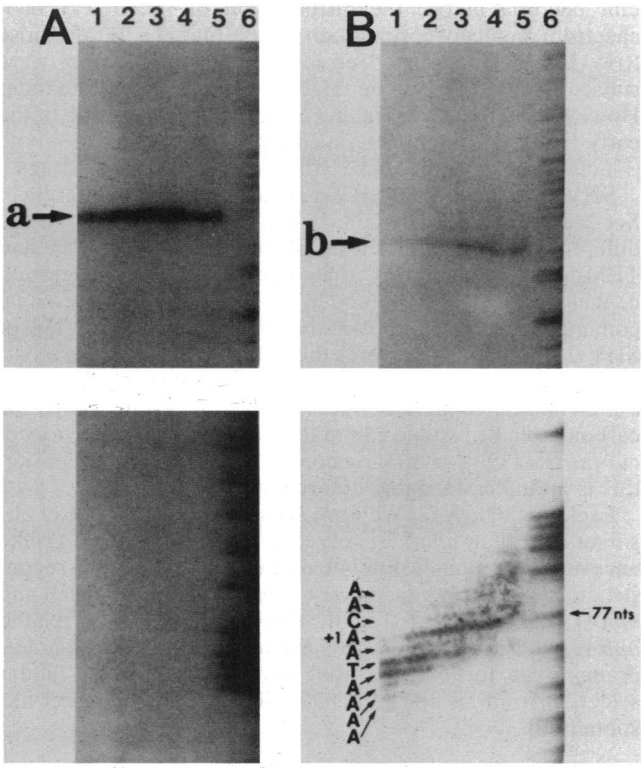


FIG. 3. S1 nuclease protection mapping of the IAB operon. The denatured DNA of γ-^{32}P-labeled probe 1, the *Hin*dIII-*Bla*I fragment (a in panel A), and of probe 2, the *Sal*I-*Xho*I fragment (B), was hybridized with mRNA of the IAB operon. The hybridized samples were treated with S1 nuclease (150 U) for 2.5 min (lane 5), 5 min (lane 4), 10 min (lane 3), 20 min (lane 2), or 40 min (lane 1), and the protected fragments were separated on 6% polyacrylamide–8 M urea gels. The full-length ^{32}P-labeled probes are indicated by the arrows a and b in panels A and B, respectively. In each panel, the top and bottom portions of the gel are separated. A sequencing lane of pKL217, a subclone of *Sal*I-*Sma*I fragment into M13mp19, was used as the molecular size standard (lane 6, calibration in nucleotides [nts]).

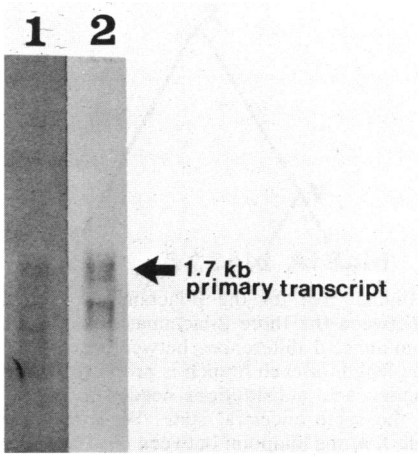


FIG. 4. In vivo primary transcript of the IAB operon. Total cellular RNA was isolated from SY203(pJH196) by the method of von Gabain et al. (36) and fractionated on a 1% agarose gel with glyoxal by the method of Maniatis et al. (16). The fractionated RNA was hybridized with ^{32}P-labeled DNA probes in a Northern blot by the method of Maniatis et al. (16), and this DNA visualized by autoradiography. For lane 1, the probe was the *Sal*I-*Xho*I fragment from the *aphA7* gene. For lane 2, the probe was a synthetic oligonucleotide primer corresponding to nucleotides 1347 to 1362 in the *blaS2A* gene. The molecular weight is based on an mRNA size standard lane (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) visualized by ethidium bromide staining (not shown).

segment of DNA that is apparently the −35 (TTGTGA) and −10 (TATTCT) regions of *len-1* and *blaS1A*.

These suggestions of the promoter organization were supported by direct physical mapping of the 5′ end of the mRNA by using an S1 endonuclease protection assay and two DNA probes. Probe 1, the *Hin*dIII-*Bal*I fragment (Fig. 1), would hybridize to mRNA from the 3′ end of *aphA7* and the 5′ end of *blaS2A*, while probe 2, the *Sal*I-*Xho*I fragment, would hybridize to RNA originating from the 5′ end of the *aphA7* gene and the IS*26* sequence. With probe 1, the assay gave a single fragment protected from S1 endonuclease digestion corresponding to the full length of the probe (Fig. 3A). This band is presumably a mixture of DNA-mRNA hybrids and reannealed DNA-DNA molecules. No shorter fragments were evident, indicating that there is no shorter transcript initiated from a promoter between *aphA7* and *blaS2A*. Using probe 2, we found that the total RNA preparation from SY203(pJH196) would protect a nucleotide fragment 77 nucleotides long (Fig. 3B). This shows that the 5′ end of the message is located in front of the *aphA7* gene at nucleotide 367; this is the expected transcript originating from the identified IS*26*-hybrid promoter. The larger fragment in Fig. 3B can be accounted for by reannealed DNA probe, although possibly there is a weak promoter lying to the left of the *Sal*I site.

To establish more thoroughly that a second promoter was not present between *aphA7* and *blaS2A*, we inserted a transcription terminator sequence into the *aphA7* gene and showed that this abolished expression of both the *aphA7* and *blaS2A* genes. In this experiment, we inserted a 600-bp DNA fragment that included the *E. coli* T1-T2 terminator sequence of *rrnB* (6) into the *Xho*I-*Sma*I site of pJH196 (Fig. 1) and examined the phenotype conferred on *E. coli* SY203 by the resulting plasmid, pKL250. Twenty-five chloramphenicol-resistant, kanamycin-sensitive transformants were analyzed. Twenty-four of these were found to be cefotaxime sensitive, while one showed a low level of cefotaxime resistance. Restriction analysis of the kanamycin-sensitive, cefotaxime-sensitive plasmids confirmed that the terminator sequence was properly inserted into the *Xho*I-*Sma*I site of the *aphA7* gene. This shows that transcription of *blaS2A* requires sequences to the left of the *Xho*I site located in *aphA7*.

Finally, we measured the in vivo primary transcript of the *aphA7* and *blaS2A* genes by performing a Northern (RNA) assay (Fig. 4). In this assay, total RNA was fractionated on an agarose gel and the gel was probed with two DNA fragments, the *Sal*I-*Xho*I fragment and a synthetic oligonucleotide extending from nucleotides 1362 to 1347. Both probes showed the presence of a 1.7-kb transcript consistent with an mRNA beginning at position 367 and continuing to 2134. Other transcripts are evident, with both probes indicating a shorter transcript with the same 5′ end as the 1.7-kb transcript. This shorter transcript may result from a weak transcription terminator within the operon or from nuclease digestion from the 3′ end of the longer transcript. Therefore, we conclude that *aphA7* and *blaS2A* constitute a single transcriptional unit.

Evolution of *blaS2A*. The above results establish that *aphA7* and *blaS2A* form the IAB operon and that the

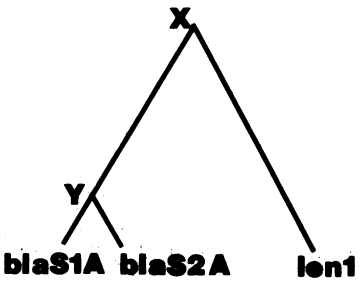


FIG. 5. Minimal tree for the β-lactamases. The phylogenetic relatedness between the three β-lactamases is determined by the number of amino acid differences between their respective gene products. The length of each branch is proportional to the minimal number of amino acid substitutions needed to relate each extant sequence to the same ancestral state. We arbitrarily placed the ancestral node X at the midpoint between *len-1* and *blaS*. Ancestral nodes X and Y contain the shared sequences at the 5′ end of *blaS1A* and *len-1* as described in the text.

promoter is, in part, provided by IS*26*. We would like to argue further that IAB has been assembled from ancestral forms of an *aphA* gene and a *blaS* gene that were initially unlinked to each other. First consider *blaS*. This argument uses the principle of parsimony in phylogenetic reconstruction and relies on comparing *blaS1A* and *blaS2A* with *len-1* as an outgroup taxon. The SHV β-lactamases are closely related to the *K. pneumoniae* chromosomally encoded LEN-1 β-lactamase (1). The evolutionary distances between *blaS1A* and *len-1* and between *blaS2A* and *len-1* are 0.11 and 0.11, respectively. (The evolutionary distance is the number of sites that are different divided by the number of sites that are being compared over the region of homology.) Moreover, the relationship between these three genes is preserved in both the 5′ and 3′ sequences flanking *blaS1A*, *blaS2A*, and *len-1* as is apparent from the sequences in Fig. 2. Therefore, these flanking sequences must have been present in the common ancestor to these three genes, which is designated as node X in Fig. 5. Because *blaS1A* and *blaS2A* are much more closely related to each other than they are to *len-1*, we can assign a common ancestor to them that excludes *len-1*, which is designated node Y in Fig. 5. Furthermore, we can say that the ancestral configuration at node Y is *blaS1A*-like rather than *blaS2A*-like. This follows because the *blaS1* gene shares at least 36 bp with *len-1*, upstream of the coding sequence and including the promoter, that are not present in *blaS2A*. In this scheme, those common sequences were deleted after divergence at node Y in the lineage leading to *blaS2A*. In assigning a *blaS1A*-like ancestral state to node Y, we are assuming that a deletion of the 36 nucleotides in the branch leading to *blaS2A* is much more probable than is the independent insertion of those same sequences in the independent branches leading to *len-1* and *blaS1A*. Therefore, we can conclude with a very high level of confidence that *blaS2A* evolved from a *blaS1A*-like ancestor.

The tree in Fig. 5 and the assignment of the ancestral nodes is based on the presence or absence of 5′- and 3′-flanking sequences and on the number of nucleotide differences between the homologous segments by using parsimony in phylogenetic reconstruction. Using a historical argument, we can also conclude that *blaS2A* probably evolved from a *blaS1A*-like ancestor. It appears that SHV-2A evolved from a SHV-1-like ancestor under the selective pressure of cefotaxime use. The SHV-1 β-lactamase was repeatedly isolated from penicillin- and carbenicillin-resistant bacteria before the introduction of the new broad-spectrum cephalosporins such as cefotaxime (25). Those SHV-1 isolates that had been saved in permanent stabs were subsequently shown to be unable to hydrolyze cefotaxime. However, after the clinical introduction of cefotaxime in the early 1980s, resistant bacteria emerged containing SHV-type β-lactamases such as *blaS2A* that hydrolyzed these drugs.

SHV-2A differs from SHV-1 by an amino acid difference, Ser-238 in place of Gly-238, that presumably results in the different catalytic properties. All the evolved SHV-type and TEM-type β-lactamases that hydrolyze third-generation cephalosporins contain Ser-238 (3, 33). Levesque (personal communication) has shown that replacing the Ser-238 of SHV-2 with Gly-238 restores the SHV-1 phenotype and that, conversely, changing the Gly-238 to Ser-238 in SHV-1 gives a β-lactamase capable of hydrolyzing cefotaxime. Since the carbon atom of residue 238 in the homologous *Staphylococcus aureus* PC1 β-lactamase contacts the enzyme active site, this is an understandable difference.

Evolution of *aphA7*. We have shown elsewhere (Lee et al., submitted) that *aphA7* evolved from a Tn*903*-*aphA1*-like ancestor. This conclusion is based on a phylogenetic reconstruction employing parsimony in which *aphA1* (Tn*903*) and *aphA7* were compared with three other closely related *aphA1* genes. In addition, we have indications, though not as dramatic as for *blaS2A*, that *aphA7* has evolved under selection from an *aphA1* (Tn*903*)-like ancestor (Lee et al., submitted).

DISCUSSION

We established that the plasmid pBWH77 carries an operon (IAB) composed of the aminoglycoside resistance gene *aphA7* and the β-lactamase resistance gene *blaS2A*. We showed that this operon is under the control of an efficient hybrid promoter, part of which consists of the IRS of an IS*26* element. The close association of ISs with antibiotic resistance genes has strongly suggested an active role for these sequences in the evolution or mobility of antibiotic resistance genes. The structure and sequence of the IAB operon allow us to strengthen this hypothesis. This follows because the three elements that make up IAB have all been encountered previously in entirely different contexts, and furthermore, we can infer that IAB evolved from these previously described elements. Thus, for example, we were able to establish the direction of descent of *blaS2A* through the application of phylogenetic tree construction principles and through the availability of sufficient sequence information to allow us to root our tree to a convenient outgroup, the *K. pneumoniae len-1* β-lactamase. Using similar arguments, we have also shown that *aphA7* evolved from an *aphA* gene that has been seen in Tn*903* and Tn*2350* (Lee et al., submitted).

Pairs of resistance genes forming a single operon have been reported before (7, 12, 17, 23). Brau et al. (6) suggested on the basis of the nucleotide sequence that IS*26* can provide the −35 region of a hybrid promoter in their example. However, in these cases it is not possible to infer ancestor-descendent relation. In the present case, we can conclude that a *blaS* gene from an ancestral form resembling *blaS1* and an *aphA* gene from an ancestral form resembling the *aphA* found in Tn*903* came together, losing their ancestral promoters and gaining the hybrid IS*26* promoter to form the IAB operon.

Not only can we infer the direction of descent for the individual elements, but also it appears that many of the

FIG. 6. Theoretical sequence of steps leading to formation of the IS*26* hybrid promoter in the IAB operon. Initially, *aphA1* is under the control of its own promoter within the transposon Tn*903*. In the intermediate stage, IS*26* is located adjacent to the *aphA* promoter, as in Tn*4352*. In the IAB operon, part of the original *aphA* promoter of Tn*903* has been deleted and the −35 region is now provided by IS*26*.

differences between IAB and its individual parts have been selected. The *blaS2A* gene encodes an enzyme that has a Ser-238 compared with the Gly-238 of its assumed ancestor, SHV-1, a change that increases affinity of the enzyme for certain third-generation cephalosporins, so that the gene confers resistance to cefotaxime and ceftizoxime. In addition, the *aphA7* gene encodes an enzyme that has four amino acid differences from its assumed ancestor, *aphA1*, changes that appear to be a consequence of aquisitive evolution (Lee et al., submitted). Presumably, the creation of a hybrid promoter with the −35 region provided by IS*26* was selected. Both *aphA1* in Tn*903* and *aphA7* have efficient promoters, but cells containing the latter gene have 2.4-fold as much aminoglycoside-3′,5″-phosphotransferase-I as cells containing Tn*903* (Lee et al., submitted), suggesting that the IS*26* insertion has produced a more efficient promoter. This more efficient promoter could be adaptively significant, either from more gene product, or possibly, the ancestral promoter found in Tn*903* may have been inadequate to drive transcription of the two-gene operon.

The actual steps in the evolution of the IAB operon from the ancestral *blaS* and *aphA* genes and IS*26* cannot be reconstructed, but it is possible to describe two possible pathways based on our knowledge of several *aphA1* transposons. One pathway would begin with the insertion of Tn*903* upstream of a *blaS1*-like gene, followed by an insertion of IS*26* between one of the IS*903*s and the 5′ end of the *aphA1* gene and a deletion extending from the 3′ end of the *aphA1* gene to 58 bp before the 5′ end of the *blaS* gene. The *aphA1* gene and the −10 region of Tn*903* are identical with those from the IAB operon sequences except for the five nucleotide substitutions within the *aphA* gene (26; Lee et al., submitted), making Tn*903* a strong candidate for the IAB operon precursor. In Tn*903*, the −35 region of the *aphA1* promoter is provided by sequences outside of the IS element (26). Tn*4352* is a possible intermediate in this pathway. It has the *aphA1* gene and the −10 and −35 promoter sequences as in Tn*903*, but with an IS*26* located immediately beyond the −35 region (37). A small deletion event within Tn*4352* would generate the hybrid promoter structure of the IAB operon (Fig. 6).

A second possible pathway is suggested by the transposon Tn*2680*, which has IS*903* and IS*26* sequences in close proximity, with the *aphA1* gene adjacent to an IS*903* variant and IS*26* elements forming the ends of the transposon (20). An internal inversion event in Tn*2680* would place the *aphA1* gene under the control of a hybrid IS*26*-Tn*903* promoter, with the IS*26* in the same orientation as the IAB operon. A second step would be the insertion of this transposon next to a *blaS* gene, followed by deletion of the sequences between the *aphAS1* and *blaS* genes. However, the nucleotide sequence of the Tn*2680 aphA1* gene and its promoter-proximal flanking region show significant divergence from Tn*903* and the IAB operon, so Tn*2680* is only suggestive of the possible structure of an IAB precursor.

Both of the genes in the IAB operon appear to have undergone recent adaptive evolution. Examples of adaptive evolution are exceedingly rare in molecular evolution, as the vast majority of cases involve neutral substitutions. Four cases of adaptive evolution can be cited: (i) the cefotaxime-hydrolyzing β-lactamases (33), (ii) *aphA7*, (iii) the convergent substitutions seen with the ruminant and leaf-eating primate lysozymes (34), and (iv) the G protein (39). It could be just a coincidence that two of these unusual cases have been encountered in this single operon. It is difficult to imagine an evolutionary mechanism to account for this, two genes under independent selective pressure becoming linked as a result, although it is certainly tempting to speculate along such lines.

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