# Hfr Mapping of Mutations in *Bordetella pertussis* That Define a Genetic Locus Involved in Virulence Gene Regulation

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We report the development of techniques for the genetic mapping of point mutations in the bacterial pathogen *Bordetella pertussis*. A plasmid vector which is self-transmissible by conjugation and which, by insertion into the *B. pertussis* chromosome, can mobilize chromosomal sequences during conjugation with a recipient *B. pertussis* bacterium has been constructed. This vector is used in conjunction with a set of strains containing kanamycin resistance gene insertions at defined physical locations in the *B. pertussis* genome. In crosses between these donor strains and a mutant recipient strain, transfer of a chromosomal segment flanking the kanamycin resistance gene insertion is selected for, and the percentage of exconjugants which reacquire the wild-type trait is scored. In this way the linkage of the mutant allele to these markers, and thus the approximate chromosomal position of the mutant allele, is determined. We have used this genetic system to map a newly described locus in *B. pertussis* involved in the regulation of the virulence genes *ptx* (pertussis toxin) and *cya* (adenylate cyclase toxin).

The genetic analysis of *Escherichia coli* may be said to have begun nearly 50 years ago upon the discovery of genetic transfer in this species, and it has continued unabated ever since (16). In contrast, the genetic analysis of most other bacteria, including pathogenic bacteria, lacking genetic tools such as Hfr mapping and bacteriophage transduction, has had to await the development of newer techniques. The molecular biology revolution has given us the ability to clone genes from virtually any bacterium and to analyze their primary structure by DNA sequence analysis. However, an equally great impact has been made by the development of new genetic tools in the form of bacterial transposable elements, both those which are naturally occurring and those which have been engineered to have special characteristics.

The great power of transposons as mutagenic agents lies in (i) the ability to directly select for mutations by selecting for transposition events, (ii) the ability to easily clone genes which have been identified by transposon insertion by virtue of the linkage of a selectable marker to those genes, (iii) the ability to easily map the sites of such mutations physically by virtue of the physical nature of the insertion mutations created, and (iv) the ability to introduce gene fusions in order to identify genes regulated in a particular fashion or encoding proteins directed to a particular subcellular compartment.

By their nature transposon-induced mutations are usually "knock-outs" that completely destroy the function of the genes they identify. They thus cannot be used to identify essential genes and cannot generally be used in the isolation of second-site suppressor mutations. This characteristic has not been a major drawback in the study of bacterial pathogens, as many of these efforts have been directed at the identification of virulence genes which are usually dispensable for a bacterium's viability when grown in vitro. However, in the case of some bacterial pathogens, our increasing knowledge and under-

standing of their pathogenesis has led us to ask increasingly subtle or detailed questions. In these cases, it would be advantageous to be able to work routinely with missense and nonsense mutations. This requires an ability to map such mutations genetically, as the physical changes associated with such mutations are undetectable under most conditions. To this end we have developed tools which allow the genetic mapping of mutations in the human bacterial pathogen *Bordetella pertussis*.

*B. pertussis* is a dramatic demonstration of a phenomenon now seen as common in pathogenic bacteria, the regulation of virulence potential in response to environmental signals. In this organism, all known protein virulence factors are regulated by a single genetic locus discovered by transposon mutagenesis and known as *bvg* (previously *vir*) (29). The *bvg* locus encodes two genes, *bvgA* and *bvgS*, which encode proteins belonging to the receiver or response regulator class and the transmitter or sensor class, respectively, of two-component regulatory systems of bacteria (1, 25, 28). At least one virulence factor, an adhesin, the filamentous hemagglutin, is regulated directly by the *bvg* locus. This judgment comes from the observations that cloning *bvg* and *fha* together in *E. coli* reconstitutes expression of filamentous hemagglutinin in response to the correct environmental signals and from biochemical studies showing direct interaction between BvgA and the *fha* promoter (18, 21, 27). For other virulence genes, such as *ptx*, encoding pertussis toxin, and *cya*, encoding adenylate cyclase toxin, this regulation appears to be indirect or to occur by a different mechanism. Efforts to reconstitute expression of *ptx* or *cya* in *E. coli* have met with failure (8, 18). A recent report suggests that *bvg*-dependent expression of *ptx* is possible under some conditions, but in this case responsiveness to environmental signals is not seen (22). Efforts to demonstrate binding of BvgA to the promoter regions of the *ptx* operon have also met with failure (21). Such findings have suggested the existence of accessory regulatory factors, present in *B. pertussis* but not in *E. coli*, which are necessary for expression of *ptx* and *cya* but not *fha*. Recently two spontaneous mutations

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TABLE 1. Bacterial strains and plasmids used in this study

| Strain or plasmid | Relevant feature(s) | Source or reference |
|---|---|---|
| **Strains** | | |
| *E. coli* K-12 | | |
| DH5α | High-efficiency transformation | BRL[a] |
| LE392 | Transduction recipient | 17 |
| | | |
| *B. pertussis* | | |
| Tohama I | Patient isolate | 14 |
| BP953 | Tohama I (*nal str fhaB-lacZ ptx-phoA*) | 24 |
| BP965 | BP953 Δ*bvg* | 24 |
| BP1000 | Tohama I(*rif*) | This study |
| BP1087 | Tohama I(*rif ksg*) | This study |
| BC7500 | *unk-1* | 5 |
| BC7501 | *unk-1 ksg* | This study |
| BC7502 | *unk-1 rif* | This study |
| RPV3 | *unk-2* | 9 |
| RPV4 | *unk-2 ksg* | This study |
| RPV5 | *unk-2 rif* | This study |
| BP953:K1 | Genetic donor constructed by using pSS1720 | This study |
| BP953:K2 | Genetic donor constructed by using pSS1722 | This study |
| BP953:K3 | Genetic donor constructed by using pSS1723 | This study |
| BP953:K4 | Genetic donor constructed by using pSS1727 | This study |
| BP953:K5 | Genetic donor constructed by using pSS1736 | This study |
| BP953:K6 | Genetic donor constructed by using pSS1738 | This study |
| BP953:K7 | Genetic donor constructed by using pSS1747 | This study |
| BP953:K8 | Genetic donor constructed by using pSS1748 | This study |
| BP953:K9 | Genetic donor constructed by using pSS1749 | This study |
| BP953:K10 | Genetic donor constructed by using pSS1765 | This study |
| BP953:K11 | Genetic donor constructed by using pSS1771 | This study |
| BP1137 | Kan$^r$ Str$^s$ transconjugant of BP953:K2 and BP1000 | This study |
| BP1138 | Kan$^r$ Str$^s$ transconjugant of BP953:K11 and BP1000 | This study |
| | | |
| **Plasmids** | | |
| pBR322 | Cloning vector | 2 |
| pEYDG1 | Source of *Bam*HI *oriT* fragment | 31 |
| pHC79 | Cosmid cloning vector, source of λ *cos Hae*III fragment | 12 |
| pRK2013 | IncP plasmid, source of *tra* genes | 7 |
| pSS1129 | Derivative of pRTP1 with gentamicin resistance gene | 23 |
| pSS1527 | pBR322 derivative with additional cloning sites | This study |
| pSS1673 | pBR322 derivative with cloned *gen* PCR fragment | This study |
| pSS1720 | Used to deliver insertion K1 to BP953 chromosome | 26 |
| pSS1722 | Used to deliver insertion K2 to BP953 chromosome | 26 |
| pSS1723 | Used to deliver insertion K3 to BP953 chromosome | 26 |
| pSS1727 | Used to deliver insertion K4 to BP953 chromosome | 26 |
| pSS1736 | Used to deliver insertion K5 to BP953 chromosome | 26 |
| pSS1738 | Used to deliver insertion K6 to BP953 chromosome | 26 |
| pSS1747 | Used to deliver insertion K7 to BP953 chromosome | 26 |
| pSS1748 | Used to deliver insertion K8 to BP953 chromosome | 26 |
| pSS1749 | Used to deliver insertion K9 to BP953 chromosome | 26 |
| pSS1765 | Used to deliver insertion K10 to BP953 chromosome | 26 |
| pSS1771 | Used to deliver insertion K11 to BP953 chromosome | 26 |
| pSS1811 | pSS1527 plus *Bam*HI *oriT* fragment | This study |
| pSS1826 | pSS1673 plus *Eco*RI linker at *Bam*HI site | This study |
| pSS1827 | Replicon fusion of pBR322 and pRK2013 at *Eco*RI and *Sal*I sites | This study |
| pSS1829 | pSS1811 plus *Eco*RI *gen* fragment from pSS1826 | This study |
| pSS1832 | pSS1829 with one *Eco*RI site destroyed by addition of a *Not*I site | This study |
| pSS1839 | pSS1827 with *Hin*dIII site destroyed by addition of a *Pme*I site | This study |
| pSS1840 | pSS1839 plus *Hae*III *cos* fragment inserted at the *Pme*I site | This study |
| pSS1853 | Replicon fusion of pSS1840 and pSS1832 at *Eco*RI and *Sal*I sites | This study |

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conferring the phenotype expected of mutations identifying such a factor (Ptx$^-$ Cya$^-$ Fha$^+$) have been reported (5, 9). In this study we report the genetic mapping of these mutations in *B. pertussis*, with the finding that they identify a new locus in this organism which is distinct from *ptx*, *cya*, *fha*, and *bvg*.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and media.** Bacterial strains and plasmids used in this study are presented in Table 1. *E. coli* DH5α, which was used as a transformation host for all cloning steps, was obtained from Bethesda Research Laboratories.

The *B. pertussis* strains BC7501 and RPV4 were spontaneous kasugamycin-resistant derivatives of BC7500 and RPV3, respectively, obtained by selection on Bordet-Gengou agar containing 200 μg of kasugamycin per ml. BC7502 and RPV5 were spontaneous rifampin-resistant derivatives of BC7500 and RPV3, respectively, selected on Bordet-Gengou agar containing 100 μg of rifampin per ml. BP1000 was isolated in an identical fashion. BP1087 was isolated in two steps, to rifampin resistance in a manner similar to that described above and to kasugamycin resistance by selection on Bordet-Gengou agar containing 200 μg of kasugamycin per ml. *B. pertussis* strains were grown on Bordet-Gengou agar (Difco) containing 1% proteose peptone (Difco) and 15% defibrinated sheep blood. Concentrations of antibiotics were as follows: gentamicin sulfate, 10 μg/ml; kanamycin sulfate, 10 μg/ml; 100 kasugamycin-HCl, 100 μg/ml; nalidixic acid, 50 μg/ml; rifampin, 50 μg/ml; and streptomycin sulfate, 100 μg/ml. *E. coli* strains were grown on L agar or in L broth (19) supplemented with antibiotics as appropriate. Concentrations of antibiotics were as follows: ampicillin, 100 μg/ml; gentamicin sulfate, 10 μg/ml; and kanamycin sulfate, 10 μg/ml.

**Construction of pSS1853.** PCR was used to generate a DNA fragment encoding gentamicin resistance. The sequence of the upstream primer was 5'-CGCGAATTCCACCGTGGAAAC GGATGAAGGCACG-3', and the sequence of the downstream primer was 5'-CGCGGATCCAAGCTTTTAGGTG GCGGTACTTGGGTCGATA-3'. The template DNA was pSS1129, which encodes a gentamicin acetyltransferase. After digestion of the PCR product with *Eco*RI and *Bam*HI, a 798-bp fragment which extends from 38 bp upstream of the reported promoter of this gene to just past the reported stop codon (30) was obtained. This fragment was ligated to *Eco*RI- and *Bam*HI-cleaved pBR322 DNA to create pSS1673. An oligonucleotide linker was added at the *Bam*HI site, which resulted in the duplication of the *Bam*HI site and the addition of an *Eco*RI site between the two *Bam*HI sites, to create pSS1826.

Complementary oligonucleotides having the sequences 5'-ATTCACTAGTTCTAGAAGATCTGGTACCCTCGAGG-3' and 5'-GATCCCTCGAGGGTACCAGATCTTCTAGAAC TAGTG-3' were annealed and ligated to *Eco*RI- and *Bam*HI-cleaved pBR322 DNA. The resulting plasmid thus had unique restriction sites at this location in the following order: *Eco*RI, *Spe*I, *Xba*I, *Bgl*II, *Kpn*I, *Xho*I, *Bam*HI; and it was named pSS1527. An approximately 760-bp *Bam*HI fragment of pEYDG1, encoding *oriT*, the origin of transfer from the R plasmid RK2, was added at the *Bam*HI site to create pSS1811. The *Eco*RI fragment from pSS1826 containing the gentamicin resistance gene was added at the *Eco*RI site of pSS1811 to create pSS1829. This plasmid was partially digested with *Eco*RI, and the linker 5'-AATTGCGGCCGC-3' was added to destroy one *Eco*RI site and add a *Not*I site at the same position. The resulting plasmid was named pSS1832.

An approximately 39-kb *Eco*RI-*Sal*I fragment from pRK2013 containing both *tra* operons was joined to *Eco*RI- and *Sal*I-digested pBR322 to create pSS1827. The single *Hind*III site in this plasmid in the pRK2013-derived sequences was destroyed and a *Pme*I was site added by the insertion of the oligonucleotide 5'-AGCTAGTTTAAACT-3' to create pSS1839. A 226-bp *Hae*III fragment containing the *cos* site of bacteriophage λ was cloned into the resulting *Pme*I site by ligation of *Pme*I-cleaved pSS1839 with *Hae*III fragments of the cosmid pHC79, packaging into phage particles in vitro (Packagene; Promega), and transduction of DH5α to ampicillin resistance. One of the resulting plasmids was named pSS1840. The final step to create pSS1853 was the ligation of the large *Eco*RI-*Sal*I
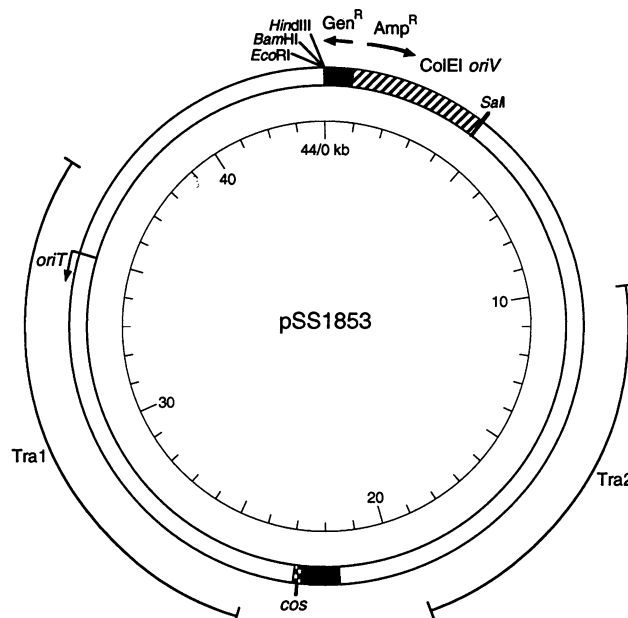


FIG. 1. Physical map of plasmid pSS1853. Coordinates for features of RK2-derived sequences are those of Pansegrau et al. (20), and the positions and extents of the phage Mu-generated deletion and the residual phage Mu sequences in pRK2013 were determined by Figurski and Helinski (7). Coordinates for pSS1832-derived sequences were predicted from the DNA sequence. The sources of different regions are indicated as follows: filled, *gen* PCR fragment; hatched, pBR322; shaded, Mu phage; checked, phage λ; and open, RK2.

fragment of pSS1840 with *Eco*RI- and *Sal*I-digested pSS1832, followed by in vitro packaging and transduction of gentamicin and ampicillin resistance. A map of pSS1853 is shown in Fig. 1.

**Construction of *B. pertussis* Hfr donor pools.** The construction of *B. pertussis* BP953 will be described elsewhere (24). This strain is nalidixic acid resistant and streptomycin resistant, and it harbors two gene fusions: *fhaB-lacZ* and *ptx-phoA*. A set of eleven insertions of a kanamycin resistance gene at different locations in the BP953 chromosome was constructed by using the linking plasmids and methods described previously (26). These insertions were named BP953:K1 through BP953:K11, and their positions are shown graphically in Fig. 2.

A library of random *B. pertussis* fragments in pSS1853 was constructed, using the strategy of Hung and Wensink (13). Chromosomal DNA of BP965 was digested with the restriction enzyme *Bst*YI, which recognizes and cleaves within the sequence R'GATCY, leaving a 4-bp 5' overhang. The ends were partially filled in with the Klenow fragment of DNA polymerase by using α-S-dATP and α-S-dGTP as substrates to prevent proofreading by the Klenow enzyme. In a similar fashion, the vector pSS1853 was digested with *Sal*I (G'TC GAC) and the sticky ends were partially filled in, using α-S-dCTP and α-S-dTTP as substrates. This approach has the combined virtues of forcing insertions in the vector without allowing insertion of multiple fragments (13). After ligation of pSS1853 vector DNA and BP965 insert DNA, ligations were packaged into bacteriophage λ particles in vitro (Gigapack XL; Stratagene). The resulting phage lysates were used to transduce *E. coli* LE392 to gentamicin and ampicillin resistance. Approximately 10^5 colonies were pooled and conjugated with each of the BP953 derivatives BP953:K1 through BP953:K11. Selection on Bordet-Gengou agar containing gentamicin, ka-
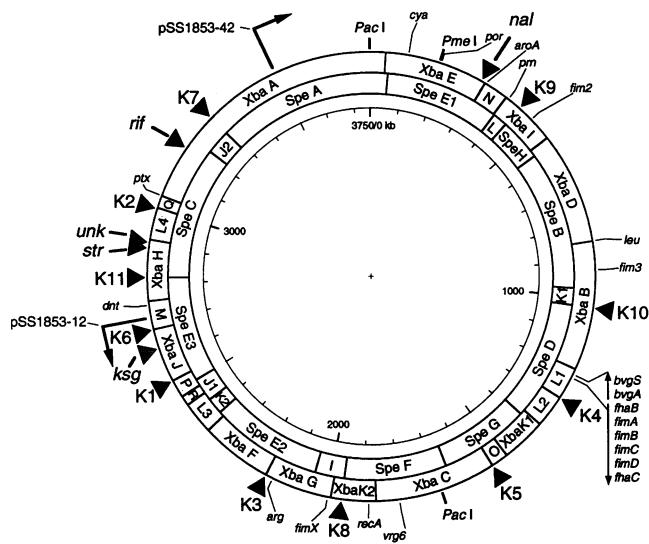
FIG. 2. Physical map of the *B. pertussis* Tohama I chromosome showing the positions of the kanamycin resistance gene insertions used as selectable markers in conjugations, indicated by filled arrowheads. Locations of genes mapped physically are depicted by thin lines. The approximate locations of loci mapped genetically are indicated by shaded arrowheads. The approximate locations and directions of transfer mediated by pSS1853 derivatives are depicted by bent arrows.

namycin, and streptomycin yielded $10^3$ to $10^4$ exconjugants, which were pooled to create the Hfr donor pools used for mapping. Similarly, Hfr donor pools for BC7500 and RPV3 were created.

**Isolation of pSS1853-12 and pSS1853-42.** Some defined donor strains having a single origin of transfer were isolated from the donor pools described above. The location of the pSS1853 insertion in these strains was mapped to a particular *Spe*I or *Xba*I restriction fragment by digestion of chromosomal DNA in agarose plugs and electrophoresis on pulsed-field gels as previously described (26). Although the pSS1853 vector does not contain these sites, fragments into which it has been inserted become approximately 50 kb larger as a result of the insertion, thus enabling mapping of the site of insertion to a particular restriction map interval. These plasmids were then recovered from the *B. pertussis* strains after conjugation with *E. coli* DH5α and selection for gentamicin resistance on L agar.

The direction of transfer for each plasmid was determined by its reintroduction into strains bearing selectable markers to either side and by determination of which marker could then be transferred with greatest efficiency. In this way, pSS1853-12 and pSS1853-42, which, after integration into *B. pertussis* Tohama I, have the approximate chromosomal locations and directions of transfer shown in Fig. 2, were isolated.

**Conjugations.** Hfr donor and recipient strains were grown on Bordet-Gengou agar and then resuspended in phosphate-buffered saline (PBS) to an $A_{600}$ of approximately 1.0. The suspensions were mixed at a ratio of 1 volume of donor to 10 volumes of recipient, and 50 μl was spotted on Bordet-Gengou agar containing 10 mM $MgCl_2$ and incubated at 37°C for 7 to 8 h. The bacteria were then recovered with a sterile Dacron swab, resuspended in 1.0 ml of PBS, and plated on Bordet-Gengou agar containing the appropriate antibiotics. Dilutions were performed when necessary. Phenotypes of exconjugants were assessed as follows: resistance to the antibiotics kasugamycin, nalidixic acid, rifampin, and streptomycin was determined by patching single colonies onto Bordet-Gengou agar containing the antibiotic indicated in Table 2, and the presence of alkaline phosphatase activity was determined by colony lifts with nitrocellulose membranes as previously described (15). β-Galactosidase activity was detected in an analogous fashion by using filters soaked in Z-buffer (19) to which 0.003% sodium dodecyl sulfate was added. Hemolysis was apparent on Bordet-Gengou agar. For the data presented in Table 2, 200 exconjugant colonies were scored for each phenotypic marker.

## RESULTS

**Construction of a plasmid vector for the mobilization of *B. pertussis* chromosomal sequences.** Hfr strains of *E. coli* are genetic donors of chromosomal loci as a result of the integration of the F plasmid in the chromosome. By analogy, we created a derivative of the conjugative R plasmid RK2 which could be directed to insert into the chromosomes of *B. pertussis* strains and thus mobilize chromosomal sequences. This plasmid, pSS1853, is depicted in Fig. 1, and the details of its construction are given above. This plasmid contains all necessary information encoded by RK2 to mediate cell-to-cell transfer, including *oriT*, the site of initiation of transfer. The RK2 vegetative origin of replication, *oriV*, has been deleted, and thus this plasmid is incapable of replication in *B. pertussis*. However, the inclusion of a ColE1-derived *oriV* enables its maintenance in *E. coli*. Following conjugative transfer to *B.*

TABLE 2. Linkage of traits to kanamycin resistance markers

| Selected marker | Rifampin resistance counterselection | | | | | Kasugamycin resistance counterselection | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Lac⁺ | Pho⁺ | Nalʳ | Strʳ | Ksgˢ | Lac⁺ | Pho⁺ | Nalʳ | Strʳ | Rifˢ |
| K1 | 0.5 | 2 | 0.5 | 9 | 53.5 | ND[a] | 1.5 | 0 | 1 | 0 |
| K2 | 0 | 66 | 0.5 | 74.5 | 7.5 | ND | 80 | 1.5 | 88 | 28.5 |
| K3 | 0.5 | 0 | 0 | 3 | 19.5 | ND | 0 | 0 | 0 | 0 |
| K4 | 75.5 | 0 | 2.5 | 0 | 0.5 | ND | 0 | 0 | 0 | 0 |
| K5 | 5 | 0 | 0.5 | 0 | 1 | ND | 0 | 0 | 0 | 0.5 |
| K6 | 0.5 | 3 | 0 | 22 | 80 | ND | 10 | 0 | 36 | 2.5 |
| K7 | 0 | 4.5 | 4.5 | 3.5 | 0.5 | ND | 40 | 5.5 | 23.5 | 56.5 |
| K8 | 0.5 | 0 | 0 | 1 | 5 | ND | 0 | 0 | 0.5 | 0 |
| K9 | 4.5 | 0 | 49.5 | 0 | 0 | ND | 0 | 52.5 | 0.5 | 0 |
| K10 | 39 | 0 | 4 | 0 | 0 | ND | 0 | 0 | 0 | 0 |
| K11 | 0 | 12.5 | 0 | 53 | 16 | ND | 14 | 0.5 | 71 | 5.5 |

*[a]* ND, not determined.

*pertussis* strains, pSS1853 is lost by segregation unless integration into the chromosome, an event which can be mediated by homologous recombination via cloned chromosomal sequences and which can be selected for by virtue of the gentamicin resistance gene of the plasmid, occurs. Transformation of *E. coli* hosts with pSS1853 is inefficient because of the large size of this vector. To enable efficient cloning of *B. pertussis* sequences, the *cos* site of λ was incorporated to allow in vitro packaging of recombinant plasmids into bacteriophage λ particles and subsequent transduction at high efficiency.

**Construction of *B. pertussis* donors.** To enable the mapping of mutations in *B. pertussis* genetically, a set of selectable markers at various chromosomal locations was required. As a physical map of *B. pertussis* Tohama I was available, the most expeditious approach was to use insertions of an antibiotic resistance gene whose physical locations could be established. The markers used in this study were a set of kanamycin resistance gene insertions which were derived as a result of a physical mapping strategy which resulted in the fusion of adjacent chromosomal *Spe*I or *Xba*I restriction fragments (26). These insertions were incorporated into the BP953 chromosome as previously described, and their locations are shown in Fig. 2. To mobilize the chromosomal sequences of the resulting donor strains, insertions of pSS1853 were made. Rather than constructing defined Hfr strains for each selectable marker which would have an insertion of pSS1853 in the proper configuration for examining linkage to that marker, we chose to use a more general approach. In this approach, pSS1853 was randomly inserted at multiple locations in each donor strain and the population of donors was pooled. Thus, in a mating with an appropriate *B. pertussis* recipient, the chromosomal sequences of the donor were transferred from a variety of origins and thus all parts of the chromosome were transferred more or less equally. Transfer of chromosomal sequences in this way is thus analogous to the process of generalized transduction mediated by some bacteriophages, except that much larger regions of the chromosome are transferred and there is not a well-defined upper limit to the size of the transferred region. We suggest the term "generalized conjugation" to describe this approach. Those recipient bacteria which received and incorporated the region of the chromosome containing the selectable marker were able to survive the selection process. A number of these survivors were then tested for the acquisiton of other genetic traits of the donor. In this way the degree of genetic linkage of that unselected trait with the selected marker was determined.

**Mapping of genes with known physical locations.** As a test of this genetic system, the *fha* and *ptx* loci, whose physical locations had previously been determined, were mapped. In the BP953-derived donor strains, each of these loci was marked by a gene fusion which provided a readily screenable phenotype. In the case of *fha* the phenotype was Lac⁺, as determined by incubation of colonies in the presence of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). In the case of *ptx*, it was Pho⁺, which was determined by incubation of colonies in the presence of BCIP (5-bromo-4-chloro-3-β-D-indolylphosphate). The recipient strain in this mating was BP1087, which is rifampin resistant and kasugamycin resistant but which lacks both gene fusions. The linkages observed are presented in Table 2. These linkages were in keeping with the known physical locations of these two loci as shown in Fig. 2. Thus, for *ptx* the level of linkage to K2 was highest, and the level of linkage to K7 or K11 was second highest, depending on the counterselection. For the *fha* locus, the level of linkage to K4 was highest and the level of linkage to K10 was second highest. The linkage of *ptx* to some selectable markers, such as

K7, differed greatly depending on the counterselectable marker used, i.e., *rif* or *ksg*. This will be discussed below.

**Mapping of loci determining antibiotic resistance.** Four loci governing resistance to the antibiotics kasugamycin, nalidixic acid, rifampin, and streptomycin were mapped. By analogy with studies of *E. coli*, resistance to these antibiotics should define the genes encoding an rRNA methylase, a DNA gyrase, the β subunit of RNA polymerase, and the ribosomal protein S12, respectively (3, 6, 10, 11). The *nal* and *str* loci were mapped in a manner similar to that in which *fha* and *ptx* were mapped, by using BP1087 as a recipient and measuring the coacquisition of nalidixic acid or streptomycin resistance with the selected kanamycin marker. The *ksg* and *rif* markers were mapped after conjugation of the donor strains, which are sensitive to both antibiotics, with BP1087, which is both kasugamycin resistant and rifampin resistant. To map *ksg*, the counterselection used in the mating was rifampin resistance, and kanamycin-resistant, rifampin-resistant exconjugants were then tested for acquisition of kasugamycin sensitivity. The *rif* locus was mapped in a similar fashion after mating of BP1087 with the donor strains and counterselection by kasugamycin resistance. The linkages seen in these matings are presented in Table 2, and the inferred locations of these loci on the *B. pertussis* Tohama I map are shown in Fig. 2. As mentioned above, different linkages were observed for some pairs of markers depending on which antibiotic resistance of the recipient was used as the counterselection. For example, K7 and *ptx-phoA* showed 40% linkage when kasugamycin resistance was used as the counterselection, but they showed only 4.5% linkage when rifampin resistance was used as the counterselection. The locations of the *rif* and *ksg* loci suggest why this is so. In the example described above, the reduced linkage seen when the *rif* marker was used was due to the fact that this marker was between the selected marker in this cross (K7) and the scored marker (*ptx-phoA*). Thus, incorporation of both K7 and *ptx-phoA* into the rifampin-resistant recipient required not two, but four crossovers, and therefore it occurred at a reduced frequency. Similarly, the linkage of K1 and *str* was reduced from 9 to 1% when the intervening *ksg* marker was used for counterselection and the linkage of K7 and *str* was reduced from 23.5 to 3.5% when rifampin resistance was used as the counterselection.

**Mapping of a locus affecting expression of the *ptx* and *cya* loci.** Two interesting mutants of *B. pertussis*, BC7500 and RPV3, in which the expression of pertussis toxin and adenylate cylase toxin, but not filamentous hemagglutinin, is affected have recently been described (5, 9). For the purposes of this paper we will refer to the mutant alleles in these strains as *unk-1* and *unk-2* (Table 1). Rifampin-resistant and kasugamycin-resistant derivatives of each of these strains were isolated and used as recipients in conjugations with the *B. pertussis* donor pools as described above. The presence of the *unk-1* and *unk-2* alleles in exconjugants was indicated by their nonhemolytic (Cya⁻) phenotype. The results of these matings are shown in Table 3. The behavior of the two mutant strains was indistinguishable in these matings, suggesting that they both defined the same gene, *unk*. For the mutant alleles, the levels of linkage to K2 and K11 were highest, suggesting a location for *unk* between these two markers. Similar results had been obtained for *str* (Table 2). As with the *str* locus, linkage of the *unk* locus to K1 and K7 was influenced by the choice of the marker used for counterselection. To measure the linkage of *str* and *unk* directly, BC7500 and RPV3 were converted into genetic donors by introduction of the pSS1853 pool and then mated with BP1000. Selection was for transfer of streptomycin resistance, and counterselection was for rifampin resistance.

TABLE 3. Linkage of *unk-1* and *unk-2* to kanamycin resistance markers

| | % of colonies hemolytic (no. hemolytic/total no.) | | | |
| Selected marker | Rifampin resistance counterselection | | Kasugamycin resistance counterselection | |
| | BC7502 (*unk-1*) | RPV5 (*unk-2*) | BC7501 (*unk-1*) | RPV4 (*unk-2*) |
|---|---|---|---|---|
| K1 | 9 (38/429) | 11 (78/730) | 0 (3/1163) | 1 (5/485) |
| K2 | 74 (214/289) | 65 (241/368) | 65 (181/279) | 70 (179/257) |
| K3 | 4 (7/165) | 4 (11/277) | 0 (2/1471) | 0 (4/1116) |
| K4 | 0 (1/283) | 0 (0/741) | 0 (0/450) | 0 (0/417) |
| K5 | 0 (0/391) | 0 (2/629) | 0 (0/289) | 0 (1/312) |
| K6 | 18 (65/370) | 22 (130/675) | 21 (113/547) | 22 (125/563) |
| K7 | 4 (5/134) | 3 (13/255) | 22 (49/224) | 21 (88/417) |
| K8 | 1 (3/595) | 0 (1/887) | 0 (1/1280) | 0 (0/219) |
| K9 | 0 (1/785) | 0 (3/1016) | 1 (13/1721) | 1 (9/1137) |
| K10 | 0 (0/575) | 0 (0/798) | 0 (2/2073) | 0 (0/469) |
| K11 | 57 (263/461) | 52 (238/454) | 60 (179/299) | 63 (259/412) |

These exconjugants were scored for acquisition of the Hly⁻ phenotype. In this way it was demonstrated that linkage of *unk-1* and *unk-2* to *str* was about 95% (data not shown).

**Three-point crosses to determine the relative locations of *str* and *unk*.** Since *unk* and *str* were very tightly linked, it was not possible to determine their relative order on the chromosomal map by determining linkages to nearby markers. In order to perform a three-factor cross to resolve this issue, the donors shown schematically in Fig. 3 were created. BP1137 was picked as a Kan$^r$ Str$^s$ exconjugant from a mating of BP953:K2 and BP1000. Similarly, BP1138 was picked from a mating of BP953:K11 and BP1000. Both were converted into donors after being mated with pSS1853-12 and pSS1853-42, respectively. These defined donors were used in conjugations with BC7501, BC7502, RPV4, and RPV5. Selection was for kanamycin resistance, and counterselection was for streptomycin resistance. In all cases the results obtained were indistinguishable, and they are presented schematically in Fig. 3. These results are explained as follows. Incorporation of either K2 or K11 by flanking crossovers requires (since selection for streptomycin resistance is imposed) that one of these crossovers occur between the marker and the *str* locus. Since Hly$^+$ Kan$^r$ Str$^r$ exconjugants were obtained only after mating with BP1137:pSS1853-12, this must mean that the *unk* locus was between K2 and *str* as depicted in Fig. 2 and 3. Had the order of *unk* and *str* been reversed, Hly$^+$ Kan$^r$ Str$^r$ exconjugants would only have been obtained after mating with BP1138:pSS1853-42.

## DISCUSSION

In this paper we describe the development and use of tools for genetic mapping of mutations in *B. pertussis* by conjugation.
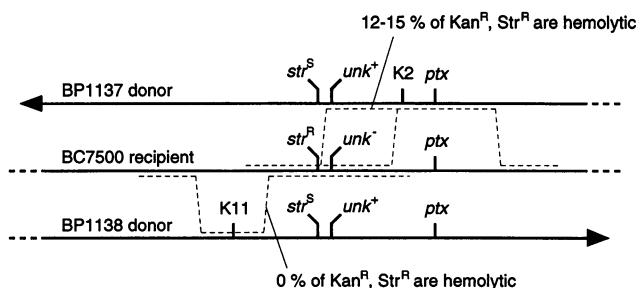


FIG. 3. Schematic representation of three-factor crosses performed to determine the relative order of *str* and *unk* loci.

These tools consist of a set of strains having selectable genetic markers at different locations in the *B. pertussis* genome and a plasmid vector capable of insertion into the *B. pertussis* chromosome and of mediating transfer of chromosomal sequences in a manner analogous to that of Hfr-mediated conjugation in *E. coli*. Insertion of this vector into a donor *B. pertussis* strain can be directed to a specific location and orientation or random locations in a pool of genetic donors. We have primarily used the latter method, which we term generalized conjugation. As with phage P1-mediated generalized transduction, linkage of two genetic markers can fail to occur in two ways. Either the fragment of the chromosome which is transferred can fail to contain both markers, or a genetic crossover can occur between the two markers. The probability of either of these events occurring increases as the separation of the markers increases, and thus genetic linkage serves as an inverse indicator of the physical distance between two markers.

We have used these techniques to map two mutations describing a new genetic locus in *B. pertussis*, one which is involved in the expression of *B. pertussis* toxin genes. The results we obtained suggest that this new locus is tightly linked to the *B. pertussis* locus conferring streptomycin resistance, which is expected to be an *rpsL* homolog. This map position proved accurate enough to direct molecular cloning efforts aimed specifically toward this part of the *B. pertussis* chromosome, which led to the successful cloning and DNA sequencing of these two alleles. Those results are described in the accompanying paper, in which it is shown that these mutant alleles affect the level of the α subunit of RNA polymerase and are thus alleles of *rpoA* (4). It is worth noting that because of the nature of these particular alleles, previous efforts at random cloning by complementation had been unsuccessful.

It should also be noted that these methods have great utility in terms of strain construction. The current method of choice for introducing known genetic markers into *B. pertussis* strains is to cross them in by using conditionally counterselectable suicide vectors donated from *E. coli* strains (23). This is a two-step process for markers for which there is not a selectable phenotype. However, since only one selection step and only one outgrowth of *B. pertussis* are required, using conjugation between *B. pertussis* strains can reduce by half, or by 3 to 5 days, the time required to introduce a marker as long as it is linked to a selectable marker. In this way the Kan$^r$ Str$^s$ derivatives BP1137 and BP1138 were isolated.

Clearly the methods we have reported can be refined. This would include the development of a more complete set of selectable markers at a greater number of locations on the *B.*

*pertussis* chromosome as well as a set of defined Hfr plasmids to allow the efficient transfer of specific regions of the genome. Efforts towards such refinements are currently under way. However, the results reported here show the overall feasibility and utility of such an approach. It is our hope that these tools will be used to further the genetic analysis and understanding of this fascinating bacterial pathogen.

## ACKNOWLEDGMENTS

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