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Circularized Copies of Amplifiable Resistance Genes from *Haemophilus influenzae* Plasmids

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Tandem repeat amplification of resistance determinants in *Haemophilus influenzae* plasmids is associated with the occurrence of separate circular DNA molecules. They were demonstrated to represent mono- and multimeric forms of the amplifiable segments of the plasmids which comprise the respective resistance transposons and an additional region designated as an amplification sequence. The latter region mediates the recombinational events involved in amplification. The DNA circles apparently lack the ability to replicate autonomously but most probably provide an effective means for the translocation of resistance genes from one plasmid to another.

We recently reported (23) that plasmids of *Haemophilus influenzae* generate multiple tandem repeats of either tetracycline or combined tetracycline-chloramphenicol resistance transposons, in adaptation to tetracycline selection. This amplification depends on host recombination pathways (23) and thus corresponds with comparable gene duplications occurring in other bacterial species (16, 20, 21, 27). In the 49-kilobases (kb) plasmid pFR16017, amplification involves a 9.3-kb tetracycline resistance transposon which is almost identical with Tn*10* (13), including two 1.4-kb IS*10* inverted repeats (23). In *Escherichia coli*, Tn*10* is known to express reverse dosage response (6, 24). However, in *H. influenzae*, the observed positive gene dosage effect is due to the unusually low level of resistance specified by Tn*10* in the nonamplified state (23). The 12.2-kb combined transposon of the 57-kb plasmid p85098 contains an additional 1.9-kb chloramphenicol resistance determinant (r-det) which is bound by two 1.3-kb inverted repeats (23). Insertion closely adjacent to the inside end of IS*10*-left affects a 1.6-kb deletion of Tn*10* (23). Besides, the amplifiable segments of both plasmids also include a 1.6-kb DNA amplification sequence (AS), which is responsible for the recombinational events involved in amplification (23).

This paper describes the isolation and characterization of circular DNA molecules that are produced in the course of the amplification process in *H. influenzae*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The *H. influenzae* strains FR16017 and 85098 used in this study harbor plasmids pFR16017 (49 kb, Tc^r) and p85098 (57 kb, Tc^r Cm^r), respectively (12, 23). The media and the growth conditions have already been listed (23).

Isolation of plasmid DNA. The methods employed for extraction and purification of plasmids were those previously specified (23). Samples were subjected to electrophoresis through 0.7% agarose gels. Plasmids R1*drd*-19 (17), RP4 (2), pW266 (14), and pRK290 (8) served as internal molecular weight standards. Several bands were excised from a gel and isolated by electroelution.

Restriction enzyme cleveage. *Bam*HI was purchased from Bethesda Research Laboratories, Neu Isenburg, Federal Republic of Germany. Digestions were carried out in REB buffer (23).

Electron microscopy. The methods used for spreading and homoduplexing have been reported (12).

RESULTS

Detection of circular DNA molecules derived from plasmid pFR16017. Samples of plasmid pFR16017 were obtained after sodium dodecyl sulfate lysis of the parent strain, followed by alkaline extraction and density gradient centrifugation. Electron microscopical observations indicated the rare occurrence of small DNA circles in such preparations. About 4 μg of plasmid DNA was concentrated and subjected to agarose gel electrophoresis. Four additional weak bands appeared below the intensive set of plasmids (Fig. 1). We assumed that those distinct size classes of DNA molecules corresponded with circular mono-, di-, tri-, and tetrameric forms of the amplifiable segment of pFR16017. Since each tandemly repeated unit consisted of the 9.3-kb tetracycline resistance transposon and the 1.6-kb AS region (23), the four bands consequently had to comprise multiples of 10.9 kb.

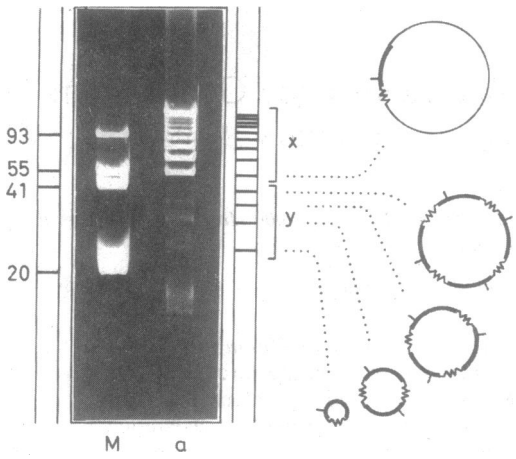

FIG. 1. Agarose gel electrophoresis (0.7%) of 4 μg of a pFR16017 sample, extracted after growth of the parent strain in the presence of 80 μg of tetracycline per ml. a, Stepwise increase in the size of the intensive bands. × indicates the gradual progress of tandem repeat amplification in the plasmid. The additional four weak bands (y) represent circular mono- and multimeric forms of the amplifiable segment of pFR16017. This characteristic is schematically illustrated. The AS region (zigzag line) and the tetracycline resistance transposon (thickened line) constitute 1 U of tandem repetition. The location of the *Bam*HI cleaving site is marked within the tetracycline r-dets. Further explanations are given in the text. M, Marker plasmids: R1*drd*-19 (93 kb), RP4 (55 kb), pW266 (41 kb), and pRK290 (20 kb).

However, these calculated molecular weights did not correlate with those inferred from the comparison with the standard plasmids (Fig. 1). Therefore, the four bands were excised from a gel, and the DNA was electroeluted and spread for electron microscopical investigation. In the presence of electroeluted plasmid pW266 (14) as a control, it was established that all intact molecules except the plasmids were present in a relaxed circular conformation, thus accounting for the observed migration properties during gel electrophoresis. By contour length measurements, we ascertained that the circles actually were equivalent in size to mono- and multimeric forms of the amplifiable segment of pFR16017. The same results were also obtained using a nonionic detergent for cell lysis. The proportion of the circles varied strongly in different preparations.

Characterization of the circular DNA molecules. After isolation from an agarose gel, the circles were digested with the *Bam*HI enzyme. Although the DNA subjected to restriction included molecules of the four disparate-size classes (Fig. 1), only one fragment was produced. It corresponded with the 10.9-kb *Bam*HI fragment of pFR16017, which constitutes a complete amplifiable segment in those plasmids which bear at least one tandem duplication (23). Accordingly, it was concluded that the different circles represented mono- and multimers of the amplifiable segment of pFR16017 (Fig. 1).

Further evidence in support of this assumption was gained by homoduplexing isolated circle DNA. Electron microscopical evaluation detected intramolecular homologous pairing within each circle. These regions were the IS*10* insertion sequences. From their opposite ends originated two single-stranded DNA loops representing the AS region and the tetracycline r-det, respectively. The circle shown in Fig. 2A comprised exactly one amplifiable segment. Figure 2B depicts a circularized dimer; Fig. 2C, a trimer. In the latter cases, the IS*10* sequences annealed in that orientation, which led to the looping out of the AS regions but not of the tetracycline r-dets, for reasons of closer proximity. Tetrameric circles were found as well. Moreover, examination of the total plasmid preparations revealed the existence of even hepta- and octomers, albeit at reduced frequencies. An approximate estimation indicated the circles to be present at a frequency of 10^{-1} per cell.

Similar DNA circles were also found in samples of plasmid p85098, which amplifies the combined transposon (23). A section of the plasmid containing two tandem repeats and the AS region between them is shown in Fig. 3A. The circles occurred only at a frequency of about 3×10^{-2} to 5×10^{-2} per cell. Therefore, they could not be efficiently isolated. Figure 3B presents a monomer, consisting of the 1.6-kb AS region and 4.9-kb tetracycline r-det, each of them forming a single-stranded loop at the opposite ends of the annealed IS*10* sequences. The additional third loop represented the chloramphenicol resistance gene, bound by two inverted repeats (23). Figure 3C shows a dimeric circle.

DISCUSSION

The results demonstrate that amplification in *H. influenzae* plasmids is associated with the occurrence of separate circular DNA molecules. They were mono- and multimeric forms of the resistance transposons and the AS region, which both constitute one unit of tandem repetition (23). As was indicated by the low and not even strictly reproducible frequency of detection, the circles are not expected to replicate autonomously. Relating to this property, they did not exhibit a supertwisted conformation, which is generally considered to be an important facet of circular duplex DNA replication (7). Rather, there is conclusive evidence that those molecules have a key function in amplification, serving as vehicles for the transmission of resistance

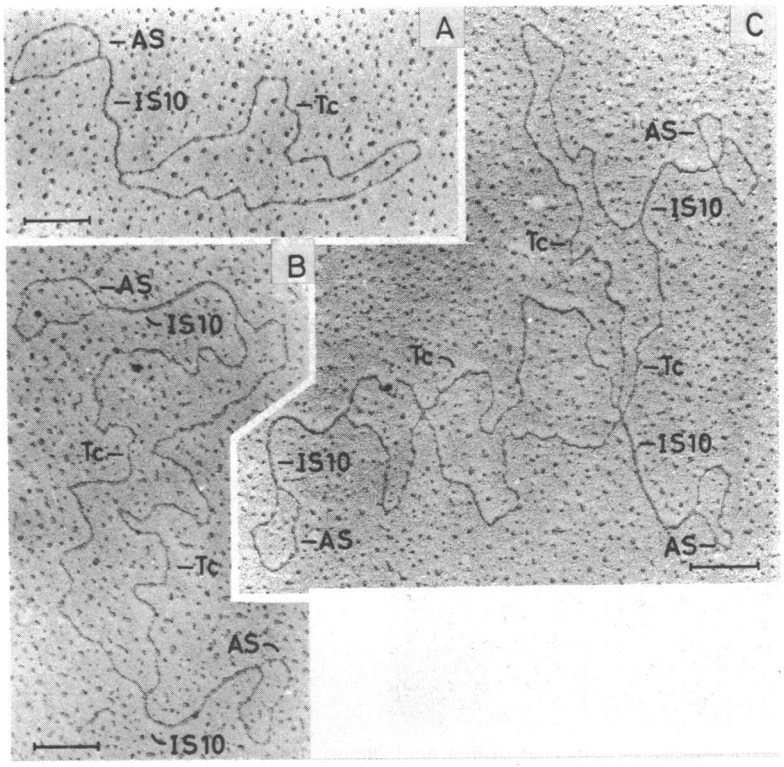

FIG. 2. Electron micrographs of homoduplexed DNA circles derived from pFR16017. An (A) mono-, (B) di-, and (C) trimeric form of the amplifiable segment of pFR16017. Tc, tetracycline. Further explanations are given in the text. Scale: 0.2 μm.

genes to another replicon. The data substantiate the hypothesis that intramolecular recombination between two AS regions gives rise to the circularization of a mono- or multimer of the amplifiable segment. The resultant circle may subsequently be capable of forming a cointegrate by reciprocal recombination with an AS region located on the recipient plasmid. This process renders possible the survival of a bacterial population under tetracycline stress conditions: the random translocation of r-dets may result in a progressive accumulation in a few plasmids, which then become selected for resistance. The characteristic features of the excisive and integrative recombination events can be explained by the Campbell model (3).

The possibility cannot be ruled out that other mechanisms also contribute to the amplification in *H. influenzae* plasmids. Concerning the amplification of a tetracycline r-det in the *Streptococcus faecalis* plasmid pAMα1, Yagi and Clewell (26, 27) stated that the recombination between direct repeat sequences located on the two daughter strands of a partially replicated plasmid was an attractive model for them. This assumption was also favored by Meyer and Iida (16) in regard to the duplication of a chloramphenicol resistance transposon in the phage P1Cm in *E. coli*. Small circular DNA molecules corresponding in size with the amplifiable segment of plasmid pAMα1 have also been detected in *S. faecalis* (26) but have not been further characterized, owing to their presence in very small amounts. Analogously, such circles have been mentioned to occur in connection with the amplification of the tetracycline resistance gene of the transposon Tn*1721* in *E. coli* (15).

In *Proteus mirabilis*, the R factor NR1 amplifies a complex of r-dets in the form of mono- and multimeric circles and of tandem multimers linked to the resistance transfer factor (10, 20). The r-det is flanked by two directly repeated copies of the insertion element IS*1* (11, 19). Evidence suggests that recombination between these IS*1* sequences produces a circular molecule containing the r-det and one IS*1* copy (19, 20). Cointegration by intermolecular reciprocal recombination with an IS*1* sequence residing on the recipient replicon generates a tandem duplication of the r-det (4, 20). Besides, transmission of the r-det may also take place by means of IS*1*-directed transposition (1, 18). The production of r-det circles from R1 and R100 in *E. coli* and their cointegration with another replicon (4, 5,

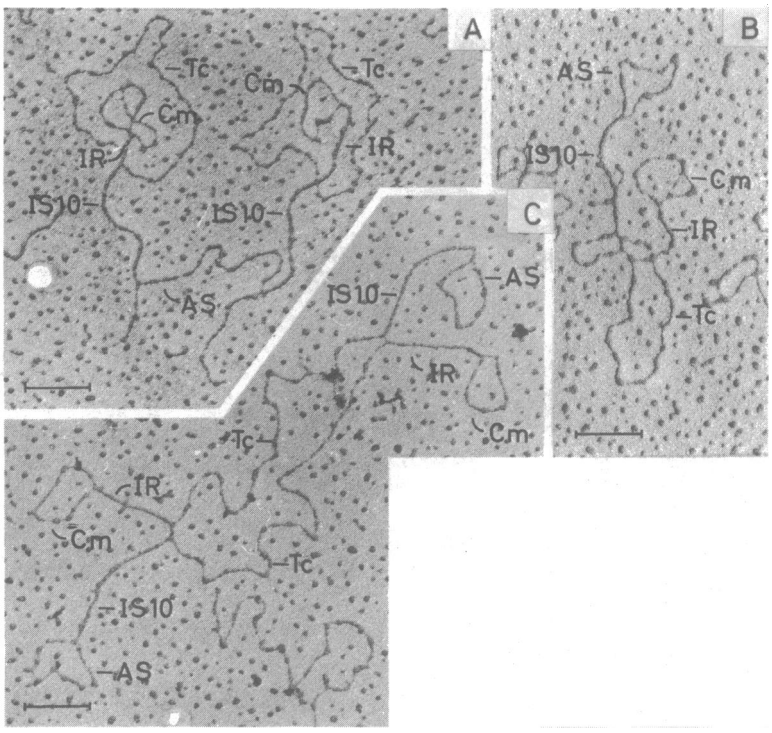

FIG. 3. Electron micrographs selected from a homoduplex preparation of p85098. This plasmid produces multiple duplications of the combined tetracycline (Tc)-chloramphenicol (Cm) resistance transposon. (A) A section of the plasmid showing two transposons in succession and the AS region between them. The chloramphenicol r-det is flanked by two inverted repeats (IR) which are inserted closely adjacent to the inside end of IS*10*-left. (B) A circle containing a monomer of the amplifiable segment. (C) A dimeric circle. Scale: 0.2 μm.

22), as well as the deletion of the r-det from R100 in *Salmonella typhimurium* (25), are closely related phenomena.

In contrast to the other gene amplification systems, the AS region does not represent the usual direct repeat in *H. influenzae* plasmids (23). Nevertheless, restricted sequence homology between the AS region and the right border of the amplifiable segments is necessary to comply with the basic supposition of the amplification models (16, 19, 26, 27). Edlund and Normark (9) proved that the amplification of the *ampC* beta-lactamase gene in *E. coli* K-12 depends on recombinational events between 12 to 13-base pair homologous repeats. We were not able to detect deleted plasmids lacking the amplifiable segment after serial subculturing of strain FR16017 in the absence of tetracycline. Thus, the initial event in amplification, which includes the formation of a deleted plasmid, may be extremely rare. Rapid amplification depends on the presence in a bacterial population of plasmids which comprise at least two copies of the r-dets and the AS region in tandem arrangement, providing extensive sequence homology for frequent recombinations.

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