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In vivo transfer of genetic information between Gram-positive and Gram-negative bacteria

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A 1427-bp DNA fragment containing the kanamycin resistance gene, *aphA-3*, of plasmid pIP1433 from *Campylobacter coli* was inserted into a shuttle vector. Full expression of *aphA-3* was obtained in *Bacillus subtilis* and in *Escherichia coli*. This DNA fragment was sequenced in its entirety and the starting point for *aphA-3* transcription in *B. subtilis*, *C. coli* and *E. coli* was determined by S1 nuclease mapping. The sequence of the promoter consists of the hexanucleotides TTGACA and TATAAT, with a spacing of 17 bp. The nucleotide sequence of the *aphA-3* gene from *C. coli* and from the streptococcal plasmid pJH1 are identical whereas they differ by two substitutions and deletion of a codon from that cloned from the staphylococcal plasmid pSH2. These results indicate a recent extension of the resistant gene pool of Gram-positive cocci to Gram-negative bacilli. From an analysis of the DNA sequences surrounding the promoter region, we concluded that the DNA fragment containing the *aphA-3* gene in plasmid pJH1 has evolved by deletions from a sequence similar to that found in plasmid pIP1433.

Key words: Gram-negative bacteria/Gram-positive bacteria/gene transfer/kanamycin/sequence homologies

Introduction

The most common form of resistance towards aminoglycosides involves the synthesis of enzymes which modify the antibiotics (Davies and Smith, 1978). Aminoglycoside-modifying enzymes are classified according to the chemical reaction catalysed (N-acetylation, O-nucleotidylation and O-phosphorylation) and the site of modification on the antibiotic molecule. 3′-Aminoglycoside phosphotransferases, APH(3′), catalyse the phosphorylation of the hydroxyl group in position 3′ of aminohexose I of kanamycin and other structurally related antibiotics. Five types of APH(3′) can be distinguished on the basis of their substrate range *in vitro* (Davies and Smith, 1978; Dowding, 1979). The APH(3′) enzymes are of special interest with regard to the origin of antibiotic resistance genes. They have been found in numerous Gram-negative (types I and II) and Gram-positive (type III) human clinical isolates, but also in aminoglycoside-producing strains of bacilli (type IV) and *Streptomyces* (type V) (Benveniste and Davies, 1973; Davies and Smith, 1978). In addition, considerable structural data concerning this group of enzymes is now available. The genes encoding APH(3′) enzymes from transposons Tn*903* *(aphA-1)* and Tn*5* *(aphA-2)*, detected in Gram-negative bacteria, from plasmids pSH2 (*aphA-3*) and pJH1 (*aphA-3*) isolated from the Gram-positive *Staphylococcus* and *Streptococcus* respectively, and from the chromosome of a butirosin-producing *Bacillus circulans (aphA-4)* and a neomycin-producing *Streptomyces fradiae (aphA-5)*, have been sequenced (Oka *et al.*, 1981; Beck *et al.*, 1982; Gray and Fitch, 1983; Trieu-Cuot and Courvalin, 1983; Herbert *et al.*, 1983; Thompson and Gray, 1983). Comparison of these genes, or of the deduced amino acid sequences, indicate that they have probably diverged from a common ancestor and that recent *in vivo* intergeneric transfer of genetic information has occurred between *Staphylococcus* and *Streptococcus*. The *aphA-1* and *aphA-2* genes are apparently confined to Gram-negative bacteria whereas the *aphA-3* gene seems specific for Gram-positive cocci. This distribution is reflected by the various degrees of homology between the three types of genes, 1 and 2 being the most closely related (Trieu-Cuot and Courvalin, 1983).

Campylobacter coli and *C. jejuni* are Gram-negative bacteria frequently responsible for bacterial acute gastroenteritis in humans. *C. coli* strain BM2509 is resistant to high levels of kanamycin and structurally related antibiotics (Lambert *et al.*, 1985). This resistance phenotype is due to the synthesis of a plasmid-encoded APH(3′) of type III, an enzyme not detected previously in a Gram-negative bacterium (Courvalin and Carlier, 1981). We report here the nucleotide sequence of the kanamycin resistance gene *aphA-3* from *C. coli* BM2509 and the study of its expression in *Escherichia coli* and *Bacillus subtilis*. The results obtained from the comparison of DNA sequences confirm our hypothesis (Lambert *et al.*, 1985) that resistance to kanamycin in *C. coli* BM2509 is due to the *in vivo* acquisition of a gene, or a plasmid, from a Gram-positive bacterium.

Results

Construction of plasmids in vitro

The *aphA-3* gene from *C. coli* plasmid pIP1433 (Km, Tc, 47.2 kb) was cloned in pBR322 after digestion with *Hind*III and subsequently subcloned after digestion with *Cla*I. The resulting recombinant plasmid, pAT95 (Figure 1), possesses a 1.42-kb insert and confers resistance to ampicillin, kanamycin and tetracycline when present in *E. coli*. A restriction map analysis (not shown) indicated that this insert was closely structurally related but not identical to the 1489-kb *Cla*I-generated DNA fragment containing the *aphA-3* gene of the streptococcal plasmid pJH1 (Trieu-Cuot and Courvalin, 1983). To study the expression of the kanamycin resistance of *C. coli* in *B. subtilis*, plasmid pAT96 was constructed by inserting the *Hind*III-linearized staphylococcal plasmid pCI94 (Horinouchi and Weisblum, 1982) into the unique *Hind*III site of pAT95 (Figure 1). Plasmid pAT96 (pAT95ΩpC194) confers resistance to chloramphenicol and kanamycin in *E. coli* and *B. subtilis* and also to ampicillin in *E. coli*.

Nucleotide sequence of the insert in pAT95

The purified 1.42-kb *Cla*I-*Hind*III fragment of plasmid pAT95 (Figure 1) was subcloned in the replicative forms of bacteriophages M13mp8 or M13mp9 after digestion with *Hpa*II, *Sau*3A and *Taq*I. In each experiment, specific clones were identified by

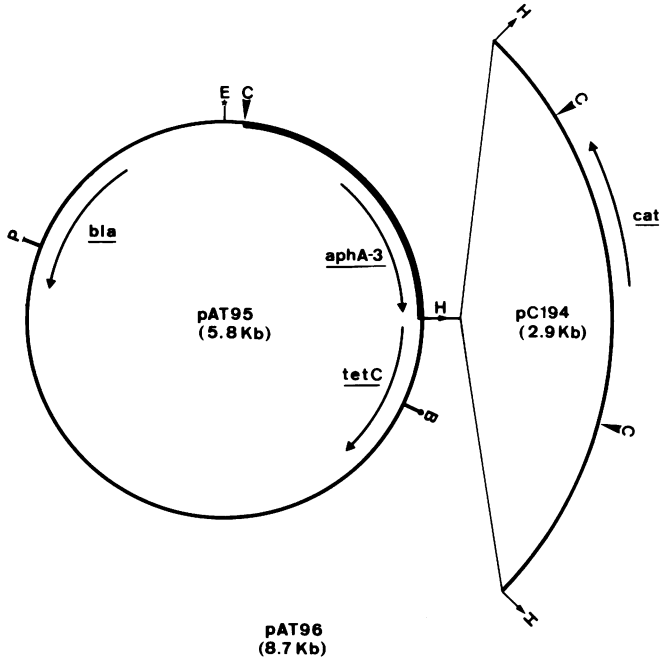

Fig. 1. Structure of plasmids pAT95 and pAT96 (pAT95ΩpC194). The 1.42-kb *Cla*I-*Hind*III *Campylobacter* insert is represented as a heavy line. *aphA-3*, 3'-aminoglycoside phosphotransferase of type III; *bla*, β-lactamase; *cat*, chloramphenicol acetyltransferase; *tetC*, tetracycline resistance gene of class C. Arrows indicate the direction and extent of transcription. B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hind*III; P, *Pst*I. Only relevant restriction endonuclease recognition sites are shown.

the dideoxy-T screening method and sequenced by the chain terminator technique. A partial restriction map and the sequencing strategy for the DNA fragment containing the *aphA-3* gene from *Campylobacter* is shown in Figure 2. The entire nucleotide sequence obtained by computer analysis is presented in Figure 3. The longest open reading frame contains 792 bp and codes for the APH(3').

Starting point for transcription in B. subtilis, C. coli and E. coli

The starting point for *aphA-3* transcription on the *Cla*I-*Hind*III DNA fragment was determined by S1 nuclease mapping. Total RNA extracted from *B. subtilis* QB666 and HB101 harbouring plasmid pAT96 (pAT95ΩpC194) and from *C. coli* BM2509 harbouring plasmid pIP1433 was hybridized with the *Cla*I-*Bss*HII DNA fragment labelled at its *Bss*HII end (Figure 3). The DNA pieces remaining after S1 nuclease treatment and those obtained by A+G chemical cleavage (Maxam and Gilbert, 1980) of the same DNA probe were electrophoresed on the same polyacrylamide gel. The patterns obtained with *B. subtilis*, *C. coli* and *E. coli* were similar (Figure 4) and showed that the longest transcripts are initiated at coordinates 197, 198, 199 and 200 after application of a 1.5-bp correction (Sollner-Webb and Reeder, 1979). The canonical −35 recognition site and −10 Pribnow box are conventionally placed upstream from the starting point. Since transcription starts preferentially at a purine rather than at a pyrimidine, the A residue at position 198 (Figure 3) is a likely candidate for the transcriptional start point. The minor transcripts initiated at coordinates 202 and 203 probably correspond to degraded mRNAs.

Comparison of the nucleotide sequences of the DNA fragments containing the aphA-3 genes of plasmids pIP1433, pJH1 and pSH2

The nucleotide sequences of the DNA fragments containing the

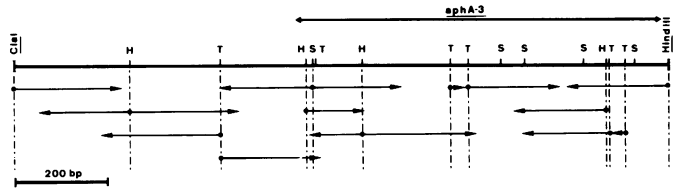

Fig. 2. Partial restriction map and sequencing strategy for the 1.42-kb *Cla*I-*Hind*III DNA fragment containing the *aphA-3* gene from *Campylobacter*. The restriction sites are: H, *Hpa*II; S, *Sau*3A; T, *Taq*I. Arrows indicate the extent and direction of the sequencing reaction. Double arrow specifies the *aphA-3* gene.

aphA-3 genes of plasmids pIP1433, pJH1 and pSH2 were compared with each other and aligned in such a way that optimal coincidence occurred (Figure 3). The *aphA-3* genes originating from plasmids pIP1433 and pJH1 were identical whereas that cloned from plasmid pSH2 differed by two substitutions located at coordinates 846 (CTG/V in pSH2 instead of CTG/L in pIP1433 and pJH1) and 1281 (CCC/P versus GCC/A), and by a deletion of a codon (GGT/C) at coordinate 721 (Figure 3). The DNA sequences upstream from the *aphA-3* genes from plasmids pIP1433 and pJH1 were also closely related although a 69-bp long gap was introduced into pJH1 to ensure optimal homology (Figure 3). In addition, these two DNA fragments differed by three substitutions located at coordinates 187 (A in pIP1433 instead of C in pJH1), 188 (A versus T), and 480 (G versus C). Interestingly, changes at coordinates 187 and 188 occurred in the −10 sequences of the *aphA-3* promoters in plasmids pIP1433 (TATAAT) and pJH1 (TATCTT) (Figure 3). The DNA sequence of the 213-bp long region upstream from the staphylococcal *aphA-3* gene has been determined (Gray and Fitch, 1983). Among the 69 bp adjacent to the structural gene, pSH2 differed from pIP1433 and pJH1 by a single base pair insertion at position 615 whereas the rest of the sequence does not exhibit any significant homology (Figure 3).

Discussion

We have determined the nucleotide sequence of the kanamycin resistance gene *aphA-3* from *C. coli* strain BM2509. This resistance determinant is located on a 1.42-kb *Cla*I-*Hind*III fragment of plasmid pIP1433 and also confers resistance to kanamycin when present in *E. coli* and in *B. subtilis*. Transcription of *aphA-3* in its original host *C. coli*, in *E. coli* and in *B. subtilis* starts at the same site located 406 bp upstream from the ATG initiator (Figure 4). The sequence of the promoter consists of the hexanucleotides TTGACA and TATAAT with a spacing of 17 bp (Figure 3). To our knowledge, no information concerning the transcriptional apparatus of *Campylobacter* sp. is available. Our results indicate that the RNA polymerases of *C. coli*, *E. coli* and *B. subtilis* recognize the same specific sequence on the DNA template. However, whether strict conservation of the prototype promoter sequence, as shown for *B. subtilis* (Moran *et al.*, 1982), is required for transcription in *Campylobacter* remains to be demonstrated. In plasmids pIP1433 and pJH1, an inverted repeat is located between the promoter and the *aphA-3* gene (Figure 3). Since the two sequences form a very weak association, $\Delta G = -4.8$ kcal/mol (Tinoco *et al.*, 1973), it seems unlikely that this structure interferes with *aphA-3* transcription. Two palindromic sequences with mirror symmetry are present upstream from the *aphA-3* gene from pIP1433 (Figure 3). Their biological role, if any, remains unknown.

The quasi-identity observed (two substitutions and deletion of

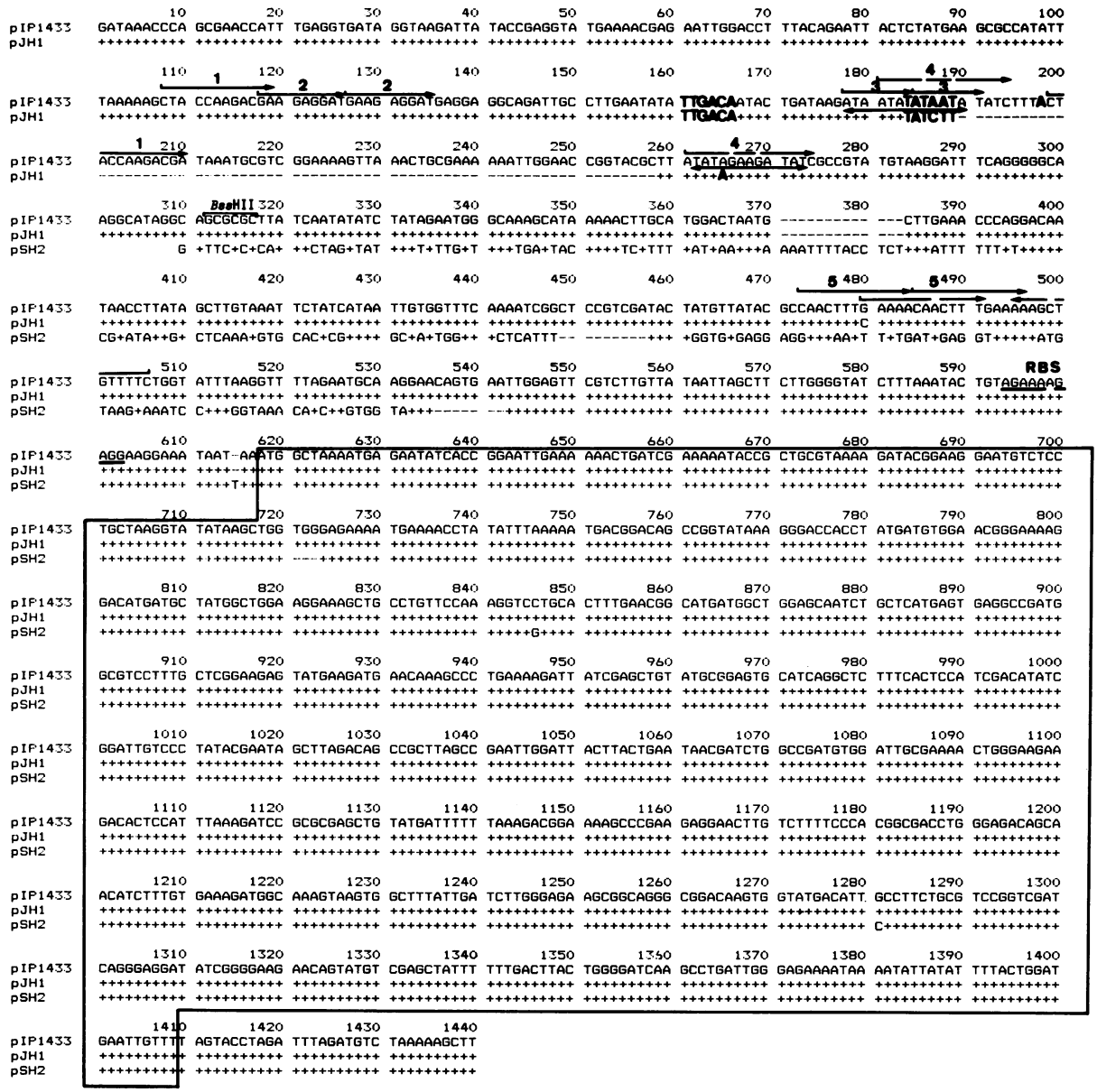

Fig. 3. Nucleotide sequences of the DNA fragments containing the *aphA-3* genes from plasmids pIP1433 (*Campylobacter*), pJH1 (*Streptococcus*) and pSH2 (*Staphylococcus*). Homology with the DNA seqeunce of *Campylobacter* is indicated by plus. Dashes represent gap introduced to ensure optimal homology. Numbering begins at the *Cla*I junction with the pBR322 vector. The *aphA-3* region is boxed by solid lines. Bases complementary to the 3′ end of the 16S rRNA of *B. subtilis* are underlined (RBS). The −35 recognition site, −10 Pribnow box and the transcription start point are indicated by darker lettering when known (Trieu-Cuot *et al.*, 1985 and Figure 4). Direct repeats, inverted repeats and palindromic sequences are depicted by numbered arrows, facing arrows and double arrows, respectively.

a codon) (Figure 3) between the *aphA-3* genes originating in *Staphylococcus* (Gray and Fitch, 1983) and *Streptococcus* (Trieu-Cuot and Courvalin, 1983) constitutes evidence for a recent intergeneric transfer of DNA between these phylogenetically remote bacteria. This finding is consistent with the fact that direct plasmid transfer has been obtained between these two genera under laboratory conditions (Engel *et al.*, 1980; Schaberg *et al.*, 1982). The nucleotide sequence of the *aphA-3* gene from *C. coli* BM2509 appeared to be identical to the corresponding gene in *Streptococcus* (Figure 3). Until now *aphA-3* genes were considered to be specific for Gram-positive bacteria (Courvalin and Carlier, 1981). Accordingly, the region upstream from the translational initiation sites of the *aphA-3* genes in plasmids pIP1433, pJH1 and pSH2 exhibits a strong complementarity ($\Delta G = -14.4$ kcal/mol) with the 3′-OH of the 16S rRNA of *B. subtilis*, a striking feature which distinguishes genes from Gram-positive and Gram-negative bacteria (MacLaughlin *et al.*, 1981). Taken together, these results substantiate our previous claim that emergence of resistance to kanamaycin in *Campylobacter* is due to recent acquisition *in vivo* of a gene from a Gram-positive bacterium (Lambert *et al.*, 1985). Antibiotic resistance determinants from Gram-positive organisms are generaly expressed in Gram-negative bacteria whereas the reverse is uncommon (Chang and Cohen, 1974; Courvalin *et al.*, 1977; Kreft *et al.*, 1978; Trieu-Cuot *et al.*, 1985). Therefore, the only apparent barrier to the acquisition of genes from a Gram-positive by a Gram-negative bacterium lies in the transfer process and in the replication of the exogenous DNA. All attempts to transfer by conjugation plasmid pJH1 from *S. faecalis* to *C. coli*, or plasmid pIP1433 from *C. coli* to *S. faecalis* were unsuccessful. Restriction endo-

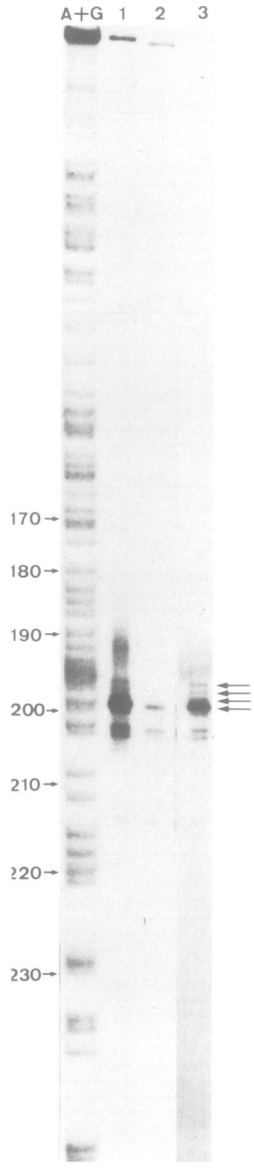

Fig. 4. Determination of the starting point for *aphA-3* transcription *in vivo*. S1 nuclease mapping was performed with a *Cla*I-*Bss*HII DNA probe protected with RNA extracted from *B. subtilis* (**lane 1**), *C. coli* (**lane 2**) or from *E. coli* (**lane 3**). Chemical degradation (A+G) was performed on the same DNA probe. Numbering refers to the *Cla*I restriction site (Figure 3). Arrows indicate putative transcriptional start sites. The smaller bands presumably represent degraded transcripts.

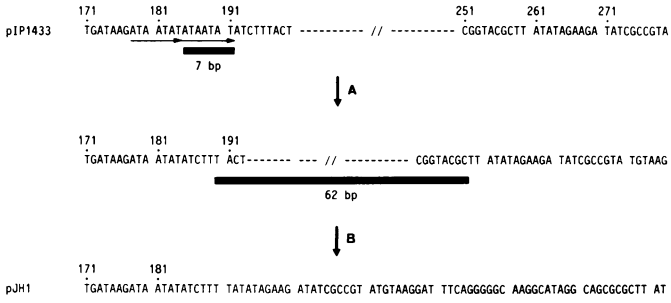

Fig. 5. Hypothetical phylogeny among the *aphA-3* genes from plasmids pIP1433 and pJH1. **A**: deletion involving slipped-mispairing or unequal crossing-over. **B**: deletion involving illegitimate recombination. Horizontal arrows indicate tandemly repeated sequences. The segments deleted are underlined. Numbering refers to the *Cla*I site (Figure 3).

nuclease analysis and Southern hybridizations (data not shown) indicated that plasmid pIP1433 shares extensive sequence homology with plasmid pMAK175, representative of the tetracycline resistance plasmids of *Campylobacter* sp. (Taylor *et al.*, 1983), but not with plasmid pJH1. These data support the notion that kanamycin resistance in *Campylobacter* results from the acquisition of a gene rather than that of a replicon *en bloc*. Our observation constitutes the first example of transfer of genetic information between Gram-positive and Gram-negative bacteria under natural conditions and extends the current notion of intergeneric exchange of resistance determinants among procaryotes.

The DNA fragments containing the *aphA-3* gene in plasmids pIP1433 and pJH1 are closely related and one could hardly doubt that one has recently evolved from the other since the only significant difference is the presence, in pIP1433, of a 69 bp long extra-sequence (Figure 3). What is the molecular process(es) responsible for this evolution? A dot matrix homology search (data not shown) revealed that the extra DNA fragment did not bear significant homology to any of its flanking sequences. Thus this sequence does not result from a duplication. The sequence CTACCAAGACGA present at coordinate 108 in plasmids pIP1433 and pJH1 is repeated at coordinate 199, i.e., within the additional fragment, in plasmid pIP1433 (Figure 3). The probability that this dodecanucleotide occurs by chance in a 69-bp-long DNA sequence is very low (2×10^{-6}). This observation argues strongly against the involvement of an intermolecular rearrangement leading to an insertion. Therefore, we hypothesize that the DNA fragment encoding the APH(3′) in plasmid pJH1 has evolved by deletion(s) from a sequence similar, or identical, to that of plasmid pIP1433. Interestingly, the region upstream from the *aphA-3* gene in plasmid pIP1433 exhibits several small, 7 – 14 bp, directly repeated sequences (Figure 3). These structures are susceptible to generate deletions or additions during replication and recombination (Streisinger *et al.*, 1966; Jeffreys and Harris, 1982). Indeed, unequal crossing-over in general recombination or slipped-mispairing occurring at the tandem repeat no. 3 in pIP1433 (Figure 3) would generate a promoter identical to that found in plasmid pJH1 (Figure 5). This mechanism, however, cannot account for the deletion of the remaining 62 bp that did not occur at repeated sequences (Figure 3). This type of deletion, already observed in the *lac*I gene of *E. coli* (Farabaugh *et al.*, 1978), could involve a gyrase-mediated illegitimate recombination (Ikeda *et al.*, 1981, 1982). The two successive deletion events proposed (Figure 5) remove most of the direct repeats present in the pIP1433 DNA (Figure 3). Consequently, these rearrangements correspond to a progression from a molecule genetically less stable to one that is more stable, as expected for an *in vivo* evolutionary process (Cohen *et al.*, 1978). Our hypothetical scheme (Figure 5) obviously implies that the divergence between the DNA fragments originating in plasmids pIP1433 and pJH1 has occurred prior to the transfer of the *aphA-3* gene from a Gram-positive bacterium to *Campylobacter*. The fact that the 69-bp long additional fragment in plasmid pIP1433 is almost entirely (61 bp) present in the non-coding region upstream from the *aphA-3* gene of the streptococcal transposon Tn*1545* (F.Caillaud, personal communication) constitutes further support to our hypothesis.

Materials and methods

Bacterial and bacteriophage strains

C. coli strain BM2509 which harbours plasmid pIP1433 (Km, Tc, 47.2 kb) was previously described (Lambert *et al.*, 1985). *E. coli* strain HB101 (Boyer and Roulland-Dussoix, 1969) and *B. subtilis* strain QB666 (Klier *et al.*, 1982) were

used for cloning experiments. Fragments of DNA to be sequenced were transfected into *E. coli* strain JM101 (Messing, 1979) using either M13mp8 or M13mp9 bacteriophages (Messing and Vieira, 1982).

Transformation of B. subtilis and E. coli

Recombinant plasmids were introduced by transformation into *B. subtilis* (Ehrlich, 1978) and into *E. coli* (Maniatis *et al.*, 1982) as described. Antibiotic concentrations for bacterial selection were: ampicillin, 100 μg/ml; chloramphenicol, 5 μg/ml; and kanamycin, 50 μg/ml.

Preparation of plasmid DNA and of total RNA

Isolation of plasmid pIP1433 DNA (Ingram *et al.*, 1973) and large-scale isolation of plasmids pAT95 and pAT96 DNA (Maniatis *et al.*, 1982) were as described. Isolation of total RNA from *B. subtilis*, *C. coli* and *E. coli* was performed according to Petit-Glatron and Rapoport (1975).

Preparation of labelled DNA probes

Plasmid pAT95 was cleaved at its unique *Bss*HII restriction site. After purification by electrophoresis in a 0.7% agarose gel, this fragment was dephosphorylated with calf intestinal phosphatase and labelled at the 5′ end with [γ-^{32}P]ATP and T4 polynucleotide kinase (Maniatis *et al.*, 1982). After a secondary cleavage, the 320-bp long *Cla*I-*Bss*HII probe labelled at one extremity was purified by electrophoresis in a 5% polyacrylamide gel (Maniatis *et al.*, 1982).

S1 nuclease mapping

Total RNA (100 μg) extracted from *B. subtilis* and *E. coli* strains harbouring pAT96 and from *C. coli* BM2509 harbouring pIP1433 were mixed with 50 000 c.p.m. of labelled DNA probe and lyophilized. The freeze-dried nucleic acids were dissolved in 30 μl of Hepes buffer (Debarbouille and Raibaud, 1983), heated at 90°C for 10 min and incubated at 42°C for 3 h. The mixture was then diluted 10-fold with S1 nuclease buffer and 500 units of S1 nuclease were added. After 1 h of incubation at 37°C, the DNA was extracted twice with phenol-chloroform, ethanol precipitated and electrophoresed.

Nucleotide sequencing

DNA fragments were cloned in bacteriophages M13mp8 and M13mp9 and sequenced by the chain terminator technique (Sanger *et al.*, 1977). The complete DNA sequence was arranged using DBCOMP and DBUTIL computer programs (Staden, 1980). Nucleotide sequences (A+G reactions) of the 5′ end-labelled DNA probes were determined as described (Maxam and Gilbert, 1980). DNA fragments were electrophoresed in 8% polyacrylamide gels containing 7 M urea.

Comparison of nucleotide sequences

The nucleotide sequences were compared using a computer and the algorithm of Wilbur and Lipman (1983). The K-tuple size was 3, the window size 20 and the gap penalty 7. All computations were carried out at the 'Centre de Calcul', Institut Pasteur.

Enzymes and chemicals

Restriction endonucleases *Acc*I, *Bam*HI, *Cla*I, *Hind*III, *Hpa*II, *Sau*3A, *Taq*I, calf intestinal phosphatase, DNA polymerase I (large fragment) and T4 ligase were from Boehringer Mannheim. *Bss*HII and M13 pentadecamer primer were from Biolabs. T4 polynucleotide kinase, S1 nuclease, deoxynucleoside triphosphates and dideoxy nucleoside triphosphates were purchased from PL-Biochemicals. Deoxyadenosine 5′-[α-^{32}P]triphosphate, triethylammonium salt and adenosine 5′-[γ-^{32}P]triphosphate, triethylammonium salt were obtained from Amersham International.

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