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Derivation of a Physical Map of the Chromosome of *Bordetella pertussis* Tohama I

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We have used pulsed-field gel electrophoresis to derive a restriction map of the chromosome of *Bordetella pertussis* for the enzymes *Xba*I, *Spe*I, *Pac*I, and *Pme*I, which cleave 25, 16, 2, and 1 times, respectively. The apparent size of the genome is 3,750 kb. The positions of genes for major virulence determinants in the *vir* regulon and of some housekeeping genes were determined. Apart from the previously known linkage of the *vir* and *fha* loci, no significant linkage of virulence genes was demonstrated.

The genus *Bordetella* currently contains four species: the human pathogens *Bordetella pertussis* and *Bordetella parapertussis* and the veterinary pathogens *Bordetella bronchiseptica* and *Bordetella avium*. In recent years, *B. pertussis*, the causative agent of the human disease whooping cough, has been the subject of intense genetic analysis. Transposon mutagenesis led to the identification of the genes for several of the major virulence determinants of this organism, i.e., *ptx* (pertussis toxin), *cya* (adenylate cyclase-hemolysin), and *fha* (filamentous hemagglutinin), as well as an important regulatory locus, the *vir* locus (38). The *vir* locus contains the genes *bvgA* and *bvgS*, encoding a two-component regulatory system which is required for the expression of the aforementioned virulence genes and which regulates their expression in response to environmental signals, a phenomenon known as phenotypic or antigenic modulation. The aforementioned genes have been cloned and subjected to DNA sequence analysis (1, 12, 22, 28, 30), as have the genes for a 69-kDa outer membrane protein termed pertactin (*prn*) and two genes for fimbrial subunits of different serotypes (*fim2* and *fim3*) which are also positively regulated by the *vir* locus (6, 20, 26, 40). One apparently silent gene for a fimbrial subunit, *fimX*, has also been characterized (29), and the gene for dermonecrotic or heat-labile toxin (*dnt*) has been identified (39).

In addition to those genes which are positively regulated by the *vir* locus, another class of genes called *vrg* for *vir*-repressed genes has been identified through the use of Tn*phoA* mutagenesis (15). Fusions of *phoA* to *vrg* genes are regulated in a fashion the inverse of that for genes such as *ptx*, *fha*, and *cya*, etc., being turned on under modulating conditions and off under nonmodulating conditions. Recently, *vrg-6* has been demonstrated to be required for full virulence of *B. pertussis* in a mouse infection model (4).

With the exception of *B. avium*, *Bordetella* spp. appear to be a closely related group. Of the *vir*-regulated genes described above, *B. bronchiseptica* and *B. parapertussis* have been demonstrated to possess *vir*, *fha*, *prn*, *cya*, and *dnt* (3, 7–9, 13, 16, 18, 31). Although immunologically related fimbriae are also expressed in these species, the serotypes are distinguishable from *B. pertussis* serotypes (27). Interestingly, although *B. bronchiseptica* and *B. parapertussis* do not express pertussis toxin, many strains contain silent *ptx* genes, apparently inactivated through the accumulation of single-base-pair mutations (2). *B. avium* has been shown to express a dermonecrotic toxin but apparently lacks *ptx*, *fha*, and *cya* (11).

In addition to *vir*-regulated genes, some *B. pertussis* genes whose products are involved in normal cellular processes and which are analogous to proteins described for other bacteria have been characterized. These include the *recA* and *aroA* homologs and *por*, the gene for the major porin protein of *B. pertussis* (10, 19, 23). Cosmid clones which complement *Escherichia coli* mutations in *arg* and *leu* have also been isolated (35).

The *vir* and *fha* loci are known from previous studies to be contiguous (35). More recently, genes involved in *B. pertussis* fimbrial biosynthesis, with counterparts in other piliation systems, have been found within the *fha* operon (21, 41). Apart from these examples, no linkage between *B. pertussis* loci has been demonstrated. Thus, the genomic organization of *B. pertussis* is largely unknown. The size of the *B. pertussis* genome is also unknown. To address these issues and to provide a basis for comparison of the genomic organization of *Bordetella* spp., we undertook to construct a map of the *B. pertussis* chromosome.

B. pertussis has proved to be amenable to several methods of genetic transfer and genetic manipulation and is also proficient at homologous recombination. Thus, the construction of a map based on genetic linkage and cotransfer frequencies is a possibility. However, the relative scarcity of genetic markers in *B. pertussis* would seriously hinder such an approach. Moreover, the development of pulsed-field gel electrophoresis apparatus capable of performing distortion-free separation of large DNA fragments, together with methods for the isolation and subsequent cleavage of intact chromosomal-size DNA, has made the construction of physical maps of bacterial genomes much easier and faster than the construction of genetic maps. We therefore chose to construct a physical map.

MATERIALS AND METHODS

Bacterial strains and media. Bacterial strains and plasmids used in this study are presented in Table 1. *E. coli* strains were grown on L agar or in L broth (25) supplemented with antibiotics as appropriate. Concentrations of antibiotics were 100 μg of ampicillin per ml and 15 μg of kanamycin sulfate per ml. *B. pertussis* strains were grown on Bordet-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant features	Source or reference
E. coli K-12		
DH5α	High-efficiency transformation	BRL[a]
SM10	Tra functions of IncP plasmids integrated into chromosome	32
B. pertussis		
Tohama I	Patient isolate	14
BP536	Str^r Nal^r derivative of Tohama I	36
Plasmids		
pRTP1	Gene replacement vector	34
pKanπ	Source of kanamycin resistance cassette	5
pSS1424	Derivative of pKanπ with *Xho*I site at one flanking *Pst*I site	This study
pSS1456	Derivative of pRTP1 and pSS1424 with *Spe*I and *Xba*I sites	This study
pSS1577	Derivative of pSS1456 with *Pac*I site at *Spe*I site	This study
pSS1680	Source of kanamycin resistance cassette with *Pac*I site and flanking *Xba*I sites	This study
pSS1682	Source of kanamycin resistance cassette with *Pac*I site and flanking *Spe*I sites	This study

[a] BRL, Bethesda Research Laboratories.

Gengou agar (Difco) containing 1% Proteose Peptone (Difco) and 15% defibrinated sheep blood. Concentrations of antibiotics were 10 μg of kanamycin sulfate per ml, 100 μg of streptomycin sulfate per ml, and 50 μg of nalidixic acid per ml. *E. coli* DH5α was obtained from Bethesda Research Laboratories.

Cloning and plasmid construction. Restriction enzymes and T4 DNA ligase were purchased from Bethesda Research Laboratories or New England Biolabs and used according to manufacturer's instructions. DNA cloning was performed by using established procedures. pSS1456 was constructed as follows. pSS1424 was derived from pKanπ by insertion of the oligonucleotide 5'-GCTCGAGCTGCA-3' at one of the *Pst*I sites flanking the kanamycin resistance gene module, resulting in the addition of an *Xho*I site. The kanamycin resistance gene was cleaved from this plasmid by partial digestion with *Bam*HI and complete digestion with *Eco*RI and cloned between the *Eco*RI and *Bam*HI sites of pRTP1. This resulted in the maintenance of one of the *Xba*I sites flanking this module. Subsequently, the oligonucleotide 5'-CTAGACTAGTCTAG-3' (United States Biochemicals), containing an *Spe*I site, was added at one of the *Sma*I sites in pRTP1 to create pSS1456. pSS1577 was created by adding the oligonucleotide 5'-CTAGTTAATTAATTTAAA-3' at the *Spe*I site of pSS1456, resulting in the addition of *Pac*I, *Ase*I, and *Dra*I sites. pSS1680 was derived from pKanπ by the addition of the oligonucleotide 5'-TCGATTAATTAA-3' at one of the *Sal*I sites flanking the kanamycin resistance gene module, resulting in the addition of a *Pac*I site and the destruction of this *Sal*I site. pSS1682 was created from pSS1680 by the addition of the oligonucleotide 5'-CTAG GACTAGTC-3' at both *Xba*I sites flanking the kanamycin resistance gene module, resulting in their destruction and the addition of flanking *Spe*I sites.

Isolation of chromosomal insertions and replacements. A gene bank was constructed by ligation of *Eco*RI-cleaved *B. pertussis* Tohama I chromosomal DNA with pSS1456, which had been cleaved with *Eco*RI and treated with calf intestinal phosphatase (Pharmacia). *E. coli* DH5α was used as the recipient for transformation, and selection was for ampicillin resistance. Colonies were pooled, and plasmid DNA was purified and used to transform SM10 to ampicillin resistance. Resulting colonies were mated en masse with BP536 as previously described (34) with selection for kanamycin and nalidixic acid resistance on Bordet-Gengou agar. Individual colonies were picked, restreaked under selection for kanamycin resistance, and used to isolate chromosomal DNA in agarose plugs as described below. Insertions of the vector pSS1577 were isolated in an identical fashion.

A partially complete linking library for *Xba*I was isolated as follows. A gene bank in pRTP1 was constructed as for pSS1456. Plasmid DNA from this library was cleaved with *Xba*I and ligated to the kanamycin resistance gene cassette from pKanπ generated by *Xba*I cleavage. After transformation of DH5α and selection for kanamycin resistance, individual colonies were picked and subjected to agarose gel electrophoresis of minipreparations of plasmid DNA cleaved with *Eco*RI and *Xba*I in order to screen out identical clones. A similar linking library was constructed for *Spe*I by using the *Spe*I-flanked kanamycin resistance cassette from pSS1682. The *Spe*I-flanked cassette from pSS1682 was subsequently cloned into the *Xba*I site of *Xba*I-linking clones to destroy the *Xba*I site and provide a selectable marker. In a similar fashion, the cassette from pSS1680 was inserted into *Spe*I-linking clones. The resulting plasmids were transformed into SM10 and transferred by conjugation to BP536 with simultaneous selection for kanamycin and streptomycin resistance. *B. pertussis* colonies resulting from these matings were examined by pulsed-field gel electrophoresis of *Xba*I and *Spe*I digests of chromosomal DNA for desired characteristics (fusion of presumably adjacent fragments).

Chromosomal DNA preparation and pulsed-field gel electrophoresis. *B. pertussis* cells were removed from Bordet-Gengou agar, embedded in low-melting-point agarose (SeaPlaque; FMC), and processed as described by Smith and Cantor (33) with slight variations. After suspension in 10 mM Tris HCl–20 mM NaCl, pH 7.2, the bacteria were mixed with an equal volume of 2.0% (wt/vol) low-melting-point agarose and pipetted into casting molds. We found that the higher concentration of agarose made the plugs more resilient during manipulations without inhibiting further processing steps. The solidified plugs were placed in 12-well tissue culture plates to facilitate the parallel processing of multiple samples. All further treatments were done with agitation by rocking, in a volume of 3.0 ml. The plugs were treated with lysozyme buffer (10 mM Tris HCl, 50 mM NaCl, 0.2% sodium deoxycholate, 0.5% sodium *N*-laurylsarcosine, 1 mg of lysozyme per ml [pH 7.2]) for 2 h at 37°C. After being

washed for 15 min at room temperature in 20 mM Tris HCl–50 mM EDTA, pH 7.2, plugs were treated with proteinase K buffer (100 mM EDTA, 1.0% sodium *N*-laurylsarcosine, 0.2% sodium deoxycholate, 1 mg of proteinase K per ml) at 42°C overnight. Proteinase K was removed by washing for 1 h at room temperature in 20 mM Tris HCl–50 mM EDTA, pH 7.2, and residual proteinase K was inactivated by washing an additional hour in the same buffer plus 1.0 mM phenylmethylsulfonyl fluoride. Plugs were then equilibrated in TE buffer (10 mM Tris HCl, 1.0 mM EDTA [pH 8.0]) with at least five changes of buffer prior to restriction enzyme digestion. For restriction enzyme digestion, slices of the plugs were placed in 200 μl of restriction buffer supplied by the manufacturer, enzyme was added, and the digestion was allowed to proceed overnight. After digestion, the plugs were equilibrated in TE buffer and chilled on ice prior to loading in a 1.0% agarose gel. Gels were cast and run in pulsed-field TBE buffer (75 mM Tris, 25 mM boric acid, 0.1 mM EDTA [Bethesda Research Laboratories]) at 0 to 4°C in a contour-clamped homogeneous electric field–DRII apparatus (Bio-Rad) at 200 V. Switch times varied depending on particular applications and are given in the figure legends.

Determination of the map location of known genes. Map locations were determined by examining *Xba*I, *Spe*I, and in some cases, *Pac*I digests of strains harboring chromosomal insertions of pSS1577 directed by homology to the cloned gene in question. Plasmids containing the genes for *cya*, *por*, *recA*, *aroA*, *ptx*, and *fimX* were obtained by screening the library of *Eco*RI fragments in pSS1577 by colony hybridization with ^{32}P-end-labelled oligonucleotide probes synthesized from published DNA sequence information. The identity of clones derived in this way was verified by restriction analysis, polymerase chain reaction analysis, or both. Clones of other genes in pSS1577 were obtained by using fragments derived from plasmids kindly provided by other workers, as follows: *arg*, *leu*, and *dnt* from Allison Weiss, *vrg-6* from David Beattie, *prn* from Ian Charles, and *fim2* from Frits Mooi. The gene for *fim3* was cloned as a polymerase chain reaction product synthesized with primers based on the DNA sequence (26). Clones of *vir* and *fha* were derived from pUW21-26 (35). In each case, three independent matings were performed with each donor plasmid. In all cases, all three insertions appeared identical.

RESULTS

Selection of restriction enzymes for mapping. In order to be useful in the generation of a physical map, restriction enzyme cleavage sites must be relatively rare and must cleave the chromosome into fragments which are well separated on gels. Logic would seem to indicate that those enzymes with 8-bp recognition sequences would be more useful than those which recognize a 6-bp sequence. However, at the time this project was initiated, the only commercially available enzymes which recognized an 8-bp sequence were *Not*I and *Sfi*I, which have all-GC recognition sequences. An examination of *B. pertussis* DNA sequences in the GenBank data base indicates that DNA from this organism has a G+C content of approximately 66%. One can calculate that *Not*I (GCGGCCGC) and *Sfi*I (GGCCN$_5$GGCC) should cleave a random sequence of this composition every 7,110 bp on average. Our observations of *B. pertussis* chromosomal DNA digested with either of these enzymes are in keeping with this prediction, with all *Not*I and *Sfi*I fragments less than 25 kb in size.

Restriction enzymes which have 6-bp recognition sequences consisting of all-AT base pairs would be predicted to cut a random 66% G+C sequence every 41,429 bp on average. This would yield approximately 113 fragments if the genome size were similar to that of *E. coli* (17). For the enzymes *Ssp*I (AATATT), *Ase*I (ATTAAT), and *Dra*I (TTTAAA), we observed approximately 80, 40, and 30 fragments, respectively.

The tetranucleotide CTAG is reported to be the rarest tetranucleotide in most bacterial DNA (24). The recognition sequences of the enzymes *Spe*I and *Xba*I both have this sequence as a core with AT base pairs flanking. Thus, they would be predicted to cut a random sequence of 66% G+C content every 10,995 bp on average to yield approximately 427 fragments for an *E. coli*-sized genome. However, *Spe*I (ACTAGT) was found to cut the chromosome into 16 fragments, and *Xba*I (TCTAGA) was found to cut it into 25 fragments, consistent with underrepresentation of the CTAG tetranucleotide. In both cases, the fragments were relatively well separated on pulsed-field gels. These enzymes were therefore chosen as markers for mapping. Pulsed-field gels of restriction digests are shown in Fig. 1a. In each case, some multiple bands occurred. *Xba*I gives two fragments the size of *Xba*I-K and four fragments of *Xba*I-L size. *Spe*I gives four fragments of approximately 330 kb (6.8 λ monomers). Of these, *Spe*I-F is distinguishable from the three *Spe*I-E fragments on gels which are underloaded. On gels run with longer switch times and for longer run times, *Spe*I-E3 can be resolved from *Spe*I-E1 and *Spe*I-E2 (data not shown). *Spe*I-J and *Spe*I-K are doublets. *Xba*I generates three additional fragments of 9.5, 8.5, and 6.5 kb which run off the bottom of gels run under conditions used for the digests shown in Fig. 1. *Spe*I generates one such fragment of approximately 5.5 kb (data not shown).

During the course of this project, the restriction enzymes *Pac*I (TTAATTAA) and *Pme*I (GTTTAAAC) became available. These were found to cut the *B. pertussis* chromosome twice and once, respectively.

Mapping strategies. The first mapping strategy used was to define overlaps of *Spe*I and *Xba*I fragments by examining random insertions of a vector containing these sites. Figure 2 shows the vectors pSS1456 and pSS1577 constructed for this purpose. These vectors differ by the presence of a *Pac*I site. They are capable of being transferred to *B. pertussis* by conjugation but are unable to replicate there. Maintenance of these vectors after conjugation is therefore dependent on recombination with the chromosome. Cloning of random *Eco*RI fragments from the *B. pertussis* genome prior to transfer leads to random insertions of the vector at locations corresponding to particular fragments cloned. As shown schematically in Fig. 3a, a given insertion of the vector will result in disruption of given *Xba*I and *Spe*I fragments, thus demonstrating that these two fragments overlap, and the resulting sizes of the new fragments reveal the size of the overlap and the position of the insertion. Approximately 200 insertions of pSS1456 were examined, and once pSS1577 was constructed, an additional 200 insertions of this vector were examined. Examples of data obtained by this method are shown in Fig. 1b, where two random insertions, P139 and P175, are shown. This type of data allowed the assignment of the *Spe*I and *Xba*I fragments to seven contiguous segments, but connections between these segments could not be inferred because of the lack of insertions in the overlaps at the ends of these segments. In most cases, this was presumably due to the fact that the overlaps were small. However, in the case of the overlap of *Xba*I-C and *Spe*I-G, no insertions were

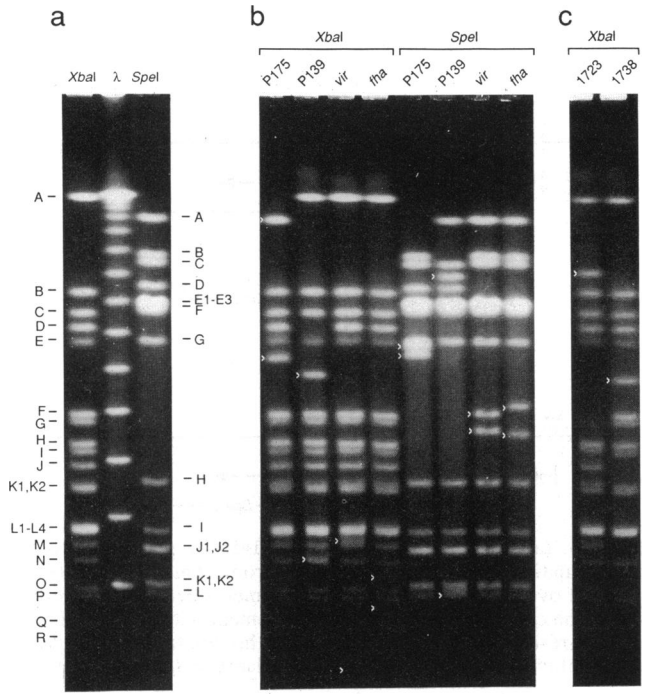

FIG. 1. Examples of pulsed-field gel electrophoresis of *B. pertussis* chromosomal digests. Gels were run at 200 V with the switch-time ramped from 1 to 50 s over the 34-h run time. (a) *Xba*I and *Spe*I digests of BP536. The lambda ladder size standard is a preparation of bacteriophage lambda concatemers (monomeric size, 48.5 kb) obtained from New England Biolabs. Fragment designations are given at the sides of the gel. (b) *Xba*I and *Spe*I digests of insertions of pSS1577 into the BP536 chromosome at different locations (Fig. 4). P175 and P139 are two random insertions. Insertions corresponding to *vir* and *fha* are described in the text. The fragments affected by these insertions are as follows: P175 affects *Xba*I-A and *Spe*I-A, P139 affects *Xba*I-D and *Spe*I-B, and *vir* and *fha* insertions affect *Xba*I-L1 and *Spe*I-D. Resulting fragments in each case are indicated by arrowheads. (c) *Xba*I digests of linking insertions which result in destruction of *Xba*I sites and the resulting fusion of adjacent fragments. Fragments *Xba*I-F and *Xba*I-G are fused in 1723, and *Xba*I-J and *Xba*I-M are fused in 1738. The resulting fragments are indicated by arrowheads.

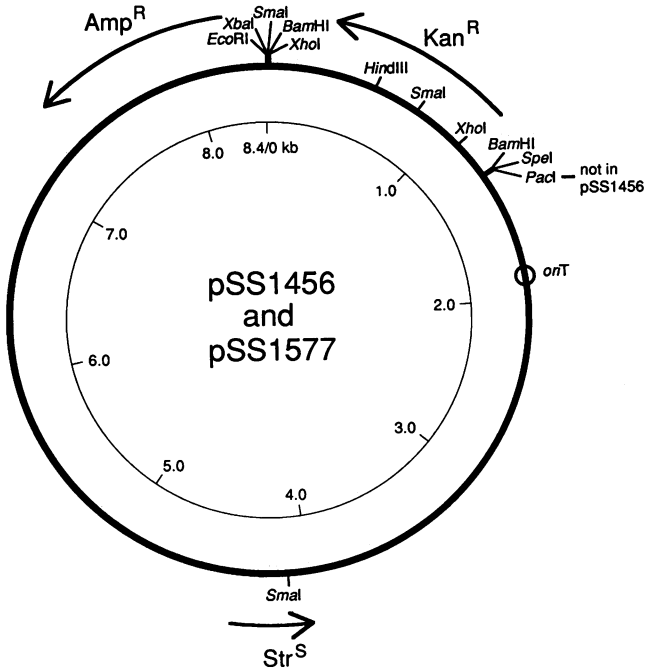

FIG. 2. Restriction map of pSS1456 and pSS1577. These two plasmids are identical with the exception of an oligonucleotide linker containing a *Pac*I site which was introduced into pSS1456 to create pSS1577.

obtained even though this is a sizable overlap, as shown in Fig. 4.

Further data were obtained by the method depicted in Fig. 3b. *Xba*I and *Spe*I linking libraries in the vector pRTP1 were constructed as described in Materials and Methods. Replacement of the kanamycin resistance cassette in *Xba*I-linking clones with a cassette flanked by *Spe*I sites and vice versa produced clones in which the *Xba*I or *Spe*I sites were no longer present and which were marked with kanamycin resistance and a *Pac*I site. These clones were crossed into the *B. pertussis* chromosome by using the ability of pRTP1 to achieve allelic replacements in a single step. After allelic replacement, two contiguous fragments generated by one enzyme should be fused, with the point of fusion marked by the presence of a *Pac*I site. Representative data obtained by this method are shown in Fig. 1c. The contiguities which were demonstrated by this method are listed in Table 2. Although only 11 contiguities were established, in several cases they provided crucial data which allowed the completion of the map.

The data generated in this fashion, along with data from double digests of either *Xba*I or *Spe*I together with *Pac*I or *Pme*I, allowed the completion of the map with one exception. The position of an overlapping *Xba*I-L and *Spe*I-K fragment could not be distinguished between two possible locations: between *Xba*I-F and *Xba*I-R (as shown in Fig. 4) or between *Xba*I-I and *Xba*I-N. To resolve this issue and to provide a final check on the validity of the map, pSS1577 insertions were digested with *Pac*I, which cuts the *B. pertussis* chromosome into two fragments. A subset of insertions around the map, which defined all major overlaps, was selected. These are shown in Fig. 4 for those contained within *Pac*I-B, the smaller of the two *Pac*I fragments. If the map were correct, upon digestion with *Pac*I, these insertions should have resulted in the cleavage of *Pac*I-B into two new fragments and the difference in size between these new fragments should have decreased with the decreasing distance of the insertion point from the center of *Pac*I-B and then have increased again with decreasing distance from the opposite end of *Pac*I-B. As shown in Fig. 5, the expected result was obtained. A similar analysis was completed for insertions in *Pac*I-A with similar results (data not shown). A *Pac*I digest of insertion P140, which is within the *Xba*I-L and *Spe*I-K segment, is also shown in Fig. 5. From this, it can clearly be seen that this insertion is within *Pac*I-A, and the sizes of the fragments generated are consistent with the placement of these two fragments as shown in Fig. 4.

Determining the map location of known genes. Previously characterized genes were located on the map in a manner analogous to that shown in Fig. 3a. In these cases, the homology by which insertion occurred was that of a known gene. Examples of the gel data obtained are shown in Fig. 1b, where insertions corresponding to *vir* and *fha* are shown. In this case, knowledge of the *vir* and *fha* loci was used to

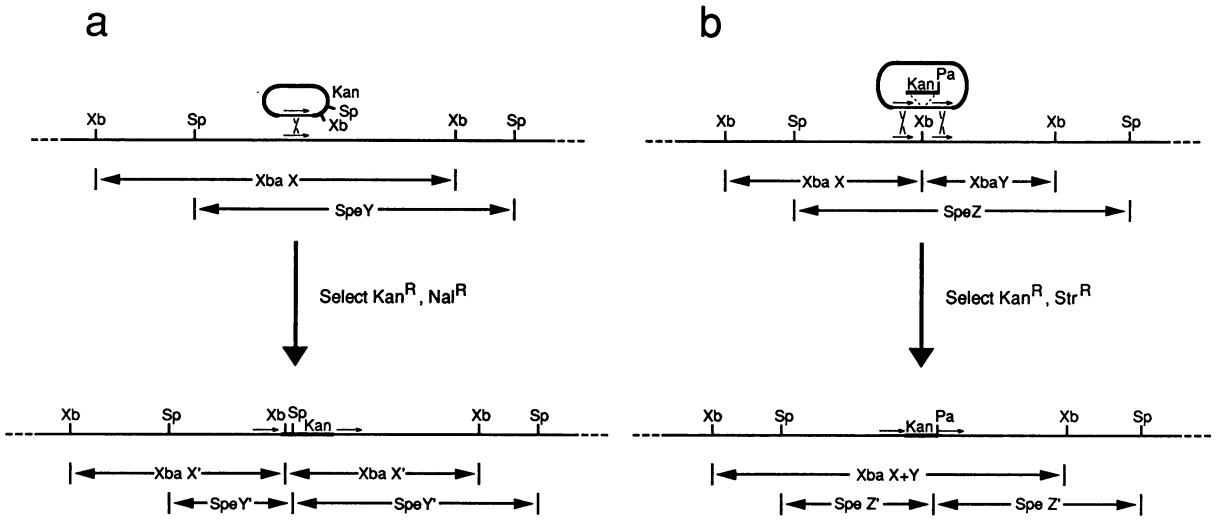

FIG. 3. Diagrammatic representation of strategies used to derive the map. (a) Random insertion of pSS1456 or pSS1577 into the chromosome of *B. pertussis* BP536 (Nal^r Str^r) directed by homologous recombination with a random cloned chromosomal segment (small horizontal arrows indicate regions of homology). This results in the disruption of overlapping *Xba*I and *Spe*I fragments because of insertion of new restriction sites contained in the vector. (b) Removal of an *Xba*I site in the chromosome by allelic replacement with an *Xba*I-linking clone modified in vitro to remove the *Xba*I site while marking it with kanamycin resistance and a *Pac*I site (small horizontal arrows indicate regions of homology). As a result, adjacent *Xba*I fragments are fused and the point of fusion is marked on the overlapping *Spe*I fragment with a *Pac*I site. Pa, *Pac*I; Sp, *Spe*I; Xb, *Xba*I.

maximize the apparent difference between these two insertions by using a 4.0-kb *Eco*RI fragment for *vir* that is actually downstream of the *bvgA* and *bvgS* genes and thus further from the *fhaB* structural gene, which was represented by a 10-kb *Eco*RI fragment (35). In addition, the two fragments were cloned in opposite orientations in pSS1577. Since the *Xba*I sites and *Spe*I sites in pSS1577 are off center with respect to the *Eco*RI cloning site, the difference in orientation enhanced the apparent distance between two insertion sites. In this case, the predicted apparent distance between the two insertions is 41.2 kb for the *Xba*I digest and 36 kb for the *Spe*I digest. As shown in Fig. 1b, observed sizes were consistent with this. The apparently small difference observed between the *Spe*I digests is due to the fact that the two insertion sites straddle the center of the *Spe*I-D fragment.

Other genes were mapped in a similar fashion after being cloned into pSS1577. Several such clones were obtained by screening the library in pSS1577 with ^{32}P-end-labelled radioactive oligonucleotide probes synthesized from published DNA sequence data. Others were subcloned from plasmids kindly provided by other investigators. For all genes except *aroA*, map positions could be determined in a straightforward manner from the fragments affected and the sizes of the resulting novel fragments in *Spe*I and *Xba*I digests alone. In this case, an additional *Pac*I digest provided the necessary information to assign the location of *aroA* unambiguously. The map positions of these genes are shown in Fig. 4. In all cases, insertions at known genes gave results, in terms of the fragments affected and the sizes of the resulting novel fragments, which were consistent with the previously determined physical map.

DISCUSSION

We have derived a physical map, i.e., a restriction map, of the chromosome of *B. pertussis* Tohama I. From this it can be seen that the size of the *B. pertussis* genome is approximately 3,750 kb or about 80% of the size of the *E. coli* genome (17). The locations of the most studied virulence genes have been determined. For the most part, these are scattered around the chromosome. Apart from *fha*, *vir*, and *fim* accessory genes, which are known from other studies to be contiguous, the closest linkage shown is between *fim-2* and *prn*, which are separated by approximately 100 kb (21, 35, 41). In the absence of other data, we would not judge this proximity to be significant in an evolutionary sense.

The absence of linkage invites speculation about the evolution of *B. pertussis* as a human pathogen. It would appear that the virulence determinants were not acquired in a single genetic event such as the insertion of an episome carrying multiple virulence determinants. Rather, we would suggest that they have been acquired stepwise either through discrete genetic events such as insertions of transposable elements or by the adaptation, perhaps following gene duplication events, of other bacterial genes to play new roles in host-parasite interaction.

On the other hand, the *vir* and *fha* loci and, it is now known, *fim* accessory genes are closely associated, both spatially and in the functional sense that it appears that *vir* is able to regulate *fha* and the *fim* accessory genes directly. In contrast, mounting evidence from several laboratories suggests that *vir*-dependent regulation of other virulence determinants such as *ptx* and *cya* may require additional factors. We may thus speculate further that the role of the *vir* locus was once restricted to regulation of the expression of filamentous hemagglutinin and fimbriae but that as genes for other virulence factors were acquired through the course of evolution, their regulatory regions evolved to come under the control of the *vir* locus.

The process we used to obtain this map is somewhat different from that used previously. To date, the most commonly used method for constructing physical maps, in cases where no genetic map information is available, has

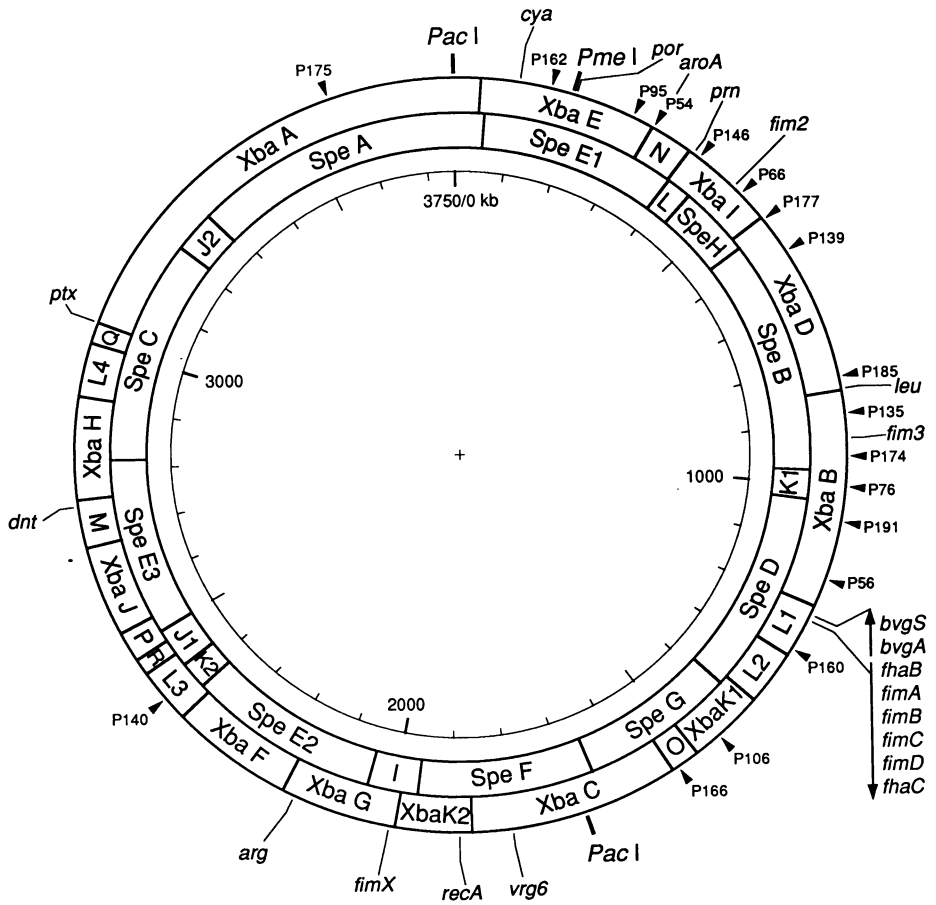

FIG. 4. Restriction map of the *B. pertussis* chromosome showing the locations of the *Xba*I and *Spe*I fragments shown in Fig. 1a. Positions of specific insertions of pSS1577 referred to in the text and figure legends are denoted by arrowheads. Positions of *Pac*I and *Pme*I sites are shown by thick lines. Positions of known genes are denoted by thin lines. The relative order and orientation on the chromosome of the *bvgA*, *bvgS*, *fhaB*, *fimA*, *fimB*, *fimC*, *fimD*, and *fhaC* genes as shown are inferred from the location and orientation of the *vir* and *fha* loci determined as described in the text and other studies on the organization of this region (1, 21, 35, 41). Coordinates are given in kilobase pairs, with one of the *Pac*I sites being used as the origin.

involved Southern hybridization of chromosomal DNA digested with one restriction enzyme with radiolabelled fragments generated by another enzyme. Such a method can reveal which fragments overlap and thus will allow their ordering into a restriction map. Differences in our approach compared with this and other methods, which may be seen by some as advantages, are as follows. (i) The point of insertion of pSS1577 in a given derivative is marked physically by the introduction of restriction sites and thus not only gives information on which two fragments overlap at this point but, by the sizes of the resulting fragments, reveals the size of that overlap. (ii) This approach avoids the complication of repeated sequences, which are more likely to be present in large DNA fragments and which can confound interpretation of Southern hybridization data. Even if a particular clone contains a repeated sequence and integrates via this homology, it still results in a single insertion and thus will give useful information. (iii) Mapping of known genes is easily performed and is capable of giving two types of information not possible if genes are mapped to intervals of a restriction map by hybridization. One is a more precise location of the gene by virtue of the fact that its location is marked on a restriction fragment by the introduction of new sites, and the other is the direction of transcription. This, if known for a cloned gene, can be determined on the map by examining insertions of the vector directed by the gene in question cloned in two different orientations. This is due to the fact that the *Xba*I, *Spe*I, and *Pac*I sites in pSS1577 are off center with respect to the *Eco*RI cloning site. Thus, insertions in two orientations will show a difference in apparent

TABLE 2. Restriction fragment contiguities established by recombination with linking clones

Linking plasmid	Fragments linked
pSS1720	*Xba*I-J with *Xba*I-P
pSS1722	*Xba*I-L4 with *Xba*I-Q
pSS1723	*Xba*I-F with *Xba*I-G
pSS1727	*Xba*I-L1 with *Xba*I-L2
pSS1736	*Xba*I-C with *Xba*I-O
pSS1738	*Xba*I-J with *Xba*I-M
pSS1747	*Spe*I-A with *Spe*I-J2
pSS1748	*Spe*I-F with *Spe*I-I
pSS1749	*Spe*I-H with *Spe*I-L
pSS1765	*Spe*I-D with *Spe*I-K1
pSS1771	*Spe*I-C with *Spe*I-E3

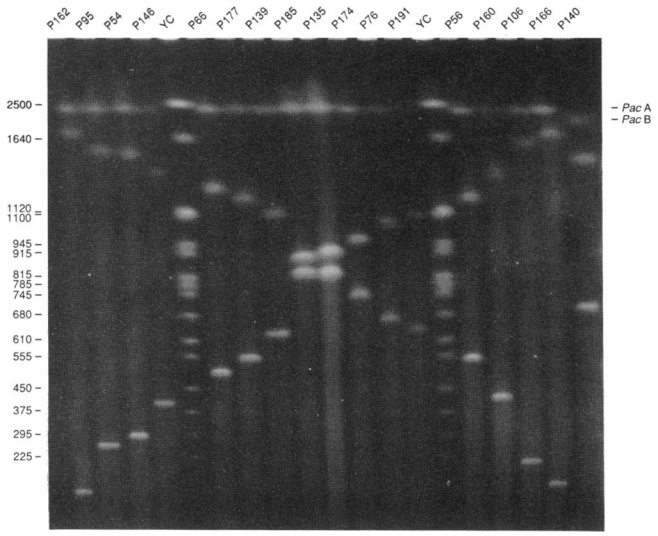

FIG. 5. Pulsed-field gel electrophoresis of *Pac*I chromosomal digests of *B. pertussis* derivatives with insertions of pSS1577. Gels were run at 200 V with the switch-time ramped from 50 to 200 s over the 40-h run time. The position of each insertion on the chromosomal map is shown in Fig. 4. The migration of fragments *Pac*I-A and *Pac*I-B is shown to the right, and the sizes of the yeast chromosomes run in lanes YC are given to the left in kilobase pairs.

map position of at least 5.8 kb in an *Spe*I digest and 8.4 kb in an *Xba*I digest. This translates into a change in the difference between the sizes of the novel fragments of a minimum of 11.6 kb for *Spe*I digests and 16.8 kb for *Xba*I digests, an amount easily discernible within the size range of these fragments. (iv) Because pSS1577 contains the origin of transfer for IncP plasmids, one can, by introduction of an IncP plasmid which supplies Tra functions in *trans*, render any strain containing an insertion of pSS1456 or pSS1577 capable of conjugative transfer of chromosomal markers. The point of initiation and the direction of transfer will depend on the site and orientation of the particular vector insertion. This should allow the use of these strains in the genetic mapping of mutations with interesting phenotypes whose genes have not been or cannot easily be cloned in *E. coli*. The feasibility of using chromosomal insertions of IncP derivatives to create donors of *B. pertussis* chromosomal markers in conjugation experiments has previously been demonstrated (37).

As in most chromosome mapping projects, no one technique gave all the information needed to complete the *B. pertussis* map. We, like others, have used the considerable power of linking clones, i.e., cloned segments which span the junction point of two fragments, to complete this map. In keeping with the genetic approach used in this study, these were used in recombination experiments to accomplish fusion of adjacent fragments and demonstrate in this way the adjacency of some fragments.

It is our hope that construction of this map will help to further the genetic analysis of *B. pertussis*, and to this end we are developing new tools to facilitate genetic mapping of mutations in this species. The map we have constructed for *B. pertussis* Tohama I will also allow the comparison of the chromosomal organization of this bacterium with other members of the *Bordetella* genus and other strains of *B. pertussis*. Efforts to map the chromosomes of *B. parapertussis* and *B. bronchiseptica* in the hopes of shedding new light on the evolutionary relationship of these closely related companion species are ongoing.

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