Isolation of a Repeated DNA Sequence from *Bordetella pertussis*

By WILLIAM L. MCPHEAT* and TERESA MCNALLY†
National Institute for Biological Standards and Control, Holly Hill, Hampstead, London NW3 6RB, UK

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A repeated DNA sequence in the genome of *Bordetella pertussis* has been demonstrated. At least 20 copies of this sequence could be observed in either BamHI or EcoRI restriction enzyme digests of chromosomal DNA; fragments carrying the repeated DNA sequence ranged in size from about 1-5 to 20 kbp. The repeated DNA sequence was cloned from two separate regions of the *B. pertussis* genome, as shown by restriction enzyme site maps of the two clones and by hybridization studies. A small number of differences in the pattern of hybridization of the repeated DNA sequence to chromosomal DNA from several strains of *B. pertussis* was observed. No repeated DNA sequences were observed in one strain each of *B. parapertussis* and *B. bronchiseptica*, and there was no hybridization of *B. pertussis* DNA to *Escherichia coli* chromosomal DNA. The repeated DNA sequence was subcloned on a 2·54 kbp BamHI fragment from one of the two original clones. Restriction enzyme digests and hybridization studies showed that the repeated DNA sequence was about 1 kbp in size and had a single, internal *ClaI* site.

INTRODUCTION

Repeated DNA sequences have been shown to be present in many bacterial genomes, but not previously in *Bordetella* species. Such sequences include the 11 bp sequence involved in the uptake of DNA by *Haemophilus influenzae* (Smith et al., 1981), the 35 bp repetitive extragenic palindromic sequence described in *Escherichia coli* and *Salmonella typhimurium* (Higgins et al., 1982; Stern et al., 1984), and a variety of transposable elements (insertion sequences, transposons or transposing bacteriophages), ranging in size from 700 to >10000 bp (Kleckner, 1981; Iida et al., 1983). The number of copies of such repeated DNA sequences in bacterial genomes varies from 2→40 for insertion sequences (Iida et al., 1983) to an estimated 500–1000 copies of the repetitive extragenic palindromic sequence (Stern et al., 1984). The precise function of many of these repeated DNA sequences is unclear, but would appear to include mediating DNA rearrangements and the control of the level of gene expression (Kleckner, 1981; Iida et al., 1983; Stern et al., 1984).

In the course of our research into the molecular genetics of the virulence determinants of *Bordetella pertussis*, we isolated a DNA fragment which hybridized to multiple bands in digests of *B. pertussis* chromosomal DNA. This repeated DNA sequence bearing fragment was isolated from a region of the genome adjacent to that thought to encode the genes for the extracellular adenylate cyclase and haemolysin (Weiss et al., 1983; Wolff, 1985). This is the first demonstration of repeated DNA sequences in the genus *Bordetella* and this paper presents our data on the isolation from *B. pertussis* of two independent recombinant clones bearing a repeated DNA sequence.

† Present address: Department of Biochemistry, University of Edinburgh, Medical School, Hugh Robson Building, George Square, Edinburgh EH8 9XD, UK.
ethanol, pelleted, washed once with 80% (v/v) ethanol, vacuum dried and resuspended in Tris pH 7.5, 1 mM-EDTA, 100 mM-NaCl) in an Eppendorf tube. Lysozyme (Sigma; 10

precipitations. First, 31 precipitate, which formed immediately was transferred to a second tube to which sufficient TE (10 mM-Tris pH 7.5, 1 mM-EDTA) was added to give a final volume of about 400 μl. After resuspension, the DNA was collected by two

sodium dodecyl sulphate (Sigma; 125

Isolation of DNA. Plasmid DNA was isolated by the method of Ish-Horowicz & Burke (1981) and was further purified by centrifugation to equilibrium in a caesium chloride/ethidium bromide gradient (Maniatis et al., 1982). Chromosomal DNAs were prepared by our modification of the methods of Marmur (1961) and Meade et al. (1982). A 2 ml portion of the culture was pelleted, washed once with, and resuspended with, 500 μl TES (10 mM-Tris pH 7.5, 1 mM-EDTA, 100 mM-NaCl) in an Eppendorf tube. Lysozyme (Sigma; 10 μl of 25 mg ml⁻¹ in TES) was added and the tubes were incubated for 10 min at room temperature. Protease K (Sigma; 12.5 μl of 5 mg ml⁻¹) and sodium dodecyl sulphate (Sigma; 125 μl of 10%, w/v) were added and the tubes incubated first at 65 °C for 2–5 min, until the suspension had cleared, and then at 37 °C for 1 h. The DNA was collected by two precipitations. First, 31 μl 7.5 M-ammonium acetate and 375 μl 2-propanol were added and the large, viscous precipitate which formed immediately was transferred to a second tube to which sufficient TE (10 mM-Tris pH 7.5, 1 mM-EDTA) was added to give a final volume of about 400 μl. After resuspension, the DNA was reprecipitated by the addition of 40 μl 3 M-sodium acetate and 1 ml 96% (v/v) ethanol (precooled to −20 °C). The precipitate, which formed immediately on mixing, was transferred to another Eppendorf tube containing 1 ml 80% (v/v) ethanol and was collected by centrifugation at 4 °C for 20 min, vacuum dried and resuspended in 1 ml 20 mM-Tris pH 7.5, 1 mM-EDTA, 200 mM-NaCl overnight at 4 °C. The DNA was then purified by passage through a disposable filter holder FP 030/20 ‘White Rim’ and Elutip-d Minicolumn (both from Schleicher and Schüll) as recommended by the manufacturer. The eluted DNA was precipitated by the addition of 1 ml 96% (v/v) ethanol, pelleted, washed once with 80% (v/v) ethanol, vacuum dried and resuspended in 100 μl TE buffer overnight at 4 °C. This procedure was convenient for preparing chromosomal DNA from a large number of samples. The DNA recovered was essentially free of RNA, readily digested by restriction enzymes, and was estimated to be at a concentration in the range 25–100 μg ml⁻¹. On some occasions, the method was scaled up to prepare chromosomal DNA from 20 ml of B. pertussis BPH30.

Preparation of a gene library. A gene library of B. pertussis BPH30 was prepared in the cosmid pMMB33 essentially as described in method A of Frey et al. (1983). In brief, high molecular mass B. pertussis BPH30 DNA (70–130 kbp) was partially digested with Sau3AI and fragments in the size range 20–40 kbp were isolated. These were ligated to pM MB33 which had been digested with HpaI/BamHI and with Smal/BamHI, packaged in vitro (DNA Packaging Kit, Boehringer Mannheim), and transfected into E. coli HB101.

DNA manipulations and related methods. Restriction enzymes were purchased from Gibco BRL and used according to their instructions. Agarose gel electrophoresis was as described by Maniatis et al. (1982). Ligations were done as described by King & Blakesley (1986), except that polyethylene glycol 6000 was used.

Transformations of E. coli HB101 with plasmid DNA preparations were done as described by Maniatis et al. (1982) except that the cultures were shaken during the incubation period prior to plating on selection media. Plasmid DNAs and restriction fragments were radiolabelled with [α-³²P]dATP (Amersham) by the random primer method of Feinberg & Vogelstein (1983, 1984). Colony blots (Colony/Plaquescreen, New England Nuclear) and Southern blots (Genescreen, New England Nuclear) were prepared, hybridized and washed according to the manufacturer's instructions, except that colony blots were washed after cell lysis in the presence of 0-1% (w/v) sodium dodecyl

Table 1. Bacterial strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype/description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. pertussis 18-323</td>
<td>Phase I, mouse virulent</td>
<td>NIBSC</td>
</tr>
<tr>
<td>B. pertussis BP338</td>
<td>nal-'I derivative of phase I Tohama</td>
<td>Weiss et al.1983</td>
</tr>
<tr>
<td>B. pertussis BP348</td>
<td>hly-'I:: Tn5 derivative of BP338</td>
<td>Weiss et al.1983</td>
</tr>
<tr>
<td>B. pertussis BPH30</td>
<td>Phase I Wellcome 28</td>
<td>NIBSC</td>
</tr>
<tr>
<td>B. pertussis BPH37</td>
<td>Clinical isolate, 1985</td>
<td>Mark Thomas, St George's Hospital, London, UK</td>
</tr>
<tr>
<td>B. pertussis BPH39</td>
<td>Clinical isolate, 1985</td>
<td></td>
</tr>
<tr>
<td>B. pertussis BPH40</td>
<td>Clinical isolate, 1985</td>
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</tr>
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<td>B. parapertussis BPAH1</td>
<td>NCTC 8250</td>
<td>NIBSC</td>
</tr>
<tr>
<td>B. bronchiseptica BBRH1</td>
<td>NCTC 8344</td>
<td>NIBSC</td>
</tr>
<tr>
<td>E. coli HB101</td>
<td>F⁻ hsdS20 recA13 ara-14 proA2 lacY1</td>
<td>NIBSC</td>
</tr>
<tr>
<td></td>
<td>galK2 rpsL20 xyl-5 mtl-1 supE44 λ⁻</td>
<td></td>
</tr>
<tr>
<td>E. coli 12652</td>
<td>O164 H⁻, enteroinvasive</td>
<td>NIBSC</td>
</tr>
</tbody>
</table>
Repeated DNA sequence in Bordetella pertussis

Isolation of a DNA probe for the B. pertussis adenylate cyclase region

B. pertussis BP348 is a strain containing a single Tn5 insert which inactivates both the adenylate cyclase and the haemolytic activities (Weiss et al., 1983). Chromosomal DNA from this strain was digested with EcoRI (which has no sites in Tn5), ligated to EcoRI-cut pBR322 (Maniatis et al., 1982) and transformed into E. coli HB101. Three kanamycin-resistant transformants were obtained and plasmid DNA, pIL60, from one was prepared. A restriction enzyme site map of pIL60 is shown in Fig. 1(a). The EcoRI insert of B. pertussis BP348 DNA was 19.6 kbp, of which 13.8 kbp was B. pertussis DNA and 5.8 kbp was Tn5. The B. pertussis DNA flanking the Tn5 insert should be specific to the adenylate cyclase/haemolysin region.

Isolation of a cosmid clone of B. pertussis BPH30 DNA by hybridization to pIL60

A gene library of B. pertussis BPH30 DNA, consisting of 546 recombinant clones, was prepared in the cosmid pMMB33 (Frey et al., 1983) and transfected into E. coli HB101. A portion of this library, 90 recombinant clones, was screened for colonies hybridizing to pIL60. Two positive clones were identified and recombinant cosmid DNA was prepared from both. On hybridization of pIL60 to Southern blots of these two recombinant cosmids, only one (pC347) gave a positive result. From the restriction enzyme site maps of pIL60 and pC347 (Fig. 1a, b) and from the hybridization of pC347 against pIL60 (Fig. 2), it was clear that pIL60 and pC347 did not originate from overlapping genomic fragments.

![Restriction enzyme site maps of pIL60, pC347 and pWT5.](image)

Fig. 1. Restriction enzyme site maps of pIL60, pC347 and pWT5. Each of the maps is shown in linear configuration, the plasmids having been 'opened' at an arbitrary site. Restriction sites for Tn5, pBR322, pMMB33 and pUC8 were taken from Jorgensen et al. (1979), Sutcliffe (1979), Frey et al. (1983), and Vieira & Messing (1982), respectively. The six BamHI fragments of pIL60 are numbered 1–6 in order of decreasing length. The region of each plasmid to which the repeated DNA sequence has been mapped to date is indicated by the dashed line. V, non-reconstituted BamHI sites during construction of pC347. B, BamHI; C, ClaI; E, EcoRI; H, HindIII; Hp, HpaI; P, PstI; S, SmaI.
The B. pertussis DNA inserts of pIL60 and pC347 have a repeated DNA sequence

When used as probes against EcoRI-digested B. pertussis BPH30 chromosomal DNA, we had expected pC347 to hybridize to two bands (>1.4 and >6.8 kbp) and pIL60 to one band (13.8 kbp) on the basis of their restriction site maps (Fig. 1a, b). However, both pIL60 and pC347 hybridized to more than 20 fragments, ranging in size from about 1.5 to 20 kbp, on Southern blots of EcoRI-digested B. pertussis BPH30 chromosomal DNA (Fig. 2). These results suggested the presence of a repeated DNA sequence on both pIL60 and pC347.

It can also be seen from Fig. 2 that pC347 hybridized to only one BamHI fragment of pIL60. To confirm that this was the only fragment with a repeated DNA sequence on pIL60, each of the six BamHI fragments from a digest of pIL60 was excised from an agarose gel, radiolabelled, and used as a probe against Southern blots of EcoRI-digested B. pertussis BPH30 chromosomal DNA. Only the Bam4 (2.54 kbp) fragment of pIL60 (Fig. 1a) gave the multiple band pattern; the remaining five BamHI fragments hybridized to a single band at 13.8 kbp (data not shown).
Repeated DNA sequence in Bordetella pertussis

Presence of repeated DNA sequences in species of Bordetella

The adjacent Bam4 and Bam2 fragments from pIL60 (Fig. 1a) were used as probes against Southern blots of BamHI digests of chromosomal DNA from several strains of B. pertussis (laboratory strains and recent clinical isolates), one strain each of B. parapertussis and B. bronchiseptica, and two strains of E. coli (Fig. 3). The Bam2 fragment hybridized to a single BamHI band of 4.8 kbp in all three species of Bordetella, but gave no hybridization to the two E. coli strains. The Bam4 probe showed the presence of repeated DNA sequences only in the B. pertussis strains, and did not hybridize to the two E. coli strains. The Bam4 fragment hybridized to only a single band (1.45 kbp) in the B. parapertussis digest and hybridized to the same 1.45 kbp band, in addition to a band at 10.1 kbp, in the B. bronchiseptica strain. Repeated DNA sequences were found in all six B. pertussis strains tested. The patterns of hybridization of B. pertussis chromosomal DNA to the repeated DNA sequence probe were very similar, although a small number of differences were observed between some strains (Fig. 3). The clinical isolates used in this study had a maximum of three subcultures since primary isolation. The size range distribution of bands hybridizing to the repeated DNA sequence probe was very similar for either EcoRI- or BamHI-digested B. pertussis chromosomal DNA (about 1.5–20 kbp). When ClaI-digested DNA was tested, the size distribution shifted markedly (to about 1–10 kbp) and there was a very prominent band at about 1.1 kbp (Fig. 3).

Sub-cloning of the repeated DNA sequence from pIL60

To further characterize the repeated DNA sequence, the Bam4 fragment from pIL60 was sub-cloned into pUC8 (Vieira & Messing, 1982; purchased from Pharmacia). A restriction site map of this subclone, pWT5, is shown in Fig. 1(c). By digesting pWT5 with a variety of restriction enzymes and probing the Southern blots of those gels with pC347, we have shown that the repeated DNA sequence has no internal sites for AvaI, BamHI, BglII, HindIII, HpaI, PstI, PvuII, SacI or Smal. However, the single ClaI site of pWT5 lies within the repeated DNA sequence; this sequence is present on a single HinfI band of about 840 bp (data not shown).

DISCUSSION

We have shown that B. pertussis contains a repeated DNA sequence present in more than 20 copies in the genome. The nature of this repeated DNA sequence is unknown at present. Both the size estimated for the repeated DNA sequence, about 1 kbp, and its estimated number of copies in the genome would be compatible with its being an insertion sequence, which is one class of repeated DNA sequence (Iida et al., 1983). Further preliminary evidence in favour of the repeated DNA sequence being an insertion sequence comes from firstly, the different patterns of repeated DNA sequence probe hybridization seen with some B. pertussis strains, which implies DNA rearrangements (known to occur with insertion sequences), and secondly, the data on hybridization to B. parapertussis and B. bronchiseptica. The 2.54 kbp Bam4 fragment hybridized to a single 1.45 kbp band in these two species. The difference of about 1.0 kbp in size represented that of the prominent band seen with ClaI digests of B. pertussis DNA probed with the Bam4 fragment. This latter prominent band could conceivably have arisen as a result of there being tandemly repeated units of the repeated DNA sequence in the B. pertussis genome. We have shown that the repeated DNA sequence has a single internal ClaI site, so that digestion of the tandemly repeated units with ClaI would give a prominent band at a size equivalent to that of the repeated DNA sequence unit. The presence of tandemly duplicated repeated DNA sequences has been shown in Vibrio cholerae (Goldberg & Mekalanos, 1986).

The absence of repeated DNA sequences in the B. parapertussis and B. bronchiseptica strains tested, which hybridize to the DNA fragment from B. pertussis, is of interest in view of the close genetic relationship of the three Bordetella species (Kloos et al., 1981; Musser et al., 1986). Musser et al. (1986) further suggested that B. pertussis strain 18-323 was more closely related to B. parapertussis and B. bronchiseptica than to other B. pertussis strains, on the basis of a comparison of the electrophoretic mobility of 15 enzymes. Our results show a very distinct difference in the content of repeated DNA sequences in the genome of the three species of Bordetella. We have
Fig. 3. Autoradiographs showing hybridization of the Bam2 and Bam4 fragments of pIL60 to various bacteria. The top line drawing shows the relative positions of the six BamHI fragments of pIL60 (E, EcoRI, B, BamHI). Digests of chromosomal DNA from various bacteria were used to load two 0.5% (w/v) agarose gels. These gels were Southern blotted and hybridized to either the Bam4 (gel a) or the Bam2 (gel b) fragment. For size markers, separate BamHI, ClaI and BamHI/ClaI digests of pIL60 were mixed and loaded onto the gels; the sizes of hybridizing fragments were calculated from the restriction site map of pIL60 (Fig. 1a). Lanes: 1, markers; 2, B. pertussis BPH30, ClaI; 3, B. pertussis BPH30, EcoRI; 4, B. pertussis BPH30, BamHI; 5, B. pertussis 18-323, BamHI; 6, B. pertussis BP338, BamHI; 7, B. pertussis BPH37, BamHI; 8, B. pertussis BPH39, BamHI; 9, B. pertussis BPH40, BamHI; 10, B. parapertussis BPAH1, BamHI; 11, B. bronchiseptica BBRH1, BamHI; 12, E. coli HB101, BamHI; 13, E. coli 12652, BamHI; 14, markers. Tracks 10–13 in gel a were exposed for longer than tracks 1–9 to make the bands more visible.
also shown (Fig. 3) that _B. pertussis_ strain 18-323, in common with other _B. pertussis_ strains, contains repeated DNA sequences and that the pattern obtained is indistinguishable from that of _B. pertussis_ BPH30. We are at present preparing chromosomal DNA from several more strains of _B. pertussis, B. parapertussis_ and _B. bronchiseptica_, and from the newly described species _B. avium_ (Kersters _et al._, 1984) to analyse further the presence of repeated DNA sequences in the genus _Bordetella_ and to compare patterns of hybridization to the repeated DNA sequence probe.

A possible role for the repeated DNA sequence would be its involvement with the pathogenic determinants of _B. pertussis_. Repeated DNA sequences have been reported to be associated with gene(s) encoding virulence determinants such as cholera toxin (Mekalanos, 1983), the heat-labile and heat-stable enterotoxins of _E. coli_ (So _et al._, 1979; Yamamoto & Yokota, 1981), the pili of _Neisseria gonorrhoeae_ (Haas & Meyer, 1986), and the capsule of _Haemophilus influenzae_ type b (Hoisseth _et al._, 1986). Pertussis toxin, one of the major virulence determinants of _B. pertussis_ (Robinson _et al._, 1985), is not found in either _B. parapertussis_ or _B. bronchiseptica_ (Ross _et al._, 1969), nor is the repeated DNA sequence of _B. pertussis_. However, there has been no mention of repeated DNA sequences being associated with recombinant clones bearing the pertussis toxin genes (Locht _et al._, 1986; Locht & Keith, 1986). We are now in the process of examining phase IV strains of _B. pertussis_ with the repeated DNA sequence probe described here to determine if genome rearrangements involving the repeated DNA sequences are associated with the control of the expression of the pertussis toxin gene(s) by phase variation (Weiss & Falkow, 1984; Goldman _et al._, 1984).

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**REFERENCES**


