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Physical Map of the Chromosomal Region of *Corynebacterium diphtheriae* Containing Corynephage Attachment Sites *attB1* and *attB2*

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The chromosome of *Corynebacterium diphtheriae* C7 was recently shown to contain two equivalent attachment sites (*attB1* and *attB2*) for lysogenization by corynephages (R. Rappuoli, J. L. Michel, and J. R. Murphy, J. Bacteriol. **153**:1202–1210, 1983). Portions of bacterial chromosome containing each *attB* site, as well as a 3.5-kilobase (kb) *Eco*RI fragment containing both *attB1* and *attB2* sites, were cloned in the pUC8 plasmid vector. Restriction endonuclease mapping and Southern blot hybridization analysis of restriction endonuclease fragments showed that *attB1* and *attB2* are 2.25 kb apart on the chromosome. Furthermore, a 0.85-kb *Hin*cII-*Eco*RI restriction endonuclease fragment containing *attB1*, a 0.77-kb *Hin*cII-*Bam*HI fragment containing *attB2*, and a 1.2-kb *Eco*RI-*Bam*HI fragment containing *attP* share short homologous regions. No homology was detected between the sequences flanking the two *attB* sites. The isolation of a segregant which had lost the entire chromosomal segment contained between *attB1* and *attB2* suggests that this region is not essential for growth.

Genetic studies and Southern blot hybridization experiments (11, 17, 24) have demonstrated that corynephages β^{tox+}, ω^{tox+}, and γ^{tox-} integrate into the chromosome of the gram-positive *Corynebacterium diphtheriae* according to the model proposed by Campbell (5) for the bacteriophage λ and its gram-negative host, *Escherichia coli*. This process involves homologous recombination between the phage attachment site (*attP*) and the bacterial attachment site (*attB*). In the λ-*E. coli* system, *attP* and *attB* share a common 15-base pair core (13).

In a recent study, it has been shown that *C. diphtheriae* strains C7, Belfanti, and PW8 contain two *attB* sites (*attB1* and *attB2*) in their chromosome and that corynephages can integrate into either *attB1* or *attB2* with equal frequency (23, 24). Lysogens containing two phage copies have also been isolated and shown to contain either two phages tandemly integrated in the same *attB* site (unstable conformation) or one phage integrated in each of the two *attB* sites (stable conformation). The production of diphtheria toxin by a given lysogen was shown to be proportional to the number of prophages integrated in its chromosome (23, 24). To our knowledge, this is the first case of two primary phage attachment sites being described for a bacterial chromosome.

Site-specific recombination in *C. diphtheriae* or other gram-positive organisms is a process largely unknown at the molecular level. To gain some information regarding the structure of the two *attB* sites, their relative position on the bacterial chromosome, and site-specific recombination, we have begun to study the bacterial and phage DNA regions involved in this process.

In the present paper we report the cloning in plasmid vectors of two *Bam*HI restriction fragments from the chromosome of the C7 strain, which contain *attB1* and *attB2* separately, and the cloning of a single *Eco*RI fragment containing both *attB* sites and some of the external flanking regions. By restriction endonuclease mapping and Southern blot hybridization analysis of the above cloned fragments, we show that a 0.85-kilobase (kb) *Hin*cII-*Eco*RI fragment containing *attB1*, a 0.77-kb *Hin*cII-*Bam*HI fragment containing *attB2*, and a 1.2-kb *Eco*RI-*Bam*HI fragment containing *attP* share a similar nucleotide sequence, whereas no homology was detected between the regions flanking *attB1* and *attB2*.

MATERIALS AND METHODS

Bacterial strains and plasmid vectors. The *C. diphtheriae* strains used in this study are listed in Table 1. The pUC8 plasmid (28) was used as a cloning vector, and *E. coli* JM101 (16) was used for transformation and propagation of the recombinant plasmids.

Media. Deferrated CY medium (20) was used for growth of corynebacterial strains and toxin production. The halo plate assay for diphtheria toxin (10, 22, 24) was performed on CY medium containing 1.2% Nobel agar which, after being autoclaved, was cooled to 55°C, and anti-diphtheria toxin serum (from the Production Control Department of Sclavo, Siena, Italy) was added to a final concentration of 3 U/ml.

E. coli strains were grown in LB medium (18). JM101 cells transformed by recombinant plasmids were plated on LB agar containing ampicillin (50 μg/ml).

Mitomycin C treatment of strain CN 2000. To cure the corynephages ω^{tox+} from *C. diphtheriae* CN2000, an overnight culture was diluted to an optical density at 590 nm of 0.15 (determined by a Perkin Elmer 35 spectrophotometer; 1-cm light path) in 10 ml of fresh CY medium and grown for 2 h at 35°C in a New Brunswick water bath rotary shaker at 200 rpm. Rabbit anti-ω^{tox+} phage serum (0.1 ml) and mitomycin C (0.2 μg/ml) were then added. The culture was then incubated with shaking for 3 more h, and after dilution in CY medium containing anti-ω^{tox+} phage serum, it was plated in CY agar containing 3 U of anti-diphtheria toxin serum per ml. Plates were then incubated for 48 h at 35°C and screened for the presence of colonies showing either a small or no precipitation halo.

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TABLE 1. *C. diphtheriae* strains

Strain	No. of integrated prophage(s)	Prophage integration site(s)	Reference(s)
C7[a]	0		1
Belfanti[b]	0		15
$C7_s(\gamma^{tox-})_{12}$	1	*attB1*	24
$C7_x(\gamma^{tox-})_{13}$	1	*attB2*	24
$C7_s(\omega^{tox+})_1$	1	*attB1*	24
$C7_s(\omega^{tox+})_5$	1	*attB2*	24
CN2000 substrain of PW8	2	*attB1*, *attB2*	21, 23
CN1 through CN11 derivatives of CN2000	1	*attB1-attB2*[c]	This study

[a] C7: *C. diphtheriae* $C7_s(-)^{tox-}$.
[b] Belfanti: *C. diphtheriae* Belfanti $1030(-)^{tox-}$.
[c] The phage is integrated in a hybrid *attB1-attB2* site. See the text and Fig. 4 for details.

Methods for corynebacterial strains. Diphtheria toxin production was quantitated by rocket immunoelectrophoresis (19). Corynephages were induced from CN1 through CN11 derivatives with mitomycin C as previously described (24) and plated on lawns of the *C. diphtheriae* Belfanti indicator strain. Phage DNA and chromosomal DNA from lysogenic and nonlysogenic strains of *C. diphtheriae* were purified as previously described (24, 26).

Cloning procedures. Chromosomal DNA restriction fragments from lysogenic and nonlysogenic strains of *C. diphtheriae*, suitable for cloning, were purified as follows: after digestion with a given restriction endonuclease, the chromosomal DNAs were electrophoresed on a 3-mm thick 1.3% agarose vertical slab gel with molecular weight markers; fragments of the desired size were then electroeluted into a dialysis bag and further purified on a column of DNB-cellulose (Serva, Heidelberg, Federal Republic of Germany) (14).

Two hundred nanograms of purified restriction endonuclease fragment DNA were then ligated to 500 ng of restriction endonuclease- and alkaline phosphatase-treated pUC8 vector in a volume of 30 μl with 0.1 U of T4 DNA ligase at 18°C for 18 h. After transformation of competent *E. coli* JM101 and plating on ampicillin plates (50 μg/ml) containing isopropyl-β-D-thiogalactoside and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (20 μl from a 1-mg/ml solution), white recombinant colonies were selected, and the desired clones were identified by colony-blot hybridization (14).

DNA procedures. Restriction endonuclease enzymes, T4 DNA ligase, calf alkaline phosphatase, and DNA polymerase were purchased from Bethesda Research Laboratories (Gaithersburg, Md.) or Boehringer (Mannheim, Federal Republic of Germany) and used according to the specifications of the manufacturers. Plasmid purification (2, 6, 8, 14), restriction mapping, Southern blot hybridization (27), and nick translation (25) were performed by standard procedures (14). Autoradiography was performed on Agfa-Gevaert Curix RP1 film at −70°C with Curix MR800 intensifying screens.

RESULTS

The corynebacteriophage attachment site POP′ is contained in the fragments *Bam*HI-3 of phage ω^{tox+} (23) and *Bam*HI-4 of phages β^{tox+} and γ^{tox-} (17). The above fragments also contain the diphtheria *tox* gene. After phage integration, the fragments are divided at the *att* site and can be recovered from the chromosome of lysogenic bacteria in two new *Bam*HI fragments: the larger one contains the P arm and the *tox* gene, and the smaller one contains the P′ arm (17, 23, 24; Fig. 1). To avoid cloning a functional *tox* gene, all the clones were derived from lysogens of the nontoxigenic bacteriophage γ^{tox-}, and only the lower-molecular-weight fragments containing the P′ site were cloned.

Cloning of *attB1*. A schematic representation of the cloning procedure is shown in Fig. 2. The fragment containing B_1OP' was identified with the fragment *Bam*HI-4 from phage β^{tox+}, which contains the phage attachment site (Fig. 2A), as a probe on Southern blots of the $C7(\gamma^{tox-})_{12}$ DNA (see Table 1) digested with *Bam*HI. The fragments comigrating with B_1OP' (about 2.7 kb) were then cloned into the *Bam*HI site of the pUC8, and the clones were screened again by the above probe. Out of 200 clones, 3 were found positive and designated pUC8-A200, pUC8-A201, and pUC8-A202 (Fig. 2B and Fig. 3).

The bacterial sequences in B_1OP' were then used to detect the size of the B_1OB' fragment in Southern blots of *Bam*HI-digested DNA from the nonlysogenic strain C7. Fragments comigrating with B_1OB_1' (about 3.5 kb) were then cloned into the *Bam*HI site of the pUC8 vector and screened by the same probe. Out of 350 clones, 7 were positive and designated pUC8-A100 through A106 (Fig. 2C and Fig. 3).

Cloning of *attB2*. The procedures were similar to those used to clone *attB1* (Fig. 2, left side), with the only difference that the bacterial DNA was obtained from the $C7(\gamma^{tox-})_{13}$ lysogen (see Table 1). Fragments comigrating with B_2OP' (about 2.2 kb) were cloned in the plasmid vector pUC8. The 120 clones obtained were screened by using the *Bam*HI-4 fragment from phage β^{tox+} as a probe: one positive clone was found and designated pUC8-A400 (Fig. 2B and Fig. 3). The bacterial sequences contained in B_2OP' were then used as a probe to identify and clone the fragment containing B_2OB_2' (1.7 kb). Of the 450 clones obtained, two positive clones were isolated and designated pUC8-A300 and pCU8-A301 (Fig. 2C and Fig. 3).

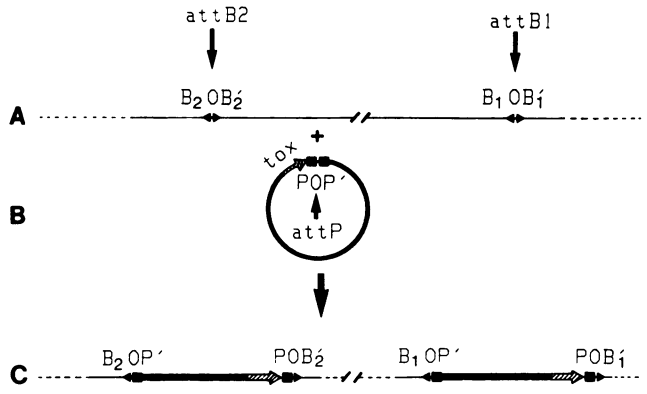

FIG. 1. Schematic representation of phage integration in *C. diphtheriae*, showing the nomenclature adopted for the loci involved in site-specific recombination. (A) Chromosomal DNA from a nonlysogenic bacterium containing *attB1* and *attB2*; (B) phage DNA containing *attP*; (C) nontandem double lysogen containing the hybrid *attB-attP* sites. Each site was divided into three parts: the left arm, the core, and the right arm. B_2, B_1, and P are the names for the left arm of *attB2*, *attB1*, and *attP*, respectively. B'_2, B'_1, and P′ are the names for the right arm of *attB2*, *attB1*, and *attP*, respectively. When the numbers 1 and 2 are omitted, we refer to both sites.

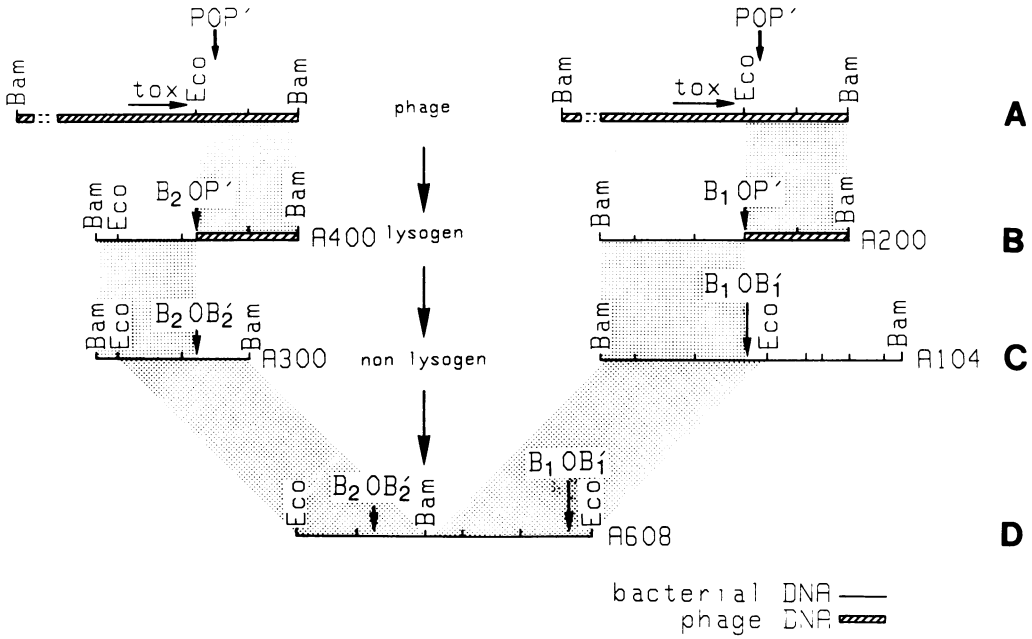

FIG. 2. Schematic representation of the strategy used for cloning a chromosomal restriction fragment containing *attB1* (right side), *attB2* (left side), and an *Eco*RI fragment containing both the bacterial attachment sites (D). The shaded areas indicate the homologies correlating A to B, B to C, and C to D which were used for the identification of cloned DNA restriction fragments. Unlabeled vertical bars indicate *Hin*cII restriction sites. (A) *Bam*HI-4 fragment from phage β'^{tox^+} which contains the *attP* site and the *tox* gene. (B) *Bam*HI fragments A400 (left) and A200 (right) containing the bacterium-phage hybrid sites B_2OP' and B_1OP', respectively. (C) *Bam*HI fragments A300 (left) and A104 (right) which contain *attB2* and *attB1*, respectively. (D) *Bam*HI fragment A608 containing both *attB1* and *attB2*.

Cloning an *Eco*RI fragment containing *attB1* and *attB2*. Clones containing B_2OB_2' and B_1OB_1' were used to probe Southern blots of DNA from strain C7 digested with several restriction enzymes. As expected, each of the probes hybridized to a band with a different molecular weight when *Bam*HI was used but to only one band after *Sal*I, *Hin*dIII, *Xba*I, *Cla*I, and *Eco*RI digestions.

Since *Eco*RI gave the smallest fragment (3.5 kb) containing both attachment sites, *Eco*RI fragments of about 3.5 kb from the nonlysogenic strain C7 were cloned in pUC8 and

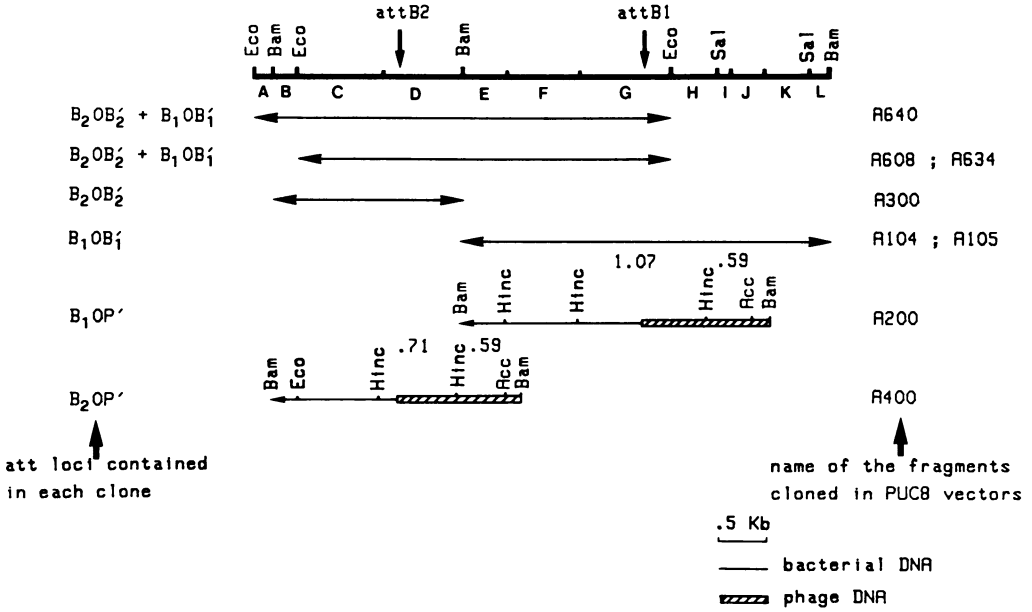

FIG. 3. Restriction map of the 5.3-kb chromosomal region containing *attB1* and *attB2*. Unlabeled vertical bars indicate *Hin*cII restriction sites (the two *Sal*I sites are also recognized by the enzyme *Hin*cII). Below the restriction map, the cloned fragments used to construct it are shown. Each fragment is identified by its name (on the right) and the sites contained (on the left). The size (in base pairs) of each restriction fragment is: A, 200; B, 250; C, 750; D, 770; E, 420; F, 690; G, 850; H, 450; I, 110; J, 350; K, 450; and L, 180.

screened with the ^{32}P-labeled B_2OB_2' fragment (A300). Out of 550 clones, 41 were positive and designated PUC8-A600 through A640 (Fig. 2D and Fig. 3).

Restriction endonuclease map of the *attB1-attB2* chromosomal region. Restriction endonucleases *Bam*HI, *Eco*RI, *Sal*I, *Hin*dIII, and *Cla*I were used to construct a restriction map of the region of the chromosomal DNA containing both the *attB1* and *attB2* attachment sites. A more detailed map was then made by using the enzyme *Hin*cII. The locations of the two *attB* sites were identified as follows: Southern blots of *Bam*HI-*Hin*cII-digested pUC8-A200 and pUC8-A400 (containing B_1OP' and B_2OP', respectively [Fig. 2B]) and blots of the 1.2-kb *Bam*HI-*Eco*RI fragment containing the POP' site (Fig. 2A), digested with *Hin*cII, were probed with the *Bam*HI-4 fragment from phage β^{tox^+} (Fig. 2A). The BOP' sites were identified in the positive *Bam*HI-*Hin*cII fragments which did not comigrate with any fragment carrying phage DNA and therefore contained a combination of phage and bacterial DNA (Fig. 2B). Since previous studies (17) indicated that the *attP* site is within 50 base pairs of the *Eco*RI site next to the end of the *tox* gene (Fig. 2A), it is possible to locate the crossover region between phage and bacterial DNA at about 1,200 base pairs from the *Bam*HI site in the P' arm of both POP' and BOP' fragments (Fig. 2A and B), and therefore at 1,500 base pairs from the *Bam*HI site in the B_1 arm and at 1,000 base pairs from the *Bam*HI site in the B_2 arm (Fig. 2B).

The *Hin*cII-*Hin*cII fragment containing the crossover point in B_1OP' (Fig. 2B, right side) was then used as a probe to detect the fragment containing B_1OB_1' in a Southern blot of *Bam*HI-*Hin*cII-digested pUC8-A104 which contains *attB1*. An identical procedure was used to identify the location of *attB2*.

The restriction map of the entire region and the clones used to construct it are shown in Fig. 3; from this map the distance between *attB1* and *attB2* was calculated to be 2,250 base pairs.

By Southern blot hybridization, a weak homology (estimated at about 10% of the hybridization one would obtain probing one fragment with itself) was detected only between the 0.85-kb *Hin*cII-*Eco*RI fragment G which contains *attB1* and the 0.77-kb *Hin*cII-*Bam*HI fragment D which contains *attB2*. Furthermore, both the above fragments showed a similar degree of homology with the 1.2-kb *Eco*RI-*Bam*HI fragment containing *attP*. No other homologies were detected, suggesting that the sites themselves have similar sequences, although the regions flanking the *att* sites are different.

The structure of the chromosomal region containing the *attB* sites was also investigated in corynebacterial strains *C. diphtheriae* Belfanti, PW8, and CN2000 (Table 1); no detectable differences from strain C7 were observed when Southern blots of *Bam*HI-digested chromosomal DNA of the above strains were probed with fragments A104 and A300.

Isolation of CN2000 derivatives which have lost the chromosomal region between *attB1* and *attB2*. To isolate a cured CN2000 strain of *C. diphtheriae*, a mitomycin C-treated culture was plated for single colonies on CY agar containing 3 U of antitoxin per ml. After treatment, most of the colonies showed a large precipitation halo, whereas 11 colonies out of 2,500 (CN1 through CN11) showed a barely detectable halo. These colonies were purified by restreaking on CY agar for single colonies and tested for growth and toxin production in liquid culture; none of the isolates showed significant differences in growth but produced exactly half the amount of toxin produced by the original CN2000 strain. When mitomycin C-induced, all 11 isolates were able to release a viable phage which plated on the *C. diphtheriae* Belfanti indicator strain.

Chromosomal DNA was then prepared from the partially cured strains, and the phage arrangement in the chromosome was determined by Southern blot hybridization by using *Bam*HI-digested chromosomal DNA probed with the *Bam*HI-3 fragment from phage ω^{tox^+}. As previously shown (23), this probe contains the ω^{tox^+} phage attachment site and gives four bands when hybridized to wild-type CN2000 DNA: bands 1 and 3 are from the phage integrated in *attB1*, and bands 2 and 4 are from the phage integrated in *attB2* (Fig. 4A, lanes 2 to 4).

The Southern blot analysis of the 11 partially cured isolates showed only bands 1 and 4 (Fig. 4A, lane 1), as if the phage was integrated in a hybrid *attB1-attB2* site. These results indicate that, after mitomycin C induction, homologous recombination between the phage integrated in *attB1* and the phage integrated in *attB2* had occurred. Furthermore, the curing of one phage by this recombination would result in the deletion of the chromosomal DNA between *attB1* and *attB2*, leaving a "hybrid" phage with B_2OP' and POB_1' junctions. A schematic representation is shown in Fig. 4B.

To test this hypothesis, we probed Southern blots of *Bam*HI-digested chromosomal DNA from the partially cured CN2000 derivatives with the fragments E and F (see Fig. 3) which are in the region between *attB1* and *attB2*. As expected, neither of the fragments was present in the partially cured CN2000 strains (Fig. 4A, lanes 5 to 8).

DISCUSSION

We have shown that the *C. diphtheriae* bacterial attachment sites *attB1* and *attB2* are very close on the chromosome. Even though their flanking regions are different, the 0.85-kb *Hin*cII-*Eco*RI fragment containing *attB1* and the 0.77-kb *Hin*cII-*Bam*HI fragment containing *attB2* show partial homology by Southern blot hybridization. Furthermore, both the above fragments show partial homology with the 1.2-kb *Eco*RI-*Bam*HI phage DNA fragment which contains the corynephage *attP* site. Under the hybridization conditions used (6× SSC [1× SSC = 0.15 M NaCl plus 0.015 M sodium citrate] and 65°C), at least 50 homologous base pairs are estimated to be necessary to detect a positive result. Since the signal obtained is rather weak, we can infer that the homology region will not be much larger than 50 base pairs.

Although nucleotide sequencing data will be needed to determine whether the hybridization detects the same homologous region in all the above three fragments, it is likely, by analogy with the coliphage λ system, that *attB1*, *attB2*, and *attP* share a common core sequence.

Although the origin of the two *attB* sites is unclear, it is very unlikely that the homologous sequences in *attB1* and *attB2* had an independent origin. Possible mechanisms to generate the two similar attachment sites could be duplication of a portion of the chromosome or transposition. However, the data presented do not support the duplication hypothesis, since the regions flanking *attB1* do not show any similarity with those flanking *attB2* either by Southern blot hybridization or restriction endonuclease mapping analysis. Alternatively it is possible that the two *attB* sites originated from an ancestral, unusual, phage integration-excision phe-

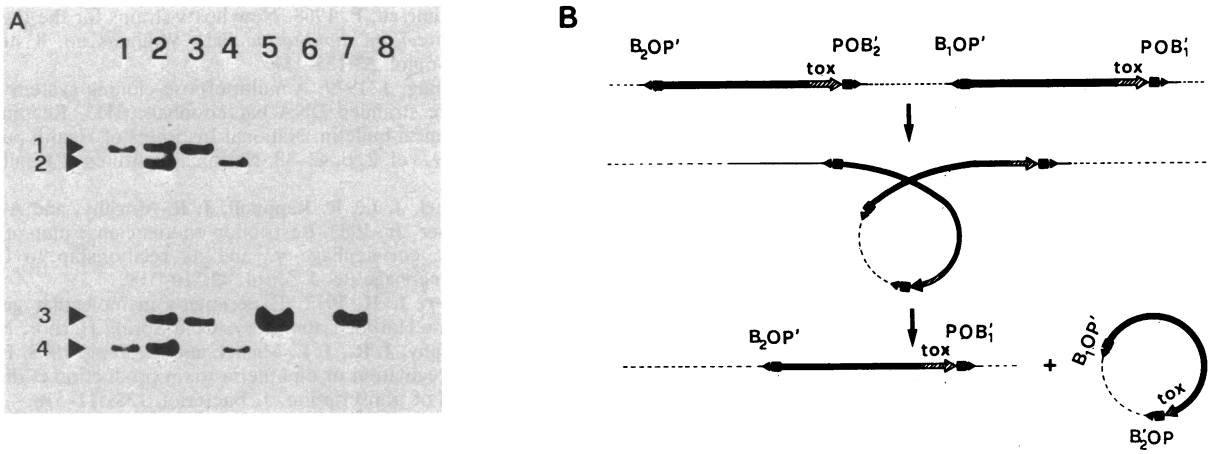

FIG.4. (A) Southern blot of the *attB* sites of partially cured isolates CN1 through CN11. *Bam*HI-digested chromosomal DNAs from (lane 1) CN4 (a typical representative of isolates CN1 through CN11), (lane 2) CN2000 (containing one ω^{tox+} phage in each *attB* site), (lane 3) C7(ω^{tox+})$_1$ (containing one ω^{tox+} phage in *attB1*), and (lane 4) C7(ω^{tox+})$_5$ (containing one ω^{tox+} phage in *attB2*) were run on 1.3% agarose gels and transferred to nitrocellulose filters which were then hybridized with the fragment *Bam*HI-3 from the ω^{tox+} phage. This probe gives four bands when hybridized to a nontandem double lysogen (lane 2); bands 1 and 3 are from the phage in *attB1* (lane 3), and bands 2 and 4 are from the phage in *attB2* (lane 4). CN4 (lane 1) contains only bands 1 (from a phage in *attB1*) and 4 (from a phage in *attB2*). *Bam*HI-digested DNAs from strains CN2000 and CN4 were then probed with the 0.41-kb *Bam*HI-*Hin*cII fragment E (lanes 5 and 6) and the 0.69-kb fragment F (lanes 7 and 8), which are in the chromosomal region between *attB1* and *attB2* (see Fig. 3). Both fragments were found in the CN2000 chromosome (lanes 5 and 7) but not on the CN4 chromosome (lanes 6 and 8). (B) Schematic representation of a process which could explain the above data. The two phages that are nontandem integrated in the chromosome of the CN2000 strain undergo homologous recombination and lose one phage which transduces the bacterial fragment contained between the two prophages. The DNA of the transducing phage has been arbitrarily drawn circularized.

nomenon which would leave in the bacterial chromosome some hybrid bacterium-phage junctions which could then evolve as two independent *attB* sites. Preliminary hybridization studies (data not shown) in fact show some homology between *attB* flanking regions and portions of phage DNA flanking the *attP* site.

β/γ, β/β, and ω/ω tandem double lysogens have been shown to be unstable and to give spontaneously monolysogenic segregants. In marked contrast, not a single monolysogenic segregant has been isolated from over 5,000 colonies of nontandem double lysogens tested (22, 24; unpublished data). The best evidence in support of the high stability of nontandem double lysogens is the fact that the highly toxigenic strain PW8 and its derivatives (which have been shown to contain two nontandem integrated ω^{tox+} phages) have been used for toxin production since 1896 and have never been reported to lose spontaneously part or all their toxigenicity. The instability of tandem double lysogens is usually explained by homologous recombination, which can easily occur in such configuration (7, 11, 12). The observation that in *C. diphtheriae* nontandem double lysogens are in fact separated by a 2.25-kb bacterial DNA fragment, which represents only 6% of the length of a phage genome, implies that in this case also homologous recombination could occur with similar frequencies. However, nontandem double lysogens have been shown to be remarkably stable, and this behavior cannot be explained merely by the increased distance between the phages. On the other hand, if the 2.25-kb fragment contained a gene essential for bacterial growth, after homologous recombination nontandem double lysogens would lose this fragment (by the mechanism shown in Fig. 4B) and die, so that one would not be able to detect them by normal procedures. We have, however, shown that the CN1 through CN11 derivatives of CN2000 which have lost this fragment grow as well as the original strain, and the reason for the differential stability must therefore be attributed to other unknown properties of the region containing the two *attB* sites.

Ishii-Kanei et al. (9) described the isolation of a cured PW8 derivative by a two-step procedure: after UV induction, they isolated a strain which produced about half of the original toxin; this was again UV induced, and a nontoxigenic strain was isolated. This strain could be converted to toxigenicity by the ω^{tox+} phage; however, they were unable to isolate strains producing more than half of the toxin produced by the original PW8 strain. If the strain of Ishii-Kanei et al. underwent the same recombination phenomenon as we report for the CN1 through CN11 strains (see Fig. 4B), it is obvious that it could be lysogenized only by one phage and would never produce more than half of the toxin produced by the original PW8 strain.

Buck and Groman (3, 4) have reported the presence of one 1.8-kb heterologous DNA fragment in 20 to 80% of the phage prepared by heat induction of the temperature-sensitive C7(γ-*tsr-1*)$^{tox-}$ lysogen. This fragment was never found when the same phage was prepared from lytic infections. However, after heat induction it is transduced at high frequency from the bacterial genome with a surprisingly high specificity. It is tempting to speculate that in the γ-*tsr-1*^{tox} prophage, one of the *attB-attP* junctions is not well recognized any more by the excising enzyme(s), which would then recognize and cut the homologous sequences found in the next (free) *attB* site. As a result, the prophage would transduce the bacterial fragment between the two *attB* sites. If this were the case, our data would predict that the transduced fragment is 2.25 kb long, which is not too far from the reported 1.8 kb; the difference between these two values is within the error of the methods used. However, further evidence of the identity of these two fragments is provided by restriction endonuclease analysis, which shows that both fragments contain a single *Bam*HI site and lack sites for *Eco*RI, *Hin*dIII, and *Sal*I.

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