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# Genome organization of the anaerobic pathogen *Clostridium perfringens*

(genome evolution/pulsed-field gel electrophoresis/indirect end-labeling/gangrene/virulence)

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ABSTRACT **A physical map of the genome of *Clostridium perfringens*, an important human pathogen, has been established by pulsed-field gel electrophoresis. Recognition sites for six rare-cutting endonucleases were situated on a single circular chromosome of approximately 3.6 million base pairs thus defining 50 arbitrary genetic intervals of between 10 and 250 kilobase pairs. This considerably facilitated the chromosomal localization of some 24 genes and loci for which probes were available and allowed the construction of the genome map of a clostridial species.**

The genus *Clostridium* consists of a diverse collection of obligately anaerobic Gram-positive bacteria that all sporulate but have a dG+dC content varying from 24 to 55% (1). Several of its members are of broad biotechnological interest whereas others produce some of the deadliest toxins known. *Clostridium perfringens*, a common pathogen of man and domestic animals, is responsible for a number of clinical conditions ranging from relatively mild food poisoning to the potentially life-threatening gas gangrene. Clinical isolates have been classified into five serotypes, A–E, differing in their tropisms and toxigenicity (2).

Genetic studies of this medically important bacterium have been hampered by the lack of classical tools, such as transducing phages, and by difficulty in introducing recombinant DNA molecules. To circumvent these problems and, in particular, to complement studies on bacterial virulence factors and their role in pathogenesis, we decided to construct a physical map of the *C. perfringens* genome using a variety of techniques based around pulsed-field gel electrophoresis (PFGE) (3–6).

In the second phase of the project, a gene map comprising more than 24 genes and chromosomal loci was superimposed on the physical map and it is now possible to locate rapidly any cloned *C. perfringens* gene to a genetic interval of 50–250 kilobase pairs (kb).

## MATERIALS AND METHODS

**DNA Preparation.** *C. perfringens* strain CPN50 was grown to midexponential phase in TYG broth (7, 8). Washed cells ($10^9$ cells per ml) were mixed with an equal volume of 1% molten low-melting-point agarose (BRL) and the mixture was allowed to solidify in 80-μl rectangular molds. Genomic DNA was prepared in the agarose plugs as described (9). Briefly, plugs were incubated for 16 hr at 37°C with gentle shaking in a solution containing 6 mM Tris·HCl (pH 7.6), 1 M NaCl, 100 mM EDTA, 0.5% sarkosyl, and lysozyme (1 mg/ml) and then plugs were deproteinized by incubating in ESP [0.5 M EDTA/0.5% sarkosyl/proteinase K (2 mg/ml), pH 8.5] for 48 hr at 50°C. Prior to restriction endonuclease digestion, the plugs, which had been stored in 0.5 M EDTA (pH 8.5), were washed in TE buffer (10 mM Tris·HCl/1 mM EDTA, pH 8.0).

**Restriction Digests.** *Apa* I, *Avi* II, *Ksp* I, *Mlu* I, *Nar* I, *Nru* I, *Sma* I, *Sph* I, and *Stu* I were provided by Boehringer Mannheim, whereas *Fsp* I, *Nae* I, *Not* I, *Sac* II, and *Xma* I were purchased from New England Biolabs. DNA samples in agarose (40 μl) were digested with 20 units of enzyme for 4 hr at 37°C (25°C for *Sma* I) in 200 μl of the buffer recommended by the manufacturer. In our hands, *Avi* II- and *Ksp* I digested DNA in agarose better than their isoschizomers *Fsp* I and *Sac* II.

**Electrophoresis Conditions.** Most of the PFGE was performed by field inversion (5) using a COPROG model 3000 microprocessor (Production Fabrication Controle, Paris) to control the pulse duration and ramp. In some experiments samples were subjected to contour-clamped homogeneous electric field (CHEF) electrophoresis (6) or orthogonal field gel electrophoresis (OFAGE) (4) using an apparatus purchased from Pharmacia L.K.B. Biotechnology.

Chilled agarose plugs were inserted into the wells of a 1% agarose gel (25 × 20 cm) containing 0.66× TBE buffer (1× TBE is 89 mM Tris/89 mM boric acid/2 mM EDTA, pH 8.3). Gels were kept at constant temperature (8°C) and the running buffer (0.66× TBE) was recirculated during electrophoresis, which was performed for 36 hr at 240 V using a ramp of 0.33 → 60 s in the forward direction and 0.11 → 20 s in the reverse. After electrophoresis, gels were stained with ethidium bromide and photographed using a Polaroid-Land model MP-4 camera. The sizes of DNA fragments were estimated by comparison with concatamerized λ genomes or yeast chromosomes (10).

**Southern Blot Hybridizations.** After electrophoresis gels were UV-irradiated for 2 min to fragment DNA, denatured in 0.5 M NaOH/1.5 M NaCl for 45 min, and then neutralized in 0.5 M Tris·HCl/1.5 M NaCl, pH 7.5 for 45 min. DNA was transferred to Hybond C-extra filters (Amersham) as described (11) and fixed by baking for 2 hr at 80°C. Hybridization with $^{32}$P-labeled probes, produced by random priming (12), and washing were as described (13). When heterologous probes were used, filters were hybridized and washed at 22°C.

**Other Techniques.** Standard cloning procedures (14) were employed in conjunction with dephosphorylated plasmid vectors pUC18 and pMTL22p (15, 16). Plaque hybridization was performed as described (17).

## RESULTS

**Estimation of the Genome Size.** The principal aims of this project were to determine the size of the genome of a type-A *C. perfringens* strain associated with human disease by PFGE and to use the information obtained to establish a physical map to facilitate the localization of cloned genes. We

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Abbreviations: PFGE, pulsed-field gel electrophoresis; Mb, megabase pair(s).

reasoned that since the genomic DNA was composed of 75% dA+dT, restriction enzymes with dG+dC-rich recognition sites of six nucleotides, or more, should cleave on average every 60–100 kb and thereby generate a small number of large restriction fragments. Furthermore, we calculated that if the restriction-site distribution was near random and the genome size was typical of a eubacterium [3–5 megabase pairs (Mb)], then the use of five or six appropriate enzymes would generate a map with arbitrary genetic intervals of 50–200 kb, defined by the distance between sites. This would be ideal for the rapid localization of genetic loci for which hybridization probes were available.

Consequently, DNA was prepared from strain CPN50, originally isolated from a patient with septicemia (7, 8), and screened with a battery of restriction enzymes. Analysis of the cleavage products by PFGE showed that *Apa* I (GGGCCC), *Fsp* I (TGCGCA), *Mlu* I (ACGCGT), *Nru* I (TCGCGA), *Sac* II (CCGCGG), and *Sma* I (CCCGGG) reproducibly generated a small number of large DNA fragments. The patterns obtained with *Nru* I and *Sac* II were virtually identical, indicating close linkage and similar distribution of the recognition sites. Of the other enzymes tested, it was found that *Nae* I (GCCGGC) and *Nar* I (GGCGCC) always gave incomplete digests, *Sph* I (GCATGC) and *Stu* I (AGGCCT) generated too many fragments, and *Not* I (GCGGCCGC) had no sites in the genome.

Typical results obtained by cleavage with the six chosen enzymes are presented in Fig. 1. The fragments generated by single digests ranged from 5 to 16 in number and from 0.03 to 2.2 Mb in size. By comparison with appropriate molecular weight markers, fragment sizes could be estimated accurately after PFGE and these are summarized in Table 1. For the larger fragments (>0.3 Mb), sizes were also determined after orthogonal field gel electrophoresis or contour-clamped homogeneous electric field electrophoresis (4, 6). However, as reported by others (18), the ethidium bromide fluorescence of fragments larger than 0.6 Mb was often stoichiometrically disproportional (see Fig. 1, lanes 1 and 8). The genome size deduced from the various single digests was 3.58 ± 0.2 Mb (mean ± SEM) (Table 1).

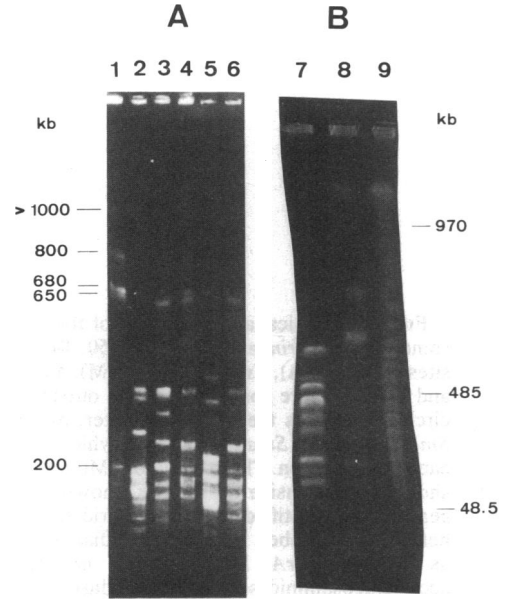


FIG. 1. PFGE separation of restriction fragments of *C. perfringens* genomic DNA. (*A*) Samples separated on a field-inversion gel were digested with *Mlu* I and the following endonucleases. Lanes: 1, alone; 2, *Sma* I; 3, *Apa* I; 4, *Sac* II; 5, *Fsp* I; 6, *Nru* I. (*B*) Samples digested with *Apa* I (lane 7) or *Mlu* I (lane 8) were resolved by orthogonal field gel electrophoresis. The deduced sizes of the *Mlu* I fragments and the positions of the λ size markers (lane 9) are indicated.

Table 1. Restriction fragments of *C. perfringens* genomic DNA

| | Fragment size, kb | | | | | |
|---|---|---|---|---|---|---|
| | *Apa* I | *Fsp* I | *Mlu* I | *Nru* I | *Sac* II | *Sma* I |
| | 650 | 450 | 1200 | 2280 | 1640 | 1460 |
| | 530 | 450 | 830 | 405 | 405 | 440 |
| | 440 | 340 | 680 | 260 | 320 | 410 |
| | 420 | 230 | 650 | 260 | 260 | 405 |
| | 410 | 230 | 200 | 160 | 260 | 170 |
| | 360 | 225 | | 100 | 230 | 170 |
| | 320 | 210 | | 70 | 160 | 170 |
| | 200 | 210 | | 45 | 100 | 120 |
| | 140 | 190 | | | 70 | 120 |
| | 100 | 180 | | | 45 | 110 |
| | 40 | 170 | | | | 75 |
| | 35 | 160 | | | | 45 |
| | | 140 | | | | |
| | | 110 | | | | |
| | | 85 | | | | |
| | | 50 | | | | |
| Total | 3645 | 3430 | 3560 | 3580 | 3490 | 3695 |

Mean fragment size from five independent measurements was rounded off to the nearest 5 kb; fragments <35 kb were not detected.

To determine the relative order of the sites, double digests were performed and these yielded valuable information about physical relationships. However, it was difficult to attribute unambiguously the smaller restriction fragments (<100 kb) to a precise genomic location so a complementary mapping approach was employed.

**Mapping by Indirect End-Labeling.** Olson and coworkers (19) have described a powerful technique for mapping large DNA fragments that involves partial digestion with restriction endonucleases, PFGE resolution of the products, and hybridization with a probe complementary to one end of the initial fragment. This allows the order of the restriction sites to be established relative to a fixed point. We decided, therefore, to generate a series of end probes by cloning fragments of CPN50 DNA bearing a rare restriction site at one end into plasmid vectors. Three such libraries carrying *Mlu* I–*Eco*RI, *Mlu* I–*Hin*dIII or *Sma* I (*Xma* I)–*Hin*dIII fragments were constructed. As will be seen below, the latter library proved to be of limited value. The *Mlu* I end probes were then hybridized to PFGE-resolved DNA that had been digested to completion with *Mlu* I and partially digested with a second enzyme. A typical result obtained with *Mlu* I and *Apa* I is presented in Fig. 2*A*. This approach confirmed and extended the results of the double-digestion analysis described above and generated an internally consistent map. Independent confirmation of the relationships of the various *Mlu* I fragments was obtained in two ways, by classical double digestions or by isolating the linking clones (20) that carried the *Mlu* I site from a λ library and using them as hybridization probes. An example of a linking analysis that demonstrates the contiguity of *Mlu* I fragments of 650 and 200 kb is presented in Fig. 2*B*. The physical map of the *C. perfringens* genome, deduced from these combined approaches, revealed a single circular chromosome (Fig. 3).

**Mapping rRNA and tRNA Genes.** When various *Sma* I–*Hin*dIII fragments were used as probes to detect homologous sequences in DNA that had been partially or completely digested and resolved by PFGE, extremely complex hybridization patterns were obtained that were suggestive of a dispersed gene family. This was shown to comprise the nine rRNA operons, *rrnA–I*, by employing probes prepared from rRNA. DNA sequence analysis revealed virtually all of the *Sma* I–*Hin*dIII fragments to be of *rrn* origin and localized the *Sma* I sites in the genes encoding the 16S and 23S rRNAs. In

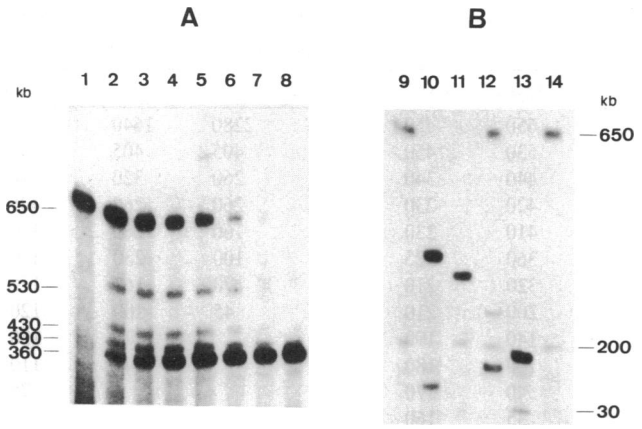


FIG. 2. (*A*) Indirect end-labeling analysis of *C. perfringens* genomic DNA. Samples (1 μg) were digested to completion with *Mlu* I then partially digested with serially diluted *Apa* I as follows: Lanes: 1, no enzyme; 2–8, 0.02, 0.05, 0.1, 0.2, 0.4, 0.6, and 4 units, respectively. After PFGE analysis, DNA was blotted and hybridized with a $^{32}$P-labeled *Mlu* I–*Eco*RI end probe. Fragment sizes are indicated; the map deduced covers the *Mlu* I fragment from position 2.3 Mb to position 3.0 Mb in the clockwise direction (Fig. 3). (*B*) Linking analysis of 0.20- and 0.65-Mb *Mlu* I fragments. The gel shown in Fig. 1*A* was hybridized with a 2.7-kb *Eco*RI linking probe, which carries the *Mlu* I site 300 base pairs from one end. This explains the relatively weak hybridization on the 0.2-Mb fragment. (Additional bands in lane 12 are due to incomplete *Sac* II digestion.)

some cases the *Hin*dIII sites were found to be associated with unique sequences and one of these was shown to correspond to the *gyrA* gene, coding for the large subunit of DNA gyrase, by homology with its counterpart in *Bacillus subtilis* (21). Additional mapping studies established the presence of a cluster of rare sites, *Sac* II–*Sac* II–*Sma* I–*Sma* I–*Nru* I–*Sma* I–*Nru* I, in each *rrn* operon (R in Fig. 3). By using probes specific for the 5′ or 3′ ends of the 16S and 23S rRNA genes, we could demonstrate that seven of the *rrn* operons were transcribed clockwise and the remaining two were transcribed in a counter-clockwise direction. When hybridization was performed with probes prepared from total tRNA, the pattern was indistinguishable from that obtained with rRNA probes, indicating tight linkage between the corresponding genes.

**Mapping Genes for Virulence Factors.** It is generally believed that the pathogenicity of *C. perfringens* is due to the secretion of toxins or virulence factors that attack the host tissue. The genes for several putative virulence factors that have been cloned in this laboratory and elsewhere (22, 23) were used as hybridization probes to test the hypothesis that a particular area of the genome might be associated with bacterial virulence. In this way the chromosomal locations of the *plc* and *pfo* genes encoding the α and θ toxins and the *nanH* and *nagH* genes encoding a sialidase and β-*N*-acetylglucosaminidase, respectively, were established. With the exception of *nanH*, three of the four virulence factor genes were localized to a 200-kb region of the chromosome. Interestingly, *nanH* is situated on the same *Fsp* I fragment as the lysogenic bacteriophage φ29.

**Mapping Housekeeping Genes.** Very few genes involved in the basic metabolism of clostridial species have been isolated. Consequently, to map some of the genes for housekeeping functions, a series of heterologous probes derived from cloned *B. subtilis* or *Escherichia coli* genes was employed. Although many of these did not cross-hybridize, probably due to differences in base composition, a number gave positive results. Of particular interest was the *gyrB* gene of *B. subtilis* (24) that detected homologous sequences on a 40-kb *Fsp* I–*Sma* I (Fig. 4) fragment known to carry the *gyrA* gene and this suggests that *gyrA* and *gyrB* are tightly linked as is the case in *B. subtilis* (21, 24). By using this heterologous approach, genes have been tentatively localized for the following cellular components: DNA gyrase (*gyrA* and *gyrB*), RNA polymerase core enzyme (*rpoA*, *rpoB*, and *rpoC*), $\sigma^{43}$ (*rpoD*), ATP synthase (*atpD*), and elongation factor Tu,

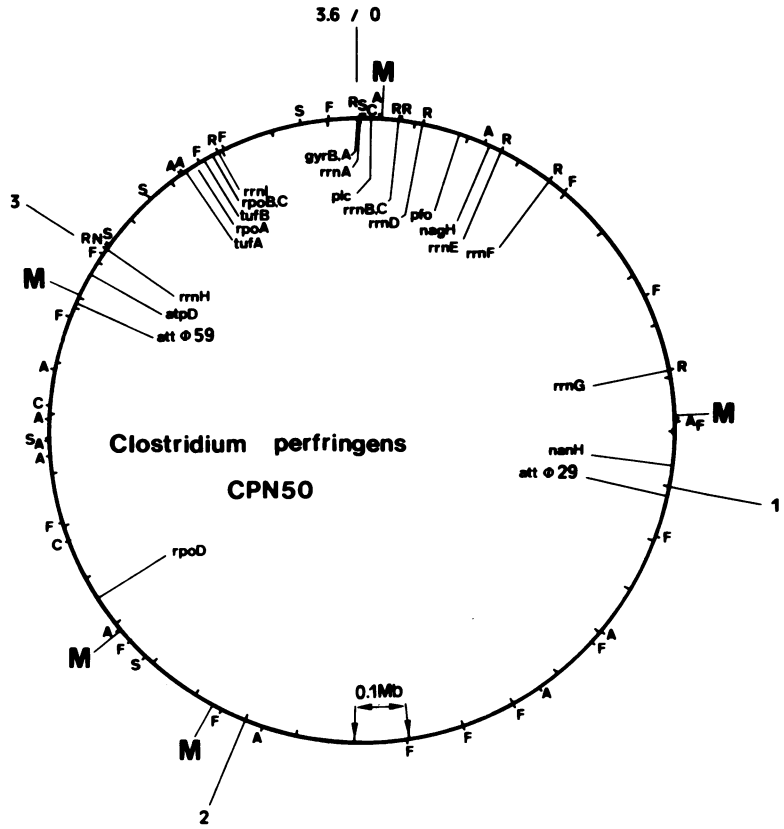


FIG. 3. Physical and gene map of the chromosome of *C. perfringens* strain CPN50. Restriction sites for *Apa* I (A), *Fsp* I (F), *Mlu* I (M), *Sac* II (C), and *Sma* I (S) are positioned on the outside of the circle; R denotes the rare site cluster, *Sac* II (2), *Sma* I (2), *Nru* I, *Sma* I, and *Nru* I, which occurs in each RNA operon. The scale in 0.1-Mb intervals is shown on the inside as well as known genes or genetic loci identified by cross hybridization with heterologous probes. Known clostridial genes were as follows: *gyrA*, DNA gyrase; *nagH*, β-*N*-acetylglucosaminidase; *nanH*, sialidase; *pfo*, perfringolysin or θ toxin; *plc*, phospholipase C or α toxin; *rrnA–I*, rRNA operons; *attφ29*, integration site for φ29; *attφ59*, integration site for φ59. Other genetic loci are as follows: *atpD*, β subunit of ATP synthase; *gyrB*, DNA gyrase; *rpoA*, α subunit of RNA polymerase; *rpoB*, β subunit of RNA polymerase; *rpoC*, β′ subunit of RNA polymerase; *rpoD*, $\sigma^{43}$ subunit of RNA polymerase; *tufA*, *B*, elongation factor Tu.

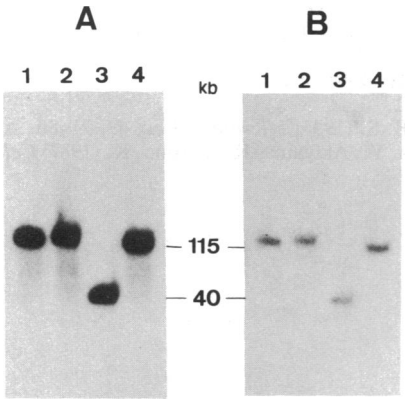


FIG. 4. Mapping DNA gyrase genes. (*A*) Hybridization with a *C. perfringens gyrA*-specific probe. Samples were digested with *Sma* I, *Sma* I plus *Apa* I, *Sma* I plus *Fsp* I, or *Sma* I plus *Nru* I, in lanes 1–4, respectively. (*B*) Same blot was hybridized with a probe derived from the *B. subtilis gyrB* gene (24).

which seems to be encoded by two genes (*tufA* and *tufB*; ref. 25).

## DISCUSSION

*C. perfringens* strain CPN50, associated with human disease, has been shown to possess a single circular chromosome of about 3.58 Mb by PFGE. More than 100 restriction sites and 24 genetic loci have been located on the genome that is slightly larger in size than that of the other Gram-positive bacterium for which physical mapping data have been published, *Staphylococcus aureus* at 2.86 Mb (18). *B. subtilis*, a distant relative and fellow member of the Bacillaceae, has a genome estimated at 5 Mb by classical genetic techniques (26), although, surprisingly, no PFGE data are available for this most intensively studied and thoroughly mapped Gram-positive microorganism.

A striking similarity between *C. perfringens* and *B. subtilis* can be seen in the number, 9 and 10, respectively, and organization of the rRNA operons, *rrn*, as in both bacteria these are clustered in a region representing about one-third of the genome (Fig. 3; refs. 26 and 27). More importantly, in *B. subtilis* the origin of replication is linked to the DNA gyrase genes, *gyrA* and *gyrB*, which precede the *rrnO* operon (21, 28). In this study, we have demonstrated linkage between *gyrA*, *gyrB*, and *rrnA* and, by analogy, it is tempting to speculate that the *C. perfringens* origin of replication is adjacent. If this is the case, there are 7 *rrn* operons located on the clockwise side of the origin, compared to 9 in *B. subtilis* (27) and 2 in an anticlockwise position. Further similarities can be seen in the compact organization of the tRNA and rRNA genes near the putative origin and this may be significant with respect to the sporulation and germination processes that both undergo (29).

One of our goals was to determine whether genes likely to be involved in bacterial virulence were clustered in a precise region of the chromosome. As shown in Fig. 3, the *plc* and *pfo* genes encoding the major cytotoxic determinants secreted by *C. perfringens* are located near the putative origin of replication. Furthermore, an operon coding for a third possible virulence factor, the β-*N*-acetylglucosaminidase is also situated nearby. Although this does not identify a "virulence domain," it does suggest that this region may merit further investigation.

It is highly significant that the genes for the α and θ toxins, which are produced by all serotypes of *C. perfringens* (2), are located near the presumed origin of replication, as this area should be among the most conserved regions of the genome. PFGE studies of genomic DNA from the five major classes of *C. perfringens* strains suggest that the bulk of the chromosome is the same in all isolates but that the 1.2-Mb *Mlu* I fragment is prone to genetic variation (unpublished results). This could in part be explained by bacteriophage-mediated rearrangements and it is noteworthy that the attachment site for the lysogenic phage φ29 (8) is on this fragment in CPN50. Strikingly, the only other genetic marker mapped to this region is *nanH* encoding sialidase, a potential virulence factor (23). These findings suggest that this particular area of the genome is susceptible to genetic variability and this might, in part, account for the different host specificities displayed by various clinical isolates of *C. perfringens*.

Finally, the physical map constructed in this study, to our knowledge, is not only the first such map for a clostridial species but also the first detailed map of any Gram-positive bacterium. Good examples of the utility and value of genome mapping are provided by pioneering work with the Gram-negative organism *E. coli* (20, 30). It is clear that this approach will not only have important ramifications for bacterial genetics but also considerably advance studies on phylogeny and molecular evolution.

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