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The Use of Molecular Techniques to Study Microbial Determinants of Pathogenicity

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The use of molecular techniques to study microbial determinants of pathogenicity

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A variety of new methods in DNA biochemistry, molecular biology and genetics have become available for the analysis of microbial determinants of pathogenicity. It has never been easier to focus upon specific genetic determinants and to manipulate them directly to meet experimental goals. Although the principles of genetic manipulation have been used with considerable success in enteric bacteria, it is not always a straightforward matter with other microorganisms. We were unable to clone *Bordetella pertussis* determinants of pathogenicity directly in *Escherichia coli* K12 by selecting for their protein products. It was possible, however, to develop a genetic transfer system and methods for the identification of specific *Bordetella* virulence genes. These studies not only provided the basis for the eventual successful genetic cloning of *Bordetella pertussis* genes but also provided an example showing that the molecular cloning of virulence genes is not always an easy task, nor even necessarily the best initial approach to take.

Introduction

Over the past 7 years the application of the newer methods of gene cloning and DNA biochemistry have provided those interested in infectious disease agents with powerful new tools to study the genetic organization of microorganisms. It has never been easier to manipulate genetic sequences of interest and to investigate the potential role of a specific gene product in the pathogenesis of infection. In theory, one attempts to perform a kind of molecular Koch's postulates in which an investigator compares two homogenic microorganisms differing from each other in only a single determinant of pathogenicity. If it is observed that the loss of a single gene product materially affects pathogenicity, then it is useful to reintroduce the appropriate genetic sequences and observe the restoration of full virulence.

Manipulation of *E. coli* K12 by molecular cloning has become commonplace and if one is dealing with recognized pathogenic determinants of *E. coli* it is possible to characterize gene segments and gene products quite precisely. Any number of cloning strategies can be used. For example, in our experience it has been most advantageous to use cosmid (Collins 1979) cloning vectors, permitting the preparation of a 'genetic library' of the total genetic complement of a microorganism within a few hundred *E. coli* K12 colonies. Once a DNA fragment carrying genetic segments of interest is identified, sequential subcloning by the use of appropriate restriction endonucleases is employed to yield the smallest DNA unit encoding the desired phenotype. Specific DNA-directed protein synthesis by the gene(s) encoded by the cloned genetic segment can usually be achieved by the use of *in vitro* translation systems such as 'minicells' (Frazer & Curtiss 1975). As a final step it is possible to determine the nucleotide sequence for a gene of interest by using well described methods (Maxam & Gilbert 1977; Sanger *et al.* 1977) and deduce the complete primary structure of the gene product.

The foregoing steps are based on a variety of assumptions that are achieved under the best

of circumstances but that under many circumstances are not so easily attainable. For example, the identification of a gene of interest within a given cosmid-cloned DNA insert is dependent upon the faithful transcription and translation of the DNA as well as its expression in a host cell, typically *E. coli* K12. This does not pose a particular problem if one is dealing with the chromosomal haemolysin of pathogenic *E. coli* strains (Welch *et al.* 1981). Moreover, the characterization of *E. coli* enterotoxins and adherence factors has been rather straightforward (Elwell & Shipley 1980). It is much more of a problem, however, if one is dealing with the IgA1 protease of the gonococcus (Koomey *et al.* 1982) and not yet attainable if one is dealing with the *Pseudomonas aeruginosa* exotoxin A gene.

Methods are available to achieve the expression of 'foreign' DNA within *E. coli* K12. When one is dealing with determinants of pathogenicity, expression is not enough. *E. coli* K12 cells (and other common host bacteria used in molecular cloning) are not inherently pathogenic, so it is necessary to devise appropriate genetic transfer systems between the non-pathogenic host carrying the cloned determinant and other appropriate microorganisms. Among the enteric bacteria the classical plasmid and bacteriophage-mediated gene transfer systems will usually suffice. But what about pathogenic microbial species that are fastidious and for which a genetic transfer system has not been described? Certainly it may be useful to isolate one or several genes of interest in *E. coli* K12 by molecular cloning. If one cannot reintroduce genetic sequences into the host of origin, however, it is unlikely that the full utility of the molecular approach can be appreciated.

In the following sections we shall describe a genetic and molecular approach that was employed to examine the genetic determinants of *Bordetella pertussis* pathogenicity. The data we shall present do not include the actual molecular cloning of any gene simply because all of our initial attempts to identify *Bordetella* pathogenic traits within *E. coli* K12 were fruitless. Rather we should like to present our strategy for developing a genetic transfer system within *Bordetella* and the means we employed to identify specific genetic sequences encoding several determinants of pathogenicity.

The scope of the problem

Bordetella pertussis, the aetiological agent of whooping cough, is a fastidious microorganism that synthesizes a fascinating array of pathogenic determinants. These include an extracellular adenylate cyclase (Adc) (Confer & Eaton 1982; Hewlett & Wolff 1976; Wolff *et al.* 1980), dermonecrotic toxin (Dnt) (Cowell *et al.* 1979), a filamentous haemagglutinin (Fha) (Cowell *et al.* 1982), a haemolysin (Hly) (Peppler 1982), and pertussis toxin (Ptx) (Katada & Ui 1982). Pertussis toxin has also been called lymphocytosis-promoting factor, islet-activating protein, histamine-sensitizing factor and pertussigen; it is believed to be responsible for many of the systemic effects accompanying *B. pertussis* infection.

The current vaccine for pertussis affords significant protection but has been shown to be the cause of rare but serious side effects (Miller *et al.* 1981; Cody *et al.* 1981). The vaccine consists of whole killed bacterial cells in which some of the toxic factors of *B. pertussis* are still active (Hewlett & Wolff 1976; Endoh & Nakase 1982). Recent efforts to develop a safe, effective vaccine have been frustrated by a lack of information on the pathogenesis of the natural infection. Indeed, the identity of the antigens necessary and sufficient to confer immunity has not been established.

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When we were unable to clone any of the determinants of *B. pertussis* directly in *E. coli* K12, it seemed worthwhile to study *B. pertussis* pathogenicity by trying to generate a series of mutants, each deficient in a single factor believed to be important for virulence. Studies with mutations in a single gene would be useful for determining the contribution of a particular microbial determinant in the disease process. Moreover these mutants would also be useful in studies designed to determine how an individual immunized with a single bacterial product would respond to an infection.

There was, however, a complicating factor. *B. pertussis* undergoes an interesting form of phase variation in which virulent bacteria lose *en bloc* their capacity to synthesize Adc, Ptx, Dnt, Fha, Hly and other pathogenic traits (Peppler 1982; Dobrogosz *et al.* 1979). This phase change occurs at a frequency of about 2.8×10^{-6} per cell per generation. The genetic mechanism underlying this phase shift is not understood. In any event, the high frequency with which this phase shift occurs has made it difficult to determine the genetic basis of *B. pertussis* pathogenicity. Consequently our first step was to develop a reliable means of genetic analysis and gene transfer within *B. pertussis*.

Transposon insertion and donor formation in *B. pertussis*

Because there has been little published information dealing with the genetic organization of *B. pertussis*, as a first step we (Weiss & Falkow 1982) examined the capacity of this microorganism to accept and maintain several well known plasmid species. It was possible to demonstrate that *B. pertussis* permitted the replication of several plasmids found in other Gram-negative bacteria, including RP4, a conjugative plasmid of the P incompatibility group known to be transferable to a wide variety of Gram-negative bacterial species (Falkow 1975). In contrast, the well known, smaller, non-conjugative plasmid, ColE1, could not be detectably maintained extrachromosomally within *Bordetella*. On this basis we utilized a chimeric plasmid containing the ColE1 replication genes and P-plasmid conjugation genetic sequences (Weiss & Falkow 1983) to introduce the antibiotic resistance transposons, Tn*7* and Tn*501*, into the *B. pertussis* chromosome (Weiss & Falkow 1983). This chimeric plasmid was transferable from an *E. coli* donor strain to *B. pertussis*. However, the plasmid, once transferred into *B. pertussis*, would not be expected to be stably replicated although any transposable element carried by this plasmid would be expected to insert into *B. pertussis* DNA at a measurable frequency.

With this approach the insertion of Tn*7* into the *B. pertussis* chromosome occurred only at a frequency of 10^{-9}. Tn*501*, which confers resistance to mercuric chloride, inserted into the *B. pertussis* chromosome about 100 times more frequently but was limited, in almost all cases, to a single genetic locus (Weiss & Falkow 1983). To our surprise, however, in some cases *B. pertussis* strains inserted the entire chimeric plasmid into their chromosome. These strains could act as genetic donors and bring about the transfer of chromosomal genes. Thus we were able to establish a reliable means to bring about gene transfer among strains of *B. pertussis*.

Recently we constructed an appropriate chimeric plasmid to introduce the transposon, Tn*5*, which confers kanamycin resistance, into the *B. pertussis* chromosome. The Tn*5*-containing 'suicide' plasmid was efficiently mobilized from *E. coli* donors into *B pertussis;* kanamycin-resistant *B. pertussis* cells that had inserted Tn*5* into their chromosome were isolated with ease (figure 1). It soon became apparent that the Tn*5* element could insert into many genetic loci, including genes encoding determinants of pathogenicity.

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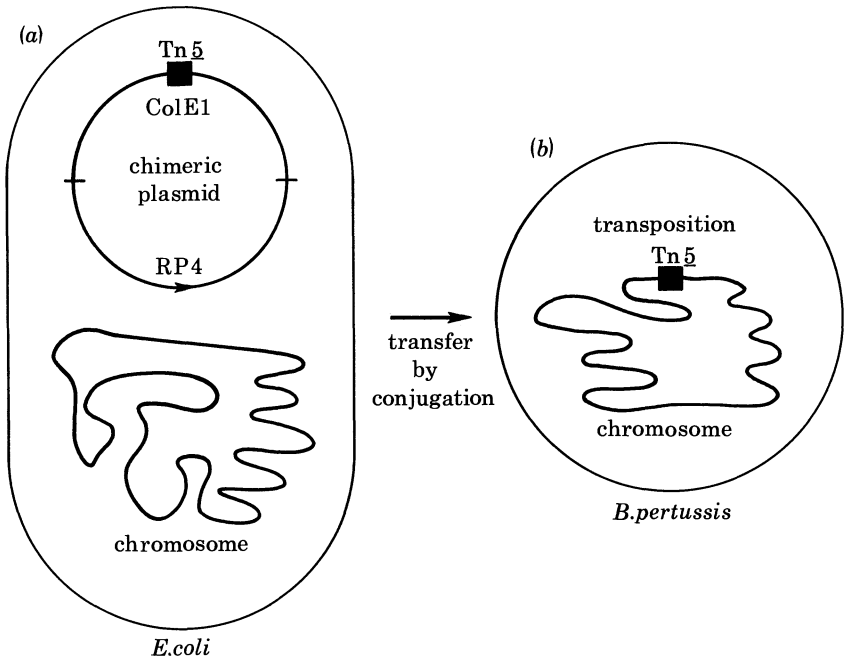

FIGURE 1. Method for introducing Tn*5* into the *B. pertussis* chromosome. The chimeric plasmid, pUW964, containing a plasmid ColE1 origin of replication and plasmid RP4 conjugate genes, as well as tranposon Tn*5*, can be maintained in *E. coli* as shown in (*a*). When this plasmid is introduced into *B. pertussis* by conjugation the plasmid is lost from *Bordetella* cells (*b*), but cells in which Tn*5* has transposed into the bacterial chromosome can be selected by plating onto kanamycin-containing media. Such kanamycin-resistant cells contain no extrachromosomal DNA or other pUW964 DNA sequences. Insertions into coding regions result in mutations for that gene, and in addition the site of the insertion is genetically marked with kanamycin resistance.

ANALYSIS OF Tn*5*-INDUCED MUTATIONS AFFECTING VIRULENCE GENES OF *B. PERTUSSIS*

Several of the insertion mutants were non-haemolytic (Hly^-) and differed from avirulent phase mutants. Phase mutants are phenotypically negative for most of the virulence determinants of *B. pertussis*, including Ptx, Fha, Adc and Dnt. The Hly^- Tn*5* insertion mutants continued to express Ptx, Dnt and Fha; variable results were seen when these mutants were assayed for Adc (table 1).

Adenylate cyclase activity of *B. pertussis* is characterized by the unusual property of being stimulated by the eukaryotic protein calmodulin (Wolff *et al.* 1980). Some of the Tn*5*-induced Hly^- mutants expressed significant levels of this specific adenylate cyclase activity whereas other Hly^- mutants were devoid of enzymic activity. Tn*5* insertion mutants unchanged in their Hly phenotype exhibited wild-type levels of adenylate cyclase, demonstrating that the changes we observed were not due to the presence of any Tn*5* product. The loss of adenylate cyclase activity in some non-haemolytic mutants could be due to the polar effects accompanying Tn*5* insertion (Berg *et al.* 1980); that is, a single insertion causes the loss of all gene activity transcribed distally from the site of insertion if the genes are encoded in a single transcriptional unit.

Further characterization of Tn*5* insertion mutants of *B. pertussis* disclosed derivatives that had lost the Fha or Ptx phenotypes. In each of these cases all other virulence determinants were retained (table 1). The availability of these mutants permitted us to ask whether the gene

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segments responsible for *Bordetella* pathogenicity were closely linked or resided within different regions of the *B. pertussis* chromosome. The location of the Tn*5* insertion mutations was determined by using a ^{32}P-labelled DNA probe specific for Tn*5* transposon sequences hybridized against *Bordetella* chromosomal DNA enzymically cleaved and separated by electrophoresis (Southern 1975). These data showed that Ptx$^-$ mutants all had a single Tn*5* insertion within the same chromosomal DNA fragment. Similarly all Fha derivatives possessed a single Tn*5* insertion within a single fragment of DNA, although this DNA fragment was

TABLE 1. Tn*5* INSERTION DERIVATIVES OF *B. PERTUSSIS*

(Alleles defined by Tn*5* are: Wlt, wild type; Vir, avirulent phase; Hly, haemolysin; Fha, filamentous haemagglutinin; Ptx, pertussis toxin; Dnt, dermonecrotic toxin; Adc, adenylate cyclase. The strain designated BP358 is a derivative which has received Tn*5* at an unknown genetic locus that does not affect its virulence properties. Symbols: + indicates that the activity or property was present; − indicates that the activity or property was not detected.)

		phenotypic characteristics				
strain	phenotype	Hly	Fha	Ptx	Dnt	Adc
BP338	Wlt	+	+	+	+	+
BP347	Vir-1	−	−	−	−	−
BP348	Hly-1	−	+	+	+	−
BP349	Hly-2	−	+	+	+	+
BP353	Fha-1	+	−	+	+	+
BP356	Ptx-1	+	+	−	+	+
BP358	zzz-1	+	+	+	+	+

electrophoretically distinct from that identified for the Ptx$^-$ insertion mutants. That the insertion within the same chromosomal DNA fragment. Similarly all Fha$^-$ derivatives possessed figure 2 for Fha. In this figure it may be seen that a 200 kDa protein specifically associated with the Fha phenotype is missing from cells that have suffered a particular Tn*5* insertion. The two Hly$^-$ mutants that have so far been examined show single Tn*5* insertions in a specific *Bordetella* DNA fragment that differs from that seen for Fha$^-$ and Ptx$^-$ mutants. These data strongly suggest that the genes governing the Ptx, Fha and Hly phenotypes occupy significantly different chromosomal locations and that, at least for Fha, the insertion has occurred directly within the structural gene governing the biosynthesis of the product.

The use of transposons as mutagens in *B. pertussis* has provided a series of homogenic strains deficient in a single virulence determinant. The different classes of mutants we have isolated provide preliminary evidence for the genetic organization of the virulence genes of *B. pertussis*. The series of mutants that have lost only a single trait demonstrate that haemolysin, Fha and pertussis toxin can be mutated independently. These mutants still retain the capacity to produce dermonecrotic toxin, indicating that this gene is also independent of the others. It is also of parenthetical interest that we have recently demonstrated that the loss of all virulence-associated traits can be brought about by a single Tn*5* insertion.

We plan to use the Tn*5*-encoded kanamycin resistance associated with each of these virulence factors as a genetic marker to clone the DNA fragment containing the gene of interest. The surrounding *B. pertussis* genetic sequences can then be used, in turn, as genetic probes to isolate the wild-type virulence genes. Although our preliminary experience indicated that *B. pertussis* DNA was not readily transcribed or translated within *E. coli*, armed with the knowledge that we have isolated the appropriate *Bordetella* sequences within *E. coli* we can use specially

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constructed 'expression vectors' and try to answer the questions concerning the molecular organization and molecular nature of the *B. pertussis* virulence determinants.

Whether we shall be successful in achieving the cloning and expression of *B. pertussis* virulence genes remains to be seen. None the less, the exploitation of the Tn*5* mutants in animal infection studies should provide useful information concerning the precise contribution of each individual virulence factor to the pathogenesis of *B. pertussis* infection. As a consequence of such studies

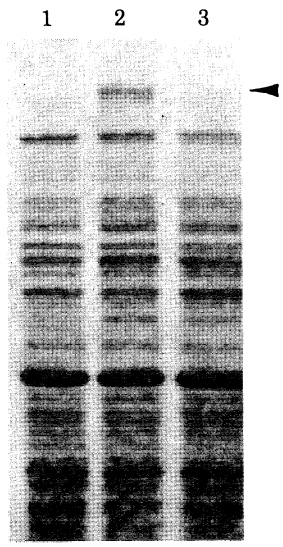

FIGURE 2. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of *B. pertussis*. Total cellular protein was isolated from: avirulent phase strain, BP326 (lane 1), virulent phase strain BP338 (lane 2), and Fha$^-$ mutant strain BP353 (lane 3). Electrophoresis and staining with Coomassie blue was performed by the method of Laemmli (1970). The uppermost band in the virulent strain is the Fha protein complex, which is missing in both the spontaneous avirulent phase mutant and the Tn*5*-induced Fha$^-$ mutant, BP353.

it may be possible to define which of the determinants of *B. pertussis* are essential for generating host immunity to disease and thus would be candidates to be included in a new, safer generation of pertussis vaccines.

We hope our experience may prove of illustrative value in other microbial systems. Molecular cloning has provided and surely will continue to provide important information about the genetic factors of infectious disease agents. Yet we hope that our experience will make the point that the use of molecular cloning for the study of microbial pathogenicity is not always an easy task, nor even necessarily the best initial approach to take.

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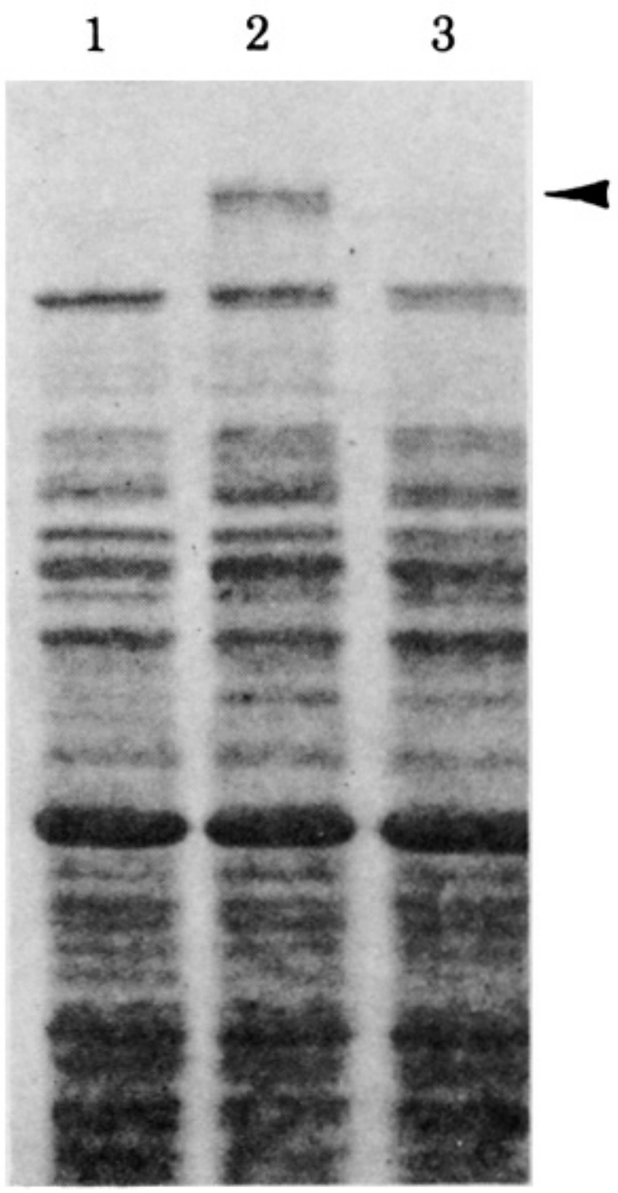

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