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Development of a New Cloning Vector for Bordetella Brochiseptica in the form of a Hitherto Unidentified Plasmid

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Dr. Bruce Wheeler
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Dear Dr. Wheeler:

This letter is to advise you that Mr. Richard Lonas McColl has satisfactorily completed the requirements for the Senior honors thesis in Microbiology, course Microbiology 402. His written thesis and oral presentation was accepted by his advising committee (Drs. Bemis, Moore and Montie) on May 11, 1993.

Sincerely,

David A Bemis

David A. Bemis, Ph.D.
Associate Professor
of Microbiology

DAB/aw

Development of a New Cloning Vector for *Bordetella brochiseptica* in the Form of a Hitherto Unidentified Plasmid

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Introduction

Bacterial plasmids are comparatively small, autonomously replicating molecules of DNA outside the main of the chromosome. They may range in size from a few thousand to a few hundred thousand base pairs, and may exist within the host cell in only one copy or in as many as several hundred copies per cell. Plasmids have been implicated in a variety of host cell functions, most notably antibiotic resistance, but their role ranges from virulence factors to metabolic functions (Hardy, 1981). Often, however, plasmids encode no known function other than self-replication and perhaps self-conjugation; such plasmids are called cryptic plasmids. Plasmids have proven remarkably useful tools to a wide range of biological fields, as the means are available wherewith one can isolate a gene and covalently insert that gene into a plasmid. It then becomes possible to study expression and function of that gene in more controlled settings. Cryptic plasmids, owing to their small size and thus ease of manipulation, are especially useful in such genetic manipulations.

Bordetella bronchiseptica is a small Gram-negative rod that is the etiological agent of kennel cough in dogs and atrophic rhinitis in pigs (Goodnow, 1980). It is related to the species B. pertussis, the causative agent of whooping cough in people, but is not normally a pathogen for humans. The virulence factors of this organism are chromosomally encoded (Charles et al, 1989; Glaser et al, 1988; Locht et al, 1986; Stibitz et al, 1988); however, plasmid-borne antibiotic resistance has been extensively documented (Graham et al, 1982; Terakado et al, 1974) and is believed to arise due to the formidable selection pressure resulting from the widespread use of antibiotics such as

sulfonamides in animal feed and drinking water (Levy et al, 1976). No other cellular function has been attributed to plasmids in B. bronchiseptica (Bemis, 1985).

However, conjugative R plasmids are not the only plasmids to be described in B. bronchiseptica. Locht et al in 1992 reported and characterized the existence of a small cryptic plasmid dubbed pBBR1 which was subsequently developed into a cloning vector. The construct developed by Locht, named pBBR122, is of limited utility, however, as the recombinant plasmid (with chloramphenicol and kanamycin resistance markers) contains a Hind III restriction site, and B. bronchiseptica makes use of a Hind III restriction system. Thus the recombinant version of this plasmid cannot be maintained in B. bronchiseptica, since the Hind III enzymes within the bacterium itself will cut the plasmid.

The overall goal of this study was contained within the context of studying fimbrial proteins in B. bronchiseptica -- specifically, to determine what role pili play in virulence of the organism. To this end the pili gene was isolated (in previous work in this lab) and its expression and structure were being studied using the commercially available plasmid pLAFR. This vector has proven unsatisfactory, however, and in this light a different cloning vector was desired. Thus the specific aim of this project was to find and develop a new plasmid into a cloning vector for Bordetella bronchiseptica. Progress toward this goal will be reported here along with a summary of difficulties that were ironed out during the process of the project and suggestions for future work.

This study contained five implicit goals: 1) to screen field isolates of B. bronchiseptica for the presence of unidentified DNA bands

on an agarose gel, 2) to prove that at least one such band is indeed a plasmid, 3) to find a single restriction site in this plasmid into which gene may be cloned without disrupting the regular functioning of the plasmid, 4) to successfully clone a gene or a marker into such a site, and 5) to stably maintain this recombinant plasmid in B. bronchiseptica.

Several difficulties were encountered in the course of the project whose resolution was necessary before the next step could be taken. As such, these problems occupied a good deal of time a prevented the eventual attainment of the goal. These difficulties will be presented here so that future workers in this area can avoid the same problems.

Materials and Methods

BACTERIAL STRAINS - The isolates of B. bronchiseptica used in this study were provided by Dr. David Bemis. They had been generally isolated from veterinary patients at the University of Tennessee Veterinary Hospital. They had all been suspended in cryopreservative media and stored at -80 Celcius. Plasmid pBBR122 was graciously provided by Camille Locht.

GROWTH AND PLASMID ISOLATION - B. bronchiseptica cultures were grown on Brucella agar or broth* for two days. Whole plasmid DNA was isolated from the cell and separated from chromosomal DNA via the alkaline-lysis ("mini-prep") method developed by Kado et al. The mini-preps were scaled up to accommodate larger initial volumes of broth. Broth cultures were grown under standard incubating conditions.

ELECTROPHORESIS - Electrophoresis was used to screen the various isolates of B. bronchiseptica. 1% agarose gels used in screening were prepared and run in TBE buffer. 1.5% gels were used whenever DNA was cut by endonucleases to prevent small fragments from running off the bottom of the gel. Separating gels were run at 20 volts for 20 hours. Electroelution gels were run at 20 volts for 24 hours, performed in TAE buffer, and polarity was reversed after 24 hours and increased to 60 volts for one minute to free the DNA from the membrane of the dialysis tubing. Mini-gels were of 0.8% agarose concentration and were run at 60 volts until the dye front was within a few centimeters of the bottom of the gel. All gels were stained in ethidium bromide solution at a concentration of 0.5ug/ml for 30 minutes and subsequently observed under transillumination.

RESTRICTION ANALYSIS - Commercially available endonucleases were purchased and used according to the manufacturer's instructions. DNA was allowed to digest for 2-3 hours before electrophoresis under the parameters described above.

* GIBCO, Grand Island, New York

Results

Screening of field isolates

A total of 21 field isolates of Bordetella bronchiseptica were screened for the presence of plasmids. These strains had been collected and stored at -80 Celcius in cryopreservative media by Dr. David Bemis over the past few years. The first twelve strains were selected to include various hosts (they ranged from dogs to koala bears to snow leopards); the remaining nine were selected completely at random. Each strain was streaked on Brucella agar, incubated for two days, and a fresh colony used to inoculate Brucella broth. The mini-prep method of DNA extraction was then used to collect plasmid DNA. Each DNA suspension was separated via electrophoresis on an agarose gel and photographed. The results of this initial screen are summarized in Table 1.

With the exception of one or possibly two strains, all isolates showed a conserved band around 23 kilobases. While this plasmid was not further characterized, it is speculated that this is the same plasmid described by Locht et al, which he named pBBR2 and showed would hybridize with the broad host range plasmid RSF1010 (Locht et al, 1992). Although antibiotic sensitivity in these isolates was not studied, it is believed that this ubiquitous plasmid is the same one that has been shown to be associated with sulfonamide resistance in previous reports (Lax et al, 1986 and Bemis, 1985).

Other than the conserved 23kb plasmid discussed above, only four strains were found to contain any other plasmid DNA, and all have been reported (though not characterized) previously. Strain 87 (which may or may not be identical to S87, from which Locht isolated pBBR1 and pBBR2)

showed four bands on the initial screen: one about 23kb, one at about 2.6kb (pBBR1), one about 2.0kb, and one around 1.5kb. Strain CD-C47 was identical to strain 87 (in a later screen; it was smeared in the first). Strain 19141 displayed two bands, both slightly above 23000 bases. And finally, strain D-1 had two clear bands, both around 23000 bases (though slightly lower than these in 19141), and possibly two more much smaller bands. (Figure 1)

It should be noted that there is often considerable variation in the pattern of bands which appears on a agarose gel; this is thought to be due to the sometime presence of different conformations of plasmids within the cell. Compare Figure 1, which shows the initial screen of strain 87, with Figure 2, which shows strains 87 and CD-C47 in a later, repeat screen. Two of these bands are pBBR1 and pBBR2, and another is a consistent band that was selected for this study. The rest are presumably supercoiled, covalently closed circular, and relaxed circular forms of the same plasmids. These, for unknown reasons, may or may not be present on any given gel.

Plasmids from a strain called S87 have been extensively studied by Locht et al, who isolated pBBR1 and pBBR2 from it. It is believed that S87 and strain 87 are identical; however, in our own labs we have consistently seen a third band in every plasmid DNA isolation performed on this strain. This band is at perhaps 1.5 kilobases and is often very bright. It was reasoned that if this was indeed a new plasmid, then it had not been described previously and would be small enough to permit easy manipulation and thus might prove a useful vector. Thus this band was selected for further investigation and was tentatively named pJRT.

Strain 87 is the same as S87 used by Locht et al.

It is likely that S87 used by Locht et al in his study is the same as our strain 87, since it was learned in communication with Dr. Locht that his source of S87 was a mutual friend of Dr. Locht and Dr. Bemis, to whom Dr. Bemis had given an isolate called "strain 87." To confirm this, however, DNA from the 2.6kb band from strain 87 was digested with Pvu II, which is known to attack pBBR1 only once (Locht et al, 1992). The position of the digested DNA on a gel was at 2.6kb, while an undigested lane of this same DNA ran above 2.6kb. This indicates that this plasmid can exist in different conformations and that its true size is 2.6kb. This data strongly suggests that strain 87 and S87 are one and the same.

pJRT is not a different conformation of pBBR1

To explore the possibility that pJRT is simply another conformation of the known pBBR1, total plasmid DNA was prepared and separated on a 1% agarose gel. The individual bands were then cut out and the DNA electroeluted from the gel fragments. A partial restriction map of pBBR1 was made by Locht et al and it is known from this map that endonuclease Pvu II cuts this plasmid at only one site. Thus a recombinant form of pBBR1 provided by Locht, known as pBBR122 (which has kanamycin and chloramphenicol resistance genes inserted into it), as well as the 1.5kb band (pJRT) were both digested separately with Pvu II. pBBR1, being hit only once by the endonuclease, would thus be linearized, and if pJRT were a conformational isomer of pBBR1, it would also be cut once and only once and thus become linear. Thus both bands would run the same distance on a gel. The results are shown in Figure

3. To interpret the results properly it is necessary to correct for the two additional genes in pBBR122; these genes are approximately 1.5kb apiece. Thus, since the band is at approximately 5.5-5.6 kilobases as compared to the markers, if about 3.0 is subtracted from that, one obtains its true size and position on the gel: 2.6kb. As can be seen, after endonuclease digestion, pJRT had not changed position, demonstrating that pBBR1 and pJRT are not conformational isomers of each other.

Restriction analysis of pJRT

A restriction map of pJRT was begun using about nine different endonucleases. However, in this gel, not enough DNA was used in the digestion to be visible after staining. Hence, only in the control lane (undigested) could a band be seen. In all of the other lanes no band was visible, either at the position of the undigested plasmid or anywhere else. At this point there was no longer enough time to repeat the experiment.

One inference can possibly be drawn from this. Since the undigested control land was visible (though barely), and the others were not, this might suggest that the plasmid was indeed cut by these enzymes in the other lanes. If that were the case, the bands would be at an even lower position than the control band, and bands that small are typically difficult to see. There is little explanation for why these bands should not be seen like the control band unless they had been cut by the enzymes. This is only speculation, however, and a repeat of this experiment would be needed to confirm this line of reasoning.

It is also worth noting that the enzyme Pvu II was used successfully in the experiment wherein it was shown that pJRT and pBBR1 are not conformational isomers. Either pJRT was not cut or it was indeed cut by the enzyme but linear forms of the plasmid do not behave any differently on an agarose gel than "usual" conformations of the plasmid.

Difficulties encountered

DNA isolation

One of the major difficulties encountered was in simply isolating enough DNA with which to do something useful. During the course of the project, increasing amounts of broth were used to culture the Bordetella. 3ml, 6ml, 20ml, 40ml, 60ml, and finally one liter of broth were grown as it became clear that it would take a lot of initial cell culture to harvest enough DNA to permit all the experiments that needed to be done with it. Some amount of DNA is lost in the initial gel and especially in the electroelution process. Afterwards, enough DNA must still be present and in sufficient concentration to allow a restriction digest or whatever experiment is to be done. This is especially true since, owing to its small size, the plasmid in question runs toward the bottom of the gel. Smaller pieces of DNA do not intercalate with as much ethidium bromide as larger pieces and hence will not fluoresce as brightly. A possible explanation for this difficulty in isolation quantity could lie in this very fact - i.e., that a greater concentration or total quantity of DNA is required because the plasmid in question is relatively small. Additionally, it is possible that the plasmid is present in a low copy number, which could account for the relatively small amounts of nucleic acid recovered from an entire liter of cells.

Volumes needed

In practical terms, the isolation of DNA from a liter's worth of broth yields about 900ul of whole plasmid DNA. 200ul of this will fill 10 lanes of a 15 lane gel for electroelution, and it is sufficiently

concentrated to yield enough isolated plasmid DNA to do some experiments but not others. The concentration of the subsequent electrelute will be dependent on the total volume in which it is resuspended. If it is suspended in about 20ul -- the amount needed if a 10-enzyme restriction digest is performed using 2ul per enzyme -- then the DNA is NOT sufficiently concentrated to be seen on the gel. This would, however, be sufficient to perform a one or two enzyme digest. To perform a restriction digest with more than one or two enzymes would require either a greater total volume of DNA or a greater concentration of the same volume. In future work on this plasmid, it is suggested that initial efforts be focused on isolation of DNA in a large enough volume to last through the course of several experiments, rather than the piecemeal approach that was inadvertently pursued here. It is suggested that several one liter mini-preps be performed and the subsequent whole-plasmid DNA separated by electroelution before other experiments are begun.

Other sources of difficulty

Other difficulties could be attributed to the inexperience of the experimenter, as some of the techniques, such as electroelution, were learned for the first time as the project was progressing. Others could be attributed to careless laboratory error, or trial-and-error guessing as to volumes and such (as witnessed by the initial culture volume problem). Some difficulties encountered were inexplicable -- such as the reason why, on occasion, a mini-prep of a strain known to have plasmids would turn up negative.

In sum, appreciable amounts of time were lost in simply fine-tuning the amounts of materials used in these procedures so that experiments could be performed and interpreted. It is hoped that anyone who works in this particular area in the future can build on what was learned here and avoid these same problems.

Outline for future study

This project began with the ambition of developing a new plasmid cloning vector for Bordetella bronchiseptica. It would be unsatisfactory to end without a suggestion for further study; an outline for what areas might be explored were there more time to do it. In this light, what follows here is a guideline for future research into the possibility of developing pJRT into a cloning vector.

pJRT may be a free minicircle

One of the earliest questions it would be necessary to consider is the possibility that pJRT is not a true plasmid. The possibility that it is merely a conformational isomer pBBR1 was explored and shown negative in this study; however, the possibility remains that pJRT is not a self-replicating plasmid but is instead a free minicircle of DNA arising from a transposable sequence. Transposons are known to be found in Bordetella (McLafferty et al, 1988; McPheat et al, 1989; Park et al, 1988), and further more, they have also been seen existing as free episomes (Scott et al, 1988). Perhaps the easiest way to investigate this possibility would be to attempt to hybridize DNA from pJRT with total DNA from B. bronchiseptica strain 87. If it is a transposon, pJRT DNA will anneal to that portion of pBBR1, pBBR2, or the chromosome which contains the transposon. If not, the Southern blot will be negative.

Finding a proper cloning site

Having demonstrated that pJRT is indeed a true plasmid, its utility as a cloning vector should be investigated. For the plasmid to be useful as a vector, two conditions must be met: 1) there must be at least one restriction enzyme that cuts the plasmid at one and only one site, and 2) this site must not be in the middle of a gene that is necessary for normal functioning of the plasmid.

Restriction mapping

The cheapest way to accomplish this task is to continue what was begun in this study: perform a restriction digest of the plasmid and map the results. Some enzymes (probably) will cut once, some more than once, some not at all. From this it should be possible to obtain a list of potential candidates for cloning sites. A potential problem foreseen here is that it is not known if single cuts will be detectable on an agarose gel. A single cut on the plasmid will simply linearize it, and it is not known if a linear conformation of this plasmid will behave differently on a gel from the one regularly seen. It is possible that two or more conformations of pJRT will run at exactly the same or close to the same distance on a gel.

Computer analysis

An easier but more expensive way to circumnavigate this potential problem would be simply to sequence or have sequenced this plasmid, and employ a computer analysis of the results. A computer can rapidly and easily scan the entire sequence for the restriction sites of a large number of different endonucleases. Thus with a computer-assisted

analysis it should be relatively simple to locate a single site, if there is one. In regard to the chances of finding such a site, it is instructive to use pBBR1 as a comparison. pBBR1 was sequenced and analyzed with a computer (Locht et al, 1992). In this 2687 nucleotide plasmid, there are 10 different single restriction sites, one of which appears twice, for a total of eleven sites. Assuming that restriction sites occur more or less at random, one can very roughly estimate that, since pJRT is about 40% smaller than pBBR1, it should have about 40% fewer sites, or about six restriction sites. Thus, a priori, it does seem that it is possible that pJRT would have a few single-hit restriction sites.

Development of the vector

Once one or several sites have been found that exist only once in the entire sequence, it is necessary to try to clone something into the site, something that will confer some means of selection. An antibiotic resistance gene is ideal, but something such as a beta-galatosidase gene would work as well. It then simply becomes a matter of systematically cloning the marker into each of the single restriction sites. Whether the sites contain sticky or blunt ends, or even if a linker is necessary to form a proper site on the marker gene, is largely irrelevant, although some difficulty may be had if a linker is necessary. It would be necessary to use a commercially available linker that does not contain a Hind III site within it, since Bordetella bronchiseptica contains a Hind III restriction-modification system. Once a recombinant plasmid is formed, it can be transformed into E.coli using the appropriate protocol to make the recipient cells competent. Then, after

plating them onto antibiotic selection agar, transformants with recombinant plasmids should be the only cells that grow. If the restriction site chosen is in the middle of a gene necessary for plasmid replication, the plasmid will not be maintained and antibiotic resistance will not be conferred on the host cell; nothing will grow on the agar. If this is the case, it will be necessary to repeat the entire procedure using a different site. This should be repeated until a site is found which does not interfere with replication or until all possible sites have been exhausted.

Potential problems with the vector

If indeed a site is found into which an antibiotic marker can be successfully cloned without disrupting plasmid function, it will be then be potentially useful as a cloning vector for Bordetella. It is not a foregone conclusion, however, that any given gene will express in this vector. The size of the insert will probably be a factor in determining utility of the vector, as the plasmid itself is relatively small. One must also be wary of restriction sites within a given insert that may not be compatible with restriction-modification systems within the host species. And, as always, there remain the problems of selection of recombinants and assaying for expression of the clone protein, both of which must be solved based on the individual situation.

Transfer of the plasmid, incompatibility, host range

Although, if all has gone well up to this point, a new cloning vector will indeed have been developed and thus the aim of this project achieved, further characterization of the plasmid will potentially add

to its utility. An obvious first step to this end would be to determine whether pJRT is conjugable or mobilizable. Simple protocols are available to test these possibilities. It is unlikely if not impossible that it will prove to be conjugable; the plasmid simply is not big enough to contain genes coding for conjugation. It is more likely that it will be mobilizable, as it is just large enough to hold genes for replication and mobilization (as compared to pBBR1 (Locht et al, 1992)). pBBR1, which does contain a mobilization gene, is conjugable when the RK2 transfer functions are provided in trans (Locht et al, 1992).

If it is found that pJRT does not contain genes for either conjugation or mobilization, then another important factor, host range, can be studied only be transformation. The range of other Gram-negative bacteria in which this plasmid can be maintained will be an important factor in its utility for cloning. Concomitant with studying host range is determination of the plasmid's incompatibility grouping. It can be reasoned a priori that, since it is stably maintained in strain 87, which contains two other plasmids -- pBBR2, which hybridizes with RSF1010 and is thus likely in the group IncQ, and pBBR1, which, inasmuch as is known about it, is in a new incompatibility group (Locht et al, 1992) -- it is not a member of IncQ, nor is it a member of whatever incompatibility grouping is given to pBBR1. Others, such as IncP and IncW, which have been found to replicate in Bordetella (Weiss et al, 1982), should be tested as well.

Genetic analysis

And finally, important information can be provided by computer analysis beyond the restriction analysis suggested earlier. There are

programs available that can compare any given sequence to a databank of known sequences and look for similarities. Such a program was used by Locht et al to find the two ORFs on pBBR1 that corresponded to mobilization and replication genes. Such an analysis would also be valuable here. It would help determine similarity and hence relationship to known replication proteins and mobilization proteins, establish ribosome binding sites, restrictions sites, conserved sequences, promoters, inverted repeats, and the like. This sort of information would be invaluable in determining replication and mobilization mechanisms, suggesting its evolutionary relationship to other plasmids or cellular genome sequences, confirming incompatibility grouping, and hinting at regulation of the plasmid's genes.

Conclusion

In brief, then, given sufficient time it would be possible to develop or at least attempt to develop pJRT into a usable cloning vector for Bordetella bronchiseptica and perhaps other Gram-negatives as well. It is regrettable that this study did not have sufficient time to accomplish this end, as such a vector could have important applications in the study of gene expression in Bordetella.

Acknowledgements

The author wishes to extend a very sincere thank-you to Dr. David Bemis for advice, guidance, and materials, and to Eugene Burns for technical assistance, instruction in lab techniques as well as infinite patience while the author tried to learn them.

Table 1 - Bacterial Strains Used and Plasmids Found

B. bronchiseptica strain	Description	Geographic source	Plasmids (size in kb)
87	dog isolate	NY	4 (23, 2.6, 2.1, 1.5)
CD-C47	dog	NY	2 (23, 1.5)
19141	dog	NY	2 (above 23, 23)
D-1	dog	IA	2 (23, 20)
UT dog	dog	TN	1 (23)
Congdon	dog	FL	1 (23)
Phase I Tuskagee	pig isolate	AL	1 (23)
DeJong	pig	Netherlands	1 (23)
633	pig	WI	indeterminate
Ct. Madrid	cat isolate	IA	1 (23)
1679a	snow leopard	TN	1 (23)
UQU193	koala bear	Australia	1 (23)
M. Bord 896	cat	W. Germany	1 (23)
JS34682	dog	KS	1 (23)
M. Bord 891	dog	W. Germany	indeterminate
495 NADL	pig	IA	indeterminate
644	pig	WI	1 (23)
6019	pig	Japan	1 (23)
315	pig	Japan	1 (23)
344	pig	Japan	1 (23)
VP1-EQ-1	horse	VA	? (23, possibly 7)
87-0600-1	rabbit	?	1 (23)

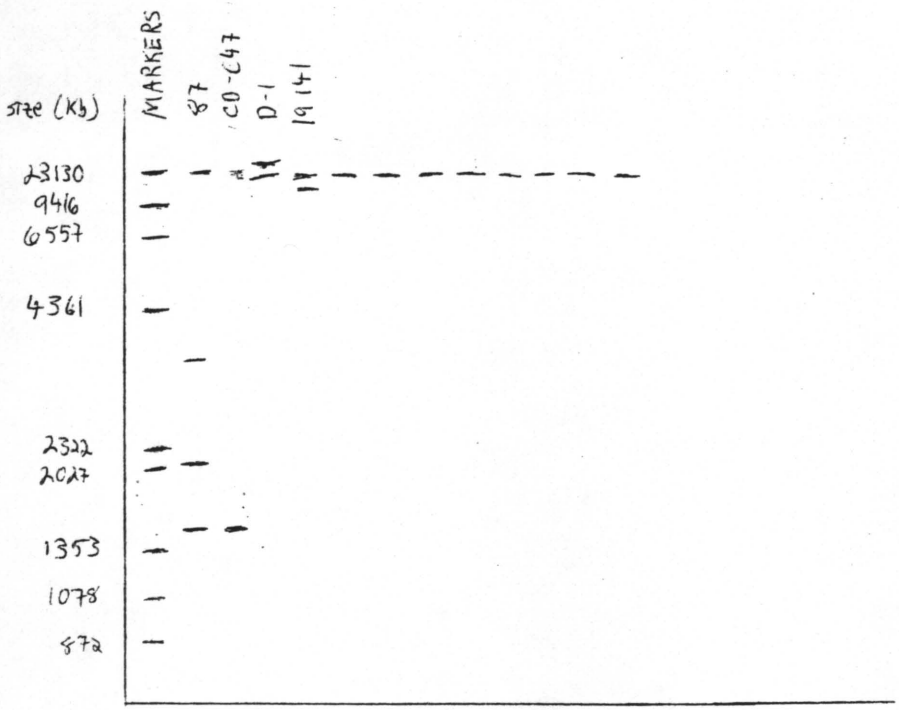

FIGURE 1

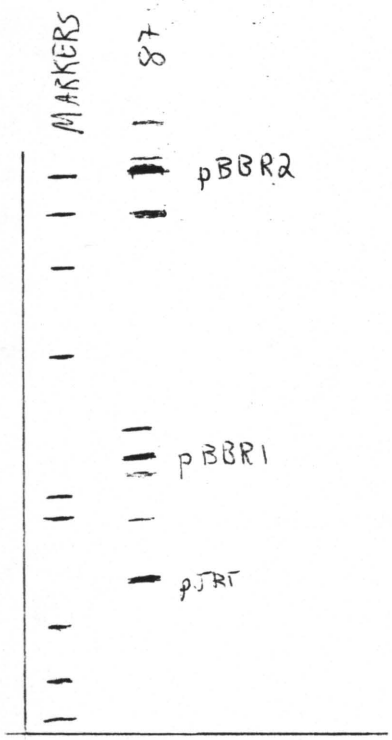

FIGURE 2

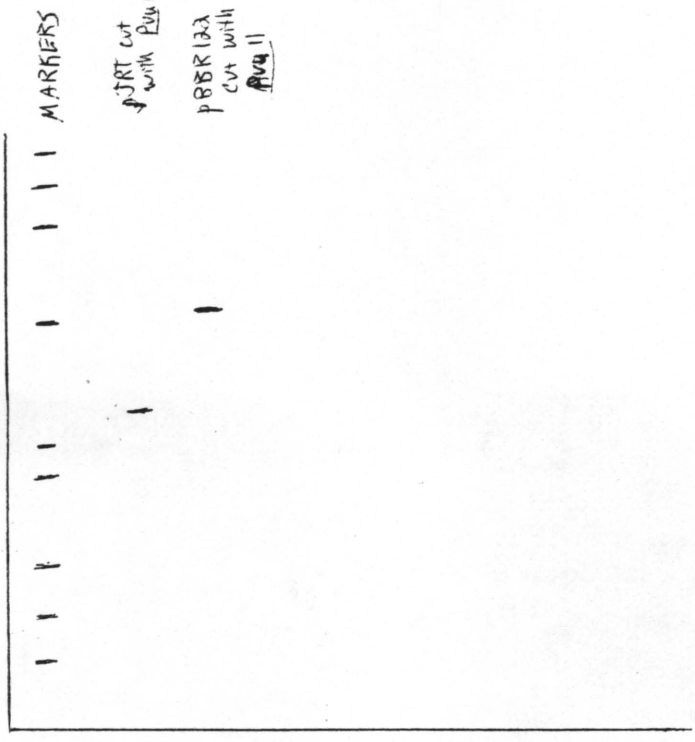

FIGURE 3

References

Bemis, David A. 1985. Mobilization of Non-Conjugative R Plasmids in Bordetella bronchiseptica and Reexamination of the Relationship Between Plasmids and Antimicrobial Resistance in this Species. Unpublished.

Charles, I.g., Dougan, G., Pickard, D., Chatfield, S., Smith, M., Novotny, P., Morrissey, P., and Fairweather, N.F. 1989. Molecular cloning and characterization of protective outer membrane protein p.69 from Bordetella pertussis. Proc Natl Acad Sci USA 86:3554-3558.

Hardy, Kimber. Bacterial Plasmids. 2nd ed. American Society for Microbiology (Hong Kong: 1981).

Hedges, R.W., Jacob, A.E., and Smith , T.J. 1974. Properties of a R Factor from Bordetella bronchiseptica. J Gen Microbiol. 84:199-204.

Glaser, P., Ladant, D., Sezer, O., Pichot, F., Ullman, A., and Danchin, A. 1988. The calmodulin-sensitive adenylate cyclase of Bordetella pertussis: cloning and expression in Eschericia coli. Mol Microbio 2:19-30.

Goodnow, R.A. 1980. Biology of Bordetella bronchiseptica. Microbiol Reviews. 44:722-738.

Graham, A.C., and Abruzzo, G K. 1982. Occurrence and characterization of plasmids in field isolates of Bordetella bronchiseptica. Am J Vet Res 43:1852-1855

Kado, C.I., and Liu, S.T. 1981. Rapid procedure for detection and isolation of large and small plasmids. J Bacteriol 145: 1365-1373.

Lax, A.J., and Walker, C.A. 1986. Plasmids related to RSF1010 from Bordetella bronchiseptica. Plasmid 15: 210-216.

Levy, S.B., Fitzgerald, G.B., and Macone, A.B. 1976. Spread of antibiotic resistant plasmids from chicken to chicken and from chicken to man. Nature 260: 40-42.

Locht, C., Barstad, P.A., Coligan, J.E., Mayer, L., Munoz, J.J., Smith, S.G., and Keith, J.M. 1986. Molecular cloning of pertussis toxin genes. Nucl Acids Res 14: 3251-3261.

Locht, C., and Antoine, R. 1992. Isolation and molecular characterization of a novel broad-host-range plasmid from Bordetella bronchiseptica with sequence similarities to plasmids from Gram-positive organisms. Mol Microbiol 6:1785-1799.

McLafferty, M.A., Harcus, D.R., and Hewlett, E.L. 1988 Nucleotide sequence and characterization of a repetitive DNA element from the genome of Bordetella pertussis with characteristics of an insertion sequence. J Gen Microbiol 134:2297-2306.

McPheat, W.L., Hanson, J.H., Livey, I., and Robertson, J.S. 1989. Analysis of separate isolates of Bordetella pertussis repeated DNA sequences. J Gen Microbiol 135:1515-1520.

Park, I., Saurin, W., and Ullmann, A. 1988. A highly conserved 530 base-pair repeated DNA sequence specific for Bordetella pertussis. FEMS Microbiol Letts 52:19-24.

Scott, J.R., Kirchman, P.A., and Caparon, M.G. 1988. An intermediate in transposition of the conjugative transposon Tn916. Proc Natl Acad Sci USA 85:4809-4813.

Stibitz, S., Weiss, A.A., and Falkow, S. 1988. Genetic analysis of a region in the Bordetella pertussis chromosome encoding filamentous hemagglutinin and the pleiotropic regulatory locus vir. J Bacteriol 170: 2904-2913.

Terakado, N., Azechi, H., Ninomiya, K., et al. 1974. The incidence of R factors in Bordetella bronchiseptica isolated in pigs. Japan J Microbiol 18:45-48.

Weiss, A.A., and Falkow, S. 1982. Plasmid transfer to Bordetella pertussis: conjugation and transformation. J Bacteriol 152:549-552.