## Description

The present invention relates to a cloned and sequenced Eco RI fragment of Bordetella pertussis chromosomal DNA containing the genes which code for the five subunits of the pertussis toxin, useful for the preparation of the pertussis toxin or of one or more subunits of the pertussis toxin.

The present invention also relates to a hybrid plasmid containing the cloned and sequenced DNA fragment or further fragments thereof and to a micro-organism transformed by the hybrid plasmid and capable of expressing the cloned DNA fragment or further fragments thereof by synthesis of the pertussis toxin or one or more subunits of the pertussis toxin.

The invention also concerns a method for the preparation of the pertussis toxin or one or more subunits of the pertussis toxin which includes the growth of the micro-organism transformed by the hybrid plasmid in a suitable culture medium.

The pertussis toxin or one or more subunits of the pertussis toxin thus obtained is useful for the preparation of vaccines and diagnostic kits.

Pertussis is an infection of the respiratory tract caused by Bordetella pertussis (B. pertussis), a Gram-negative coccobacillus which is transmitted directly through the air during a catarrhal or convulsive period from the invalid to a susceptible healthy individual.

Pertussis may cause respiratory complications, nerve damage and high mortality, particularly in children in low socio-economic groups and in new born babies without maternal, anti-pertussis antibodies. The clinical course of pertussis includes four phases: incubation, cattarhal phase, paroxysmic phase, and a convalescent phase.

During the first two phases there are symptoms comparable to those of a common cold and the B. pertussis may be isolated easily from the patients.

During the paroxysmic phase, characterised by the symptoms of pertussis itself, the bacterium is isolated only in 50% of cases.

During the convalescent phase it is no longer possible to isolate B. pertussis from the nasopharynx although the patients still have symptoms of pertussis.

It is clear from this that the more violent clinical indications of the illness occur after the disappearance of the bacteria and from this it may be inferred that pertussis is not due to invasion of the respiratory tract by the bacteria but to a toxic state induced by the bacteria but which remains even after their disappearance.

The change of B. pertussis from phase I (virulent) to phase III (non-virulent) is accompanied by a loss of capacity to synthesise certain substances such as: the pertussis toxin (PT), haemolysin (hly), adenylcyclase (Adc) and the dermonecrotic toxin (Dmt).

Tests carried out by Munoz. J.J. et al. (1981) (Inf. Immun. 32. 243) have shown that a vaccine constituted by the pertussis toxin alone, suitably detoxified with glutaraldehyde, is capable of protecting mice from death due to the intracerebral administration of bacteria in phase I.

Recent studies (Weiss, A.A. et al. (1983) Inf. Immun. 42, 33; Weiss, A.A. et al (1984) J. Inf. Dis. 150, 219) have shown that not all these five substances contribute with equal effect to the virulence of B. pertussis and Weiss has succeeded in isolating the mutants which have lost selectively only one of the factors of the virulence by the insertion of a transposable element, a transposon (TN5), into the genome of B. pertussis. From tests carried out in animals, it was found that only the mutants which had lost their capacity to synthesise PT or Adc had, at the same time, lost their virulence.

Hence the pertussis toxin (PT) is the major factor in the virulence of Bordetella pertussis.

The pertussis toxin, a protein with a molecular weight of about 100,000 daltons, is produced and released into the extra cellular environment by Bordetella pertussis during phase I.

PT has an enzymatic activity and deactivates ADP-ribosilandol, a GTP-dependent protein which is involved in the deactivation of cellular adenylcyclase.

Like other toxins, the pertussis toxin is also constituted by two different fragments: A and B.

The A fragment, which is toxic, comprises a single polypeptide S1 (subunit S1) having a molecular weight of about 28,000 daltons, which can bind an ADP-ribose group to a GI protein involved in the transmission of signals from the outside to the inside of cells.

The B fragment comprises five polypeptides S2, S3, S4 and S5 (subunits S2, S3, S4, S5) with molecular weights of 23,000, 22,000, 12,000 and 9,000 daltons respectively, disposed as two dimers S2+S4 and S3+S4 and a monomer S5.

The B fragment binds to membrane receptors of eucaryotic cells facilitating entry of S1 into the cells.

At present a pertussis vaccine is used which, although giving permanent immunity, has numerous disadvantages.

The vaccine is in fact constituted by virulent bacteria (phase I) treated at 56°C for 30 minutes to remove a toxin which is heat-labile (dermonecrotic toxin) and killed by merthiolate.

Since the bacteria are not subjected to any detoxification treatment, any toxic substance which withstands 56°C for 30 minutes is included in the vaccine.

The presence of such toxic substances, particularly from the PT, causes side effects which vary from simple flushing to permanent neurological damage and/or death.

All this has meant that over the last ten years the use of the vaccine has been reduced drastically with a consequent re-explosion of cases of pertussis.

Recently a vaccine has been prepared which is constituted essentially by fibrous haemagglutinin (FHA) and pertussis toxin detoxified with formaldehyde (Sato Y., et al: Lancet Jan. 21. 122 (1984)).

However this vaccine has disadvantages such as: presence of side effects, even though less than those of the conventional vaccine; obtaining of a product which is too crude to be used as such and extreme variability of the product from preparation to preparation.

There is thus a need to provide an effective vaccine which can be produced on a large scale and which does not have the disadvantages given above.

Thus, for example, recent developments in the biochemical field and in the field of genetic engineering have made it possible to prepare synthetic vaccines and micro-organisms capable of producing proteins useful for the preparation of vaccines with high yields.

In every case a key element for the preparation of the vaccines is a knowledge of the amino acid sequence of the protein and the nucleotide sequence of the gene and/or genes which code for the protein.

Once the gene which codes for a certain protein has been cloned and its nucleotide and amino acid sequences have been determined, the production of these on a large scale and the construction of synthetic vaccines is possible with current techniques.

At present nothing is known of the nature, structure and expression of the gene and/or genes of the pertussis toxin and no data other than the amino acid composition of the individual subunits of the pertussis toxin is available.

Accordingly, by the present invention there has been determined the aminoterminal amino acid sequence of the subunits S1, S2, S3 and S4 of the pertussis toxin and an EcoRI -fragment of Bordetella pertussus chromosomal DNA has been cloned and sequenced, the fragment having 4696 base pairs and containing the genes which code for the five subunits of the pertussis toxin, useful for the preparation of the pertussis toxin or of one or more subunits of the pertussis toxin. Thus a subject of the present invention is a cloned and sequenced 4696-base-pair EcoRI fragment of Bordetella pertussis chromosomal DNA containing the genes which code for the five subunits of the pertussis toxin or fragments thereof, useful for the production of the pertussis toxin or of one or more subunits of the pertussis toxin.

Another subject of the invention is a hybrid plasmid containing the cloned and sequenced DNA fragment or further fragments thereof.

A further subject of the present invention is a micro-organism transformed by the hybrid plasmid and capable of expressing the cloned DNA fragment or its further fragments by synthesis of the pertussis toxin or of one or more subunits of the pertussis toxin.

Another subject of the present invention is a method for the preparation of the pertussis toxin or of one or more subunits of the pertussis toxin by growth of the transformed micro-organism.

A further subject of the present invention is the use of the pertussus toxin or of one or more subunits of the pertussis toxin for the preparation of anti-pertussis vaccines and diagnostic kits.

Yet another subject of the invention is the protein of the pertussis toxin in which the subunits S1, S2, S3, S4 have the amino acid sequences given in Figures 2 and 3. Further subjects of the present invention will become apparent from the description and the experimental examples which follow.

## BRIEF DESCRIPTION OF THE TERMS USED IN THE DESCRIPTION

Genetic code: by this term is meant the relationship existing between the nucleotide sequence in DNA and the amino acid sequence in a protein.

An important characteristic of the genetic code is the fact that the synthesis of each amino acid is specified by a sequence of three nucleotides in the DNA, also called a triplet or codon.

The genetic code is universal, that is, a particular triplet codes the same amino acid in all living beings.

Reading phase or frame: by this term is meant a group of triplets used by a cell to decode the genetic message.

Cloning vectors: these are molecules of DNA which contain all the genetic information to enable them to replicate when transferred into a host micro-organism.

Examples of cloning vectors commonly used in genetic engineering are the plasmids and the DNA of several bacteriophages.

The plasmid DNA, which is circular, may be cut by suitable techniques and a heterologous DNA fragment may be inserted and the ring reclosed to form a larger molecule containing the heterologous DNA, the so-called molecule of recombinant DNA or hybrid plasmid.

The DNA of the bacteriophage may contain a segment of heterologous DNA inserted instead of several non-essential genes. Both these vectors are used for the insertion of heterologous DNA fragments and for the subsequent transformation of micro-organisms, also called host cells.

Restriction enzymes: these are hydrolytic enzymes capable of cutting a DNA molecule at specific sites, so-called recognition sites for the restriction enzymes.

Transposons: these are segments of DNA which may transpose and insert themselves at different points in the genome and give rise to the process known as transposition.

Promotor: a specific region of the DNA molecule in which the RNA polymerase starts transcription.

The promoter includes a recognition site and a binding site for the enzyme.

Termination Region: a specific region of the DNA molecule in which transcription ends.

Translation: this is the passage of genetic information from the mRNA to the protein according to the rules of the genetic code.

Expression: this term means the mechanism by means of which an organism can synthesise a protein coded by a specific gene.

In this case one says that the gene is expressed by the micro-organism.

In general, a method for obtaining a heterologous protein by recombinant DNA techniques requires the cloning of the gene which codes for the protein, where by cloning is meant the sequencing, isolation and purification of the gene and/or genes which code for the protein. Once cloned, the gene may be inserted in an expression vector and the molecule of recombinant DNA thus obtained may then be introduced into a host micro-organism where the gene will replicate simultaneously with the replication of the micro-organism, from which it may be re-isolated by conventional methods.

With this method of operation it is possible to provide a continuously renewable source of the gene which can then be manipulated further, modified and inserted in other vectors or in different sites in the same vector.

The transformed micro-organism, grown in a suitable culture medium, will enable the protein coded by the gene to be synthesised.

Accordingly by the present invention there has been cloned and sequenced an EcoRI fragment of Bordetella pertussis BP 165 chromosomal DNA containing the genes which code for the five subunits of the pertussis toxin and the aminoterminal sequence of the subunits S1, S2, S3 and S4 of the pertussis toxin has been determined. In particular, the pertussis toxin produced by Bordetella pertussis 165 has been purified by affinity chromatography and the subunits subsequently separated by electrophoresis in polyacrylamide sodium dodecylsulphate gels as shown in Figure 1.

The individual subunits were then separated and purified by electroelution (Hunkapiller M.W. et al.; Methods in Enzymology 91, 227-236, 1983) and analysed in a gas-phase microsequencer.

The aminoterminal sequence of the subunits S1, S2, S3 and S4 is given in Figure 2.

A gene library was then constructed with the use of the E. coli lambda phage EMBL4 (bought from Promega Biotec 2800 S. Fish Hatchery Road, Madison, WI 53711 USA) starting from the strain Bordetella pertussis BP356.

This strain is a mutant which does not produce an active toxin and has a single transposon TN5 inserted in its chromosome [Weiss, A.A. et al. Infect. Immun. 42, 33-41 (1983)].

The chromosomal DNA of the said strain was separated from the cells and, after purification, was partially digested with the restriction enzyme Sau3AI by the method and under the operative conditions described by Maniatis T. et al.: Molecular Cloning a Laboratory Manual Cold Spring Harbor N.Y., (1982). The fragments of chromosomal DNA with 15000 to 20000 base pairs were then separated and cloned in the E. coli lambda phage vector EMBL4 previously prepared as reported by Frischauf A. et al. [J. Mol. Biol. 170, 827-842 (1983)] with the use of the Promega Biotec "Packagene" Kit according to the method described by Maniatis T. et al. (Molecular Cloning a Laboratory Manual Cold Spring Harbor N.Y. 1982).

The recombinant phages were then used to transform E.coli NNM 539 cells (Promega Biotec).

The phages containing DNA fragments in which the transposon TN5 had been inserted were then selected from the transformed cells by the plate-hybridisation technique with a radio-active probe for the TN5 DNA.

The recombinant phage DNA was then extracted from the positive recombinant phages and, after digestion with the restriction enzyme EcoRI, the DNA fragments containing the transposon TN5 were

separated and selected by hybridisation with a probe for TN5 DNA.

In this manner it was possible to isolate an Eco-RI DNA fragment with about 10500 base pairs containing the entire sequence of the transposon TN5 flanked on the one hand by about 1100 base pairs and on the other by about 3500 base pairs of chromosomal DNA of Bordetella pertussis BP 356.

The Eco-IR fragments with 10500 base pairs were then digested with the restriction enzyme Hinc II and the DNA fragments containing the junction between the TN5 and the chromosomal DNA were isolated by hybridisation with a probe for TN5 DNA.

Two fragments were thus identified, one with about 500 base pairs and the other with 1900 base pairs.

The two fragments, purified by electroelution, were then cloned in the phage vector MI3mp8 (New England Biolabs) the DNA whereof had previously been cut by the restriction enzyme HincII .

The nucleotide sequences of the two fragments were then determined, starting from the HincII site according to the technique described by Sanger F.S.: Proc. Natl. Acad. Sci. 74, 5463 (1977).

The fragment with 1900 base pairs had, at about 400 nucleotides from the HincII site, a nucleotide sequence (Figure 3A from 3030 to 3100 bp) which, translated into the corresponding amino acids according to the genetic code, corresponded exactly to the amino acid sequence determined previously for the subunit S3 and given in Figure 2.

This result confirms that the cloned DNA fragment with 10500 base pairs contained the gene for the pertussis toxin.

The fragment with 1900 bp was then used as a hybridisation probe to identify and isolate a fragment DNA fragment containing the gene for and/or which codes for the pertussis toxin from the chromosomal DNA of B. pertussis BP 165 for which a gene library had been constructed as described above for B. pertussis BP 356.

At the end of the cloning operations, a 4696 base-pair Eco RI fragment of chromosomal DNA was isolated which we knew contained at least the gene which codes for the subunit S3 in that the fragment hybridised with the specific probe for S3.

The said fragment or parts thereof were then cloned in the phage vector MI3mp8 and MI3mp9 and the recombinant phage DNA thus obtained was sequenced.

Analysis of the sequences has enabled various open reading frames (ORFS) to be identified.

A comparison of their coding properties and the aminoterminal sequences of the subunits of the toxin have shown that four of these ORFS in fact code for the subunits S1, S2, S3 and S4 of the pertussis toxin.

Moreover, the molecular weight, the amino acid composition and the electric charges were in exact accordance with published data (Table 1). A fifth ORFS was also identified, placed between those which code for S4 and S3, which codes for a protein with a molecular weight and an amino acid composition identical to those described for the subunit S5.

These five open reading frames are grouped in a fragment with 3200 base pairs in the following order: S1, S2, S4, S5 and S3 and the ORFS reading frame which codes for S4 is superposed on those which code for S2 and S3 (Figure 3). On the basis of these results it is possible to conclude that the sequences determined contain the genes which code for the subunits of the pertussis toxin, and hence the open reading frames will be termed genes below.

In accordance with the present invention a transcription signal, very similar to the concensus sequence for the E.coli promotors, was identified before the gene which codes for S1.

In fact a region - 10, TAAAAT, which contains five of the six base pairs of the concensus sequence is associated with a region - 35, TGCTGACC, which contains six of the eight bases of the concensus sequence - 35.

The distance between the two regions - 35 and - 10 is 21 base pairs.

At the end 3' of the gene which codes for S3 there has been identified an inverted repeated sequence followed by a poly-T sequence which could represent a termination site.

Since no other promoter before the four genes S2, S3, S4 and S5 has been identified in the DNA fragment it may be deduced that these genes are organised in a single operon and are transcribed as a single polycistronic mRNA.

The presence of a single Shine-Dalgarno sequence located nine base pairs before the ATG of the gene S1, strongly suggests that this is the ribosomal binding cite which enables the translation of the S1 mRNA.

The presence of a new consensus sequence, TCC (T) GG, located eight to twelve base pairs before each ATG initiation codon for the four genes, suggests that this site is responsible for the translation of the entire mRNA.

Moreover it was found that the gene S4, which is produced in stoichiometric quantities of 2 to 1 with respect to the other genes, is the only one which is preceded by a slightly modified consensus sequence, TCCTGG, which probably increases the translation efficiency.

A characteristic common to all the subunits of the pertussis toxin is the presence, in the gene, of a sequence immediately preceding the mature protein, which codes for a 27 - 42 amino acid peptide the characteristics of which are typical of signal peptides involved in the secretion of the proteins.

This suggests that the various subunits are synthesised as proproteins, processed and secreted individually in the periplasmic space and subsequently processed, assembled and released into the extracellular space in the form of a single protein.

It has also been found that the signal peptide for S4 is unexpectedly long (42 amino acids) and has the highest aminoterminal positive charge described until now.

Since the positively-charged aminoterminal regions play an important role in the efficiency of production of the secreted proteins, the unusual structure of the signal peptide for S4 could cause increased translation of the gene S4.

It was also noted that, in the absence of the subunit S3 as occurs in the mutant BP356, the pertussis toxin is not excreted into the culture medium. Consequently, this protein is necessary for the complete assembly of the pertussis toxin.

The cloned DNA fragment or further fragments thereof, the said fragments containing at least one gene which codes for at least one subunit of the pertussis toxin, must be capable of being inserted in an expression vector and the hybrid plasmid thus obtained may be used to transform a micro-organism.

The transformed micro-organisms, grown in a suitable culture medium, are able to express the DNA fragment or fragments thereof by synthesis of the pertussis toxin or one or more subunits of the pertussis toxin.

Cloning vectors suitable for the purpose may be selected from natural plasmids known in the art or synthetic vectors obtained by recombinant DNA techniques.

In particular, the plasmid of E.coli pEMBL8 with about 4000 base pairs is used, this containing the gene for resistance to ampicillin and restriction sites useful for the cloning, such as: HindIII, PstI, AccI, HincII, SalI, BamHI, AvaI, SmaI, XmaI, EcoRI (Dente L. et al (Nucleic Acids Research 11, 1645-1655 (1983)), and the plasmids 31A, 31B and 31C derived from the vector PEX29 (Klinkert M. et al. Inf. Imm. 49, 329-335 (1985)) which contain the gene which codes for the DNA polymerase of the phage MS2 placed under the control of the inducible promoter pL and a polylinker inserted before the end of the gene of the MS2 polymerase in three possible frames, so as to be able to break each possible DNA fragment in the same frame of the MS2 polymerase.

Examples of micro-organisms used as host cells are strains of Escherichia coli, Bacillus subtilis, Saccharomyces, or eucaryotic cells.

In accordance with the present invention, there are used cells of E. coli JM 101 (New England Biolabs 32 Tozer Road, Beverly, MA 01915-9990 USA) and cells of E.coli K-12 Hl trp (described by Remant E. Gene 15: 81-93 (1981)) which produce a heat-sensitive repressor which, at 30°, completely inhibits the transcription of the gene of the MS2 polymerase preventing the production of proteins fused to it and, at 42°C, is inactivated giving good production of the polymerase and of the proteins fused to it.

The choice of the cloning vector and of the micro-organism to be transformed are not however limited by the present invention.

In accordance with the present invention, the 4696 base-pair fragment of chromosomal DNA obtained as described above, was inserted in the plasmid vector of E.coli pEMBL-8 after digestion of the plasmid DNA with the restriction enzyme EcoRI .

The hybrid plasmid obtained, designated pPT101, was then used to transform cells of E.coli JM101 (New England Biolabs) made competent by the method described by Cohen S. et al. (Proc. Natl. Acad. Sci. U.S. 69, 2110 (1972)).

The strain of E.coli (pPT101) was desposited in the American Type Culture Collection, on 6.8.85 with the number ATCC 53212.

In order to check the ability of the transformed micro-organism to express the cloned DNA, fragment, the E.coli strain (pPT101) was cultivated in a suitable culture medium.

More particularly, the strain was grown in LB medium (DIFCO) at a temperature of 37°C up to an absorbance of 0.75, measured in the culture broth at 590 nm.

The cells were then subjected to lysis and the pertussis toxin was determined directly in the cellular lysate by immunoenzymatic methods.

The biological activity of the pertussis toxin was determined by the method reported by Hewlett E.L. et al. (1983) (Infect. Immun. 40,, 1198-1203), the change in form of the CHO cells incubated with the cellular lysate under examination being analysed.

The results obtained confirm that the 4696 base-pair fragment of Bordetella pertussis chromosomal DNA contains the genes which code for the five subunits of the pertussis toxin and the said toxin can be

neutralized by antibodies against the toxin itself.

According to one embodiment of the present invention, the genes which code for the individual subunits of PT were cloned in the plasmids 31A, 31B, 31C derived from the vector PEX29 and the hybrid plasmids thus obtained and designated PTE255 (S1), PTE211 (S2), PTE221 (S3), PTE240 (S4) and PTE230 (S5) were used to transform cells of E. coli K-12 Hl trp.

The cells thus transformed were then cultivated in a suitable culture medium and the subunits, obtained as fused proteins, were recovered, purified and tested to determine their biological activities.

The results obtained show that all five subunits, when injected into rabbits, induce the formation of specific antibodies.

Moreover, the fused S1 protein shows the same enzymatic activity as the entire PT toxin, thus showing not only an immunological but also a functional identity with the natural S1.

In fact ADP-ribosylation tests carried out by incubating fused S1 with homogenised ox retina (ROS) in the presence of NAD marked with $^{32}P$, indicate that the subunit S1 binds the ADP-ribose group to the transducine present in the retina.

Hence both the pertussis toxin and the individual subunits obtained by the method of the present invention may be used for the preparation of vaccines against pertussis and diagnostic kits for determining specific antibodies in clinical samples from individuals infected with pertussis.

Analysis of the sequences given in the present invention also shows a certain similarity between the amino acid sequence in the subunit S1 of the pertussis toxin and that of the subunit A of the cholera toxin (J. Mekalanos et al. Nature 306, 551-557, 1983) (Figure 7).

There is thus a possibility of preparing a vaccine capable of neutralising cholera and pertussis simultaneously, with the use of the peptide S1 made by chemical synthesis or by recombinant DNA techniques.

## Brief Description of the Drawings

Figure 1: Electrophoresis of the pertussis toxin purified by affinity chromotography on 15% polyacrylamide (PAGE) - sodium dodecylsulphate (SDS) gel.

The toxin in column A was treated with a reducing agent before being applied to the gel.

The toxin in column B was not reduced.

S2 and S3, although having the same deduced molecular weight (table 1 - data from literature), had different mobilities on SDS-PAGE.

S5 was slightly coloured and also, having a lower molecular weight than that of S4 (table 1 - data from the literature), under reducing conditions migrated more slowly than S4.

Figure 2: Aminoterminal sequence of the subunits S1, S2, S3 and S4 determined by means of a micro-sequencer in the gaseous phase with the use of the individual subunits purified as in Figure 1.

A = alanine; C = cysteine, D = aspartic acid; E = glutamic acid; F = phenylalanine; G = glycine; H = histidine; I = isoleucine; K = lysine; L = leucine; M = methionine; N = asparagine; P = proline; Q = glutamine; R = arginine; S = serine; T = threonine; V = valine; W = tryptophan; Y = tyrosine; X = unidentified aminoacid residue.

All the sequences given are exactly in accordance with the nucleotide sequences with the single exception of the glutamine-2 in S2 which was found to be a threonine (Figure 3).

Figure 3A: Nucleotide sequence of the EcoRI fragment containing the five genes which code for the pertussis toxin.

The amino acid sequence of the five subunits of the pertussis toxin deduced from the nucleotide sequence is also given.

The arrows, before the amino acid sequences, indicate the start of the mature subunits as identified by comparison with the aminoterminal sequences in Figure 2.

In the case of S5, the arrow indicates the expected start of the mature subunit.

Before the sequence of each subunit, the amino acid sequence of the expected peptide signals is given.

Upstream of the gene which codes for S1 are indicated the expected promoter and Shine-Dalgarno sequences.

The sequences TCC (T) GG are present before S2, S3, S4 and S5.

At the end of the gene which codes for S3 the arrows above the nucleotide sequence indicate an inverted repetitive sequence followed by a poly-T sequence (underlined) which represents a possible transcription termination site.

Four open reading frames (ORFS) having the same use as the codons of the genes of the pertussis toxin are indicated by dotted lines.

Figure 3B: Schematic representation of the ORFS frames in the sequence given in Figure 3A.

The frames 1, 2 and 3 are shown from top to bottom and only the open reading frames with at least 200 base pairs are given.

P: expected promoter sequence

T: expected terminator sequence

Figure 4: Amino acid sequence of the signal peptides of the five subunits of the pertussis toxin.

The sequence (S) (P) A X A precedes the site at which cutting occurs.

Figure 5A: Translation and transcription signals. The initiation ATGs of the codons of the various ORFS are aligned and shown to the right.

Upstream of the ATG of S1 are shown the expected promoter and Shine Dalgarno sequences.

The respective sequences of E.coli are given above.

Upstream of the ATG of the other ORFS is given the sequence TCC (T) GG.

This sequence was not identified before the other ATG codons present in the entire nucleotide sequence given in Figure 3.

Figure 5B: This gives the structure of the expected termination sequence.

Figure 6: This shows the correspondence between the amino acid sequences of S2 and S3. The arrows indicate the sites at which the preproteins are cut and the start of the matured subunits.

Figure 7: Comparison of the amino acid sequence of the subunit S1 of the pertussis toxin and the subunit A of the cholera toxin. The corresponding amino acids in the two proteins are bracketed.

Figure 8: The three plasmids 31A, 31B, and 31C and the introduction of the polylinker into the three possible frames are shown.

Figure 9: This shows the cloning scheme for the genes which code for the five subunits of the pertussis toxin in the plasmids 31A, 31B and 31C and the construction of the hybrid plasmids PTE255 (S1), PTE211 (S2), PTE221 (S3), PTE240 (S4) and PTE230 (S5).

Figure 10A: This shows the electrophoresis of the total lysate of the strains which produce the polymerase of MS2 and the five subunits fused thereto (S1-S5).

Figure 10B: Electrophoresis of the partially-purified, fused proteins (S1, S2, S3, S4 and S5) on 15% acrylamide gel.

Figure 10C: Electrophoresis of the purified fused proteins (S1, S2, S3, S4, S5) on 15% acrylamide gel.

Figure 11 shows:

A): Western blot of the pertussis toxin incubated with goat serum against the entire toxin; this serum reacts with all five subunits:

B): Western blot of PT incubated with anti-fused S1 anti-serum: only the subunit S1 detected;

C): Western blot of PT incubated with anti-fused S2 anti-serum: only the subunit S2 detected;

D): Western blot of PT incubated with anti-fused S3 anti-serum: only the subunit S3 detected;

E): Western blot of PT incubated with anti-fused S4 anti-serum: only the subunit S4 detected;

F): Western blot of PT incubated with anti-fused S5 anti-serum: only the subunit S5 detected;

Figure 12: Autoradiography on polyacrylamide gel indicating the enzymatic activity of the fused S1 and of the pertussis toxin.

Figure 13: Nucleotide sequence of the DNA region which contains genes of the pertussis toxin. The sequence at the centre is that of Bordetella pertussis while above and below respectively are given the differences found in the sequences of B. bronchiseptica and B. parapertussis.

Figure 14: Southern blot showing that Bordetella pertussis may be distinguished from B parapertussis and bronchiseptica by the magnitude of the Eco RI fragment which hybridises with the clone pPT101.

Figure 15: Amino acid sequence of the five subunits of the pertussis toxin. The sequence at the centre is that of Bordetella pertussis while above and below respectively are given the differences found in B. bronchiseptica and B. parapertussis.

Figure 16: Enzymatic activity of the subunit S1 produced in E.coli as the fusion protein.

A: S1 of B. pertussis

B: MS2 polymerase from the vector pEX31a

C: Subunit S3

D: S1 of B. parapertussis

E: S1 of B. bronchiseptica

TABLE 1: Comparison of the amino acid composition in percentages, molecular weights and total charges of the five subunits of the pertussis toxin. A: experimental data given by Tamura et al. (Biochem. 21, 5516-5522 (1982)).

B: Data deduced from the nucleotide sequence.

TABLE 1

| | S1 | | S2 | | S3 | | S4 | | S5 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | A | B | A | B | A | B | A | B |
| Ala | 10.6 | 11.5 | 6.5 | 6.0 | 11.7 | 11.0 | 9.4 | 8.1 | 9.8 | 8.0 |
| Arg | 5.9 | 9.3 | 6.2 | 6.0 | 6.1 | 6.5 | 5.1 | 5.4 | 3.3 | 3.0 |
| Asp+Asn | 9.3 | 9.4 | 6.3 | 6.5 | 6.3 | 6.0 | 5.3 | 4.5 | 8.2 | 8.1 |
| Cys | 1.0 | 0.8 | 1.3 | 3.0 | 1.1 | 3.0 | 0.9 | 3.6 | 1.6 | 4.0 |
| Gly | 11.2 | 7.2 | 13.0 | 10.5 | 11.9 | 10.0 | 9.6 | 6.3 | 8.7 | 8.0 |
| Glu+Gln | 10.6 | 10.2 | 8.7 | 7.5 | 9.0 | 8.0 | 9.5 | 8.2 | 9.3 | 9.1 |
| His | 1.7 | 2.1 | 2.4 | 2.0 | 1.0 | 1.0 | 0.5 | 0.9 | 3.0 | 3.0 |
| Ile | 3.2 | 3.8 | 4.2 | 5.5 | 5.0 | 6.5 | 2.0 | 1.8 | 3.4 | 3.0 |
| Leu | 5.5 | 3.4 | 7.3 | 7.5 | 8.1 | 8.0 | 8.4 | 9.0 | 13.8 | 15.1 |
| Lys | 2.2 | 0.0 | 3.4 | 3.0 | 2.7 | 2.5 | 6.9 | 7.3 | 4.7 | 5.0 |
| Met | 1.6 | 2.1 | 1.5 | 1.5 | 1.1 | 1.5 | 5.1 | 7.2 | 1.6 | 2.0 |
| Phe | 3.5 | 3.0 | 3.2 | 2.5 | 3.2 | 2.5 | 3.6 | 4.5 | 4.9 | 5.0 |
| Pro | 4.4 | 3.8 | 4.6 | 4.5 | 5.7 | 5.0 | 9.1 | 10.0 | 5.6 | 5.0 |
| Ser | 10.6 | 9.4 | 8.5 | 8.5 | 6.3 | 5.0 | 8.0 | 5.4 | 6.9 | 6.0 |
| Thr | 7.4 | 7.2 | 10.4 | 10.0 | 8.2 | 8.0 | 5.0 | 4.5 | 6.9 | 7.0 |
| Trp | - | 0.8 | - | 1.0 | - | 0.5 | - | 0.0 | - | 1.0 |
| Tyr | 4.6 | 8.0 | 7.6 | 8.0 | 7.9 | 9.5 | 2.2 | 1.8 | 4.3 | 4.0 |
| Val | 6.7 | 7.6 | 4.9 | 6.0 | 4.7 | 5.0 | 9.4 | 10.9 | 4.0 | 3.0 |
| MW [a] | 28.0 | 26.22 | 23.0 | 21.92 | 22.0 | 21.86 | 11.7 | 12.06 | 9.3 | 10.94 |
| pI: charge [b] | 5.8 | -4.0 | 8.5 | +2.0 | 8.8 | +3.0 | 10.0 | +5.0 | 5.0 | -3.0 |

a) Molecular weight in kilodaltons

b) In order to compare the entire charge of the subunits, we have given the experimental isoelectric point at A and the nett charge calculated for each subunit at B.

The nett charge is calculated as ((Lys + Arg)-(Glu + Asp)).

The experimental examples which follow are illustrative and non-limiting of the invention.

Example 1

Determination of the aminoterminal sequence of the subunits of the pertussis toxin

A strain of B. pertussis BP165 was grown in a fermentor (Palias System N.B. App. Fabr. Van door De Bilt), provided with an agitator, with a capacity of 50 l, containing 40 l of Verwey culture medium with the following composition:

| | | |
|---|---|---|
| Bacto casamino acids (DIFCO) | g. | 14 |
| KCl | " | 0.2 |
| $K_2PO_4$ | " | 0.5 |
| $MgCl_2.6H_2O$ | " | 0.1 |
| nicotinic acid | " | 0.02 |
| glutathione | " | 0.01 |
| Starch | " | 1.00 |
| $H_2O$ | | 1 litre |
| pH 6.8 | | |

previously sterilised at 120 °C for 15 minutes, under aeration, at a temperature of 36.5 °C for 28 hours.

At the end of the said period of time, the cells were separated from the culture broth by centrifuging and the pertussis toxin was recovered from the supernatant liquor by affinity chromotography on Affi-Gel blue (100-200 mesh) by BioRad and on fetuin-sepharose as described by Sekura R.D. et al. [The J. Biol. Chem. 258, 23, 14647-14651 (1983)].

The protein obtained had a purity of more than 95%.

The protein was then subjected to electrophoresis on a 15% (p/p) polyacrylamide gel containing sodium dodecylsulphate (SDS) at 125 volts for 5 hours and the five subunits were separated, as given in Figure 1.

Each of these bands was cut and subjected to electroelution by the method of Hunkapiller M.W. et al [Methods in Enzymology 91, 227-236 (1983)].

Thus the five purified subunits were obtained.

The aminoterminal sequence of each of the subunits obtained was determined subsequently with the use of a gaseous-phase micro-sequencer model 470A (Applied Biosystems, Foster City, CA-USA) in accordance with the operating instructions.

Figure 2 shows the aminoterminal sequence of the subunits S1, S2, S3 and S4.

Example 2

Cloning of the DNA fragment containing the genes which code for the five subunits of the pertussis toxin

The strain B pertussis BP 356 is a mutant strain containing a transposon (TN5) inserted in the chromosome.

The strain, described by Weiss A.A. et al. in Infect. Immun. 42, 33-41 (1983), was produced by Stanley Falkow, Stanford University.

A culture of B pertussis BP 356 in the exponential phase (100 ml of Verwey medium) was centrifuged and the cells resuspended in 2 ml of 25% sucrose, 50 mM Tris, 1 mM EDTA (pH8).

To the suspension were then added 50µl of lysozyme (40 mg/ml) and, after 5 minutes, 10µl of proteinase K (20 mg/ml).

To the a gigated suspension were added 0.4 ml of EDTA (0.05 M).

The cells were subjected to lysis by the addition of 0.25 ml of Sarkosil (10%) at 0 °C to the cell suspension.

The lysated cells were then suspended in 35 ml of a solution containing 69.6 g of CsCl in 55.2 ml of buffer, 50 mM Tris, 1mM EDTA (pH8) containing 50µg of phenyl methyl sulphonylfluoride, an inhibitor for the proteinase K. The solution was then centrifuged at 50,000 revolutions per minute (rpm) for 16 hours in a 70 t i Beckmann SOV t i and the chromosomal DNA thus separated was then recovered as a viscous band.

500µg of chromosomal DNA thus obtained were dialysed against 100 ml of distilled water to remove the CsCl and then partially digested with five units (U) of restriction enzyme Sau 3 AI (Boehringer) in 5 ml of 50 mM NaCl, 10 mM Tris, 10 mM $MgSO_4$, 1 mM dithiothreitol buffer (pH7.4).

The digested DNA was precipitated by the addition to the solution of 12 ml of ethanol and, after separation, was resuspended in 0.5 ml of 10 mM Tris, 1 mM EDTA buffer.

This volume was loaded on to a 10% to 40% gradient of sucrose dissolved in 35 ml of 1 mM NaCl, 10 mM Tris, 1 mM EDTA buffer (pH 7.5).

The gradient was then centrifuged at 26000 rpm for 16 hours in a Beckman SW 28 rotor.

After this, 1 ml fractions were collected and the molecular weight of the DNA content of each fraction was determined by electrophoresis in agarose, as reported by Maniatis T. et al. "Molecular Cloning a Laboratory Manual", Cold Spring Harbor N.Y. (1982).

The fractions containing the DNA fragments with 15000 - 20000 base pairs (bp) were then dialysed and the DNA precipitated with ethanol as described above.

The precipitated DNA was separated by centrifuging and resuspended in 100µl of 10 mM Tris, 1 mM EDTA buffer (pH 7.5) to a final concentration of 1µg/ml of DNA.

The chromosomal DNA fragments were then cloned.

This was carried out with the use of an E. coli lambda phage vector EMBL 4 prepared as described by Frishauf A. et al. J. Mol. Biol. 170, 827-842 (1983).

1µg of DNA of the phage vector EMBL 4, previously cut with two U of restriction enzyme BamHI , and 1µl of the solution containing the fragments of DNA with 15000 - 20000 bp were mixed in 5µl of 1 mM ATP, 20 mM Tris, 10 mM $MgCl_2$, 10 mM dithiothreitol buffer (pH 7.6) in the presence of one U of T4 DNA ligase.

The ligase reaction was carried out at a temperature of 15 °C for 16 hours.

At the end of this period, the recombinant DNA obtained was inserted in lambda phages without DNA, with the use of the Packagene Kit of Promega Biotec (Maniatis T. et al. Molecular Cloning a Laboratory Manual Cold Spring Harbor N.Y. (1982)).

The recombinant phages thus obtained were used to transform the strain E.coli strain NM 539 (Promega Biotec.)

The transformed cells of E.coli NM 539 were plated on LB medium (Bacto Triptone 10 g, Bacto Y.E. 5g, NaCl 10g, $H_2O$ 1 litre pH 7.5) giving about 30000 plates of recombinent phages.

About 5000 recombinant phages were hybridised by hybridisation on a plate with a radioactive probe for the TN5 DNA, in order to identify those phages containing the DNA fragment in which the transposon TN5 was inserted.

Twelve recombinant phages were positive on hybridisation. The DNA was then extracted from these phages by the extraction methods given above.

1µg of recombinant phage DNA was cut with two U of the restriction enzyme Eco RI in 20µl of 50 mM Tris, 100 mM NaCl, 10 mM $MgSO_4$ buffer (pH 7.4), the solution being kept at a temperature of 37 °C for one hour.

The digested solution of DNA was then loaded on to a 1% agarose gel and subjected to electrophoresis for two hours at 120 volts for six hours.

The fragments of recombinant phage DNA thus separated were transferred on to nitrocellulose and hybridised with a radioactive probe for TN5 DNA in order to identify the EcoRI fragment containing the transposon TN5.

In this manner a positive EcoRI fragment of about 10500bp was isolated which contained the entire sequence of TN5 flanked on one side by about 1100 bp and on the other by about 3500 bp of chromosomal DNA of B. pertussis BP 356.

1 µg of the EcoRI fragment was cut with two U of the enzyme Hinc II in 25µl of 50 mM NaCl, 10 mM Tris, 10 mM $MgSO_4$, 1 mM dithiothreitol buffer (pH 7.4), at 37 °C for 1 hour).

At the end of this period, the solution containing the digested DNA fragments was subjected to electrophoresis on 1% agarose gel for six hours at 120 volts, transferred onto nitrocellulose and then hybridised with the radioactive probe for TN5 DNA, in order to identify the fragments containing the junction between the TN5 and the chromosomal DNA.

Thus two fragments were identified, one with about 500 bp and the other with about 1900 bp.

The two fragments were then purified by electroelution and cloned in the phage vectors MI3 mp8 and MI3 mp9 (New England) the DNA of which had previously been cut with the restricton enzyme Hinc II.

The nucleotide sequence of the two fragments was then determined starting from the HincII site with the use of the technique described by Sanger F.S. (Proc. Natl. Acad. Sci. 74, 5463 (1977)).

At about 400 nucleotides from the HincII site of the larger fragment (1900 bp), the nucleotide sequence given in Figure 3A - 2 from 3030 to 3100 bp was identified and, translated into the corresponding amino acids, gave the amino acid sequence determined by us for the subunit S3 as described in Example 1 and given in Figure 2.

This result indicates that, in the strain B. pertussis 356, the TN5 is inserted in the gene which codes for the subunit S3 of the PT and confirms that the fragment of DNA cloned by us contains the gene for the pertussis toxin.

The fragment thus identified was then used as a hybridisation probe to identify the gene for the PT present in the chromosomal DNA of B. pertussis BP165.

A gene library was again constructed for this strain in the phage vector EMBL 4, in the same manner as described for B. pertussis BP 356.

At the end of the cloning operation, an Eco RI fragment was isolated with 4696 bp which we knew contained at least the gene which codes for the subunit S3 in that it hybridised with the specific probe S3.

In order to check whether this fragment also contained the genes which code for the other PT subunits , the fragment, or parts of it, were cloned in the phage vectors MI3 mp8 and MI3 mp9 and then the nucleotide sequence of the entire fragment was determined.

Analysis of the nucleotide sequence of the entire fragment, given in Figure 3, shows that this fragment also contains the genes which code for the subunits S1, S2 and S4, in that the translation of the nucleotide sequence of the DNA fragment in the corresponding amino acid sequence corresponds to the amino acid sequences determined by us for the subunits S1, S2 and S4 and given in Figure 2.

Once the beginning of the amino acid sequence had been identified from the data given in Figure 2, it was possible to deduce the entire amino acid sequence of the said subunits.

The analyses of the chemical and physical properties of the various subunits deduced from the amino acid sequence, such as the molecular weight, amino acid composition and electric charge, are in accordance with the data in the literature(Tamura et al. (1982) Biochemistry 21, 5516-5522).

It was also noted that a common characteristic of all five subunits was the presence in the gene of a sequence immediately before the mature protein which coded for a peptide with 27-42 amino acids and which had characteristics typical of the peptides involved in the secretion of the proteins, that is the presence of one or more positive charges on the terminal amino group followed by a hydrophobic zone (Figure 4).

This shows that the subunits were produced in the form of preproteins and these were subsequently processed during secretion.

All the secretion signals also terminated with the sequence (S) (P)A X A which is typical of other secretion signals.

Among the genes which code for S4 and S3 was also identified a nucleotide sequence, from 2461 to 2862 bp, which codes for a peptide which has the same properties as the other secretion signals and terminates with the sequence SPADVA, followed by an amino acid sequence which has exactly the same amino acid composition as that given in the literature for the subunit S5 (Table 1).

This has enabled us to establish that the Eco RI fragment with 4696 bp cloned by us also contains the gene for the subunit S5 and hence has enabled us to determine the amino acid sequence of the latter (Figure 3).

Further analysis of the nucleotide sequence of the DNA fragment isolated and cloned by us has enabled the location of a promoter in the zone 440 bp to 485 bp, which has the same characteristics as those of E.coli, and of a termination sequence in the zone 3608 to 3670 bp.

This means that the five genes of the pertussis toxin are organised in a typical bacterial operon and are transcribed in a single mRNA.

## Example 3

### Construction of the hybrid plasmid pPT 101 containing the genes which code for the pertussis toxin

1µg of plasmid DNA of E.coli pEMBL-8 described by Dente L. (1983) Nucl. Acids Res. 11, 1645-1655 containing the gene which gives resistance to ampicillin were cut with two U of EcoRI enzyme in 20µl of 100 mM NaCl, 50 mM Tris, 10 mM $MgSO_4$ buffer (pH 7.4) at 37 °C for one hour.

At the end of the digestion reaction, 3µg of the EcoRI DNA fragment with 4696 bp, the sequence of which is given in Figure 3, were added to the solution containing the cut plasmid DNA and reacted in the

presence of one U of T4 DNA ligase (BRL) under the conditions recommended by the manufacturer.

The ligase mixture was then used to transform cells of ampicillin-sensitive E.coli JM 101 (New England Biolabs) rendered competent.

The transformed cells were selected on LB plates containing 100µg/ml of ampicillin in order to isolate those cells which contain the hybrid plasmid.

Among the clones of ampicillin-resistant (AmpR) E.coli thus obtained, it was possible to isolate clones containing the hybrid plasmid pEMBL 8 containing the DNA fragment which codes for PT by the technique of hybridisation with a probe for the sequence of the PT gene.

One of these hybrid plasmids was designated pPT 101 by us.

The E.coli JM 101 strain containing the plasmid has been deposited by us at the American Type Culture Collection on 6.8.85 under the number ATCC-53212.

Example 4

Construction of the hybrid plasmid PTE 255 containing the gene which codes for the subunit S1.

The construction of the hybrid plasmid was carried out in the manner given in example 3 above, by ligating the plasmid 31B, previously digested with the restriction enzymes BamHI and Xba I, with the Sau3al-$Xba_1$ from 612 to 1317 of the 4696bp fragment corresponding to the gene which codes for S1.

The ligase mixture was then used to transform cells of competent E.coli, the transformed cells being selected on LB plates (DIFCO) containing ampicillin.

The hybrid plasmid PTE 255 (S1) was separated from one of the positive clones and its sequence is given in Figure 9 where the lower case letters indicate the coding sequence for the polymerase MS2 and the upper case letters indicate the sequence which codes for S1.

The resulting protein contains all the subunit S1 apart from the first amino acid Asp.

Example 5

Construction of the hybrid plasmid PTE 211 containing the gene which codes for the subunit S2

This was carried out as in Example 3 above with the use of the plasmid 31A digested with BamHI and treated with DNA polymerase to fill the cohesive termini and the Sau96-SmaI fragment from 1433 to 2064 of the 4696 bp fragment, corresponding to the gene which codes for S2, was treated with DNA polymerase (Klenow) to fill the cohesive termini.

The hybrid plasmid PTE 211 (S2) isolated from one of the positive transformants had the sequence given in Figure 9.

The resulting fused protein contained the sequences of the polymerase of MS2 (lower case letters to the left) fused to an amino acid of the peptide leader of the subunit S2 (upper case letters), and hence to the protein S2 (lower case letters to the right).

Example 6

Construction of the hybrid plasmid PTE 221 containing the gene which codes for the subunit S3

This was carried out as in Example 3 above with the use of the plasmid 31C digested with BamHI and treated with DNA polymerase to fill the cohesive termini and the SpH1-DDE1 fragment from 3014 to 3628 of the 4696 bp fragment, corresponding to the gene which codes for S3, was treated with DNA polymerase to eliminate the cohesive termini.

The hybrid plasmid PTE 221 (S3) isolated from one of the positive transformants had the sequence given in Figure 9.

The fused protein which resulted from it contained the polymerase MS2 (lower case letters to the left) fused to five amino acids of the peptide leader of the subunit S3 (upper case letters), and hence to the natural subunit S3 (lower case letters to the right).

Example 7

Construction of the hybrid plasmid PTE 240 containing the gene which codes for the subunit S4

This was carried out as in example 3 above, with the use of the plasmid 31B cut with BamHI and treated with polymerase and the BstN1-BstN1 fragment from 2151 to 2600 of the 4696 bp fragment corresponding to the gene which codes for S4.

The sequence of the hybrid plasmid PTE 240 (S4) thus obtained is given in Figure 9.

The fused protein which results from it contains the polymerase of MS2 (lower case letters) fused to two amino acids of the peptide leader of the subunit S4 (upper case letters), and hence to the natural subunit S4.

Example 8

Construction of the hybrid plasmid PTE 230 containing the gene which codes for the subunit S5

This was carried out as in Example 3 above, with the use of the plasmid 31A cut by BamHI and treated with DNA polymerase to fill the cohesive termini and the Aat2 - SnaBI fragment from 2558 to 3210 of the 4696 bp fragment, corresponding to the gene which codes for S5.

The sequence of the hybrid plasmid PTE230 obtained is given in Figure 9.

The resulting fused protein contained the polymerase of MS2 (lower case letters to the left), two amino acids of the peptide leader of the subunit S5 (upper case letters), and hence the natural subunit S5 (lower case letters to the right).

Example 9

Production of pertussis toxin and experiment to determine its activity

The strain E.coli JM 101 (pPT 101) was grown in a 100 ml flask containing 10 ml of LB, under mild agitation, at a temperature of 37 °C for 16 hours.

0.1 ml of this culture was then used to innoculate 10 ml of LB medium and grown at 37 °C up to an absorbance $OD_{590}$ of 0.75.

The culture broth was then centrifuged at 4 °C and the cells thus separated were resuspended in 0.5 ml of 10 mM Tris (pH 7.5).

The cell suspension was subjected to lysis by ultrasonics in a Branson Sonifier-cell Disruptor 200 (Bransonsonic Power Co., a Smithkline Company).

The presence and biological activity of the pertussis toxin were then determined directly on the cellular lysate by means of CHO cells, by the method reported by Hewlett, E.L. et al. (1983) Infect. Immun. 40, 1198-1203. The CHO cells used were obtained in our laboratory by mutation of CHO ATCC CCL 61 cells. 10,000 CHO cells were incubated in 2.5 ml of medium (the composition of which is given by Hewlett E.L. et al. (1983) (Infect. Immun. 40, 1198-1203) in the presence of 5µl of cell extract of E.coli JM 101 (pPT 101), 5µl of E.coli JM 101 cells containing the unmodified plasmid PEMBL-8 and 0.1 ng of pertussis toxin as a standard.

Part of the cell extract had previously been incubated with a 1:100 dilution of ordinary goat antiserum (A) and another part with a 1:100 dilution of the same goat antiserum taken after immunisation with the pertussis toxin.

After 48 hours of incubation at 37 °C, the results were read in the manner described by Hewlett in the text indicated above.

A value of 4 (+) was attributed to a maximum form change of the CHO cells, a value of 1 (+) to a minimum form change and (-) to a lack of form change.

The results are given in table 2.

TABLE 2

Activity on CHO cells of the toxin produced by the recombinant clones

| Sample | Whole | Goat anti-toxin antibodies | Preimmunie goat antibodies |
|---|---|---|---|
| 0.1 ng Toxin | ++++ | - | ++++ |
| E. coli extract containing pPT101 ATCC 53212 | +++ | - | +++ |
| E. coli extract containing PEMBL8 | - | - | - |

0.1 ng of the purified pertussis toxin was used as a positive control. The sample was constituted by 5µl of E.coli lysate containing the plasmid PT101. The negative control was constituted by the same strain of E.coli containing the plasmid used as a vector, without the genes for the pertussis toxin (pEMBL8).

It may be seen from table 2 that the extract of cells of E.coli (pPT101) ATCC 53212 gave a positive result and the toxin could be neutralised by anti-pertussis toxin antibodies but not by antibodies from the same goat before it had been immunised.

The strain E.coli JM 101 (pEMBL8) did not have any activity in this test.

We may thus conclude that the fragment of EcoRI chromosomal DNA with 4696 base pairs cloned by us in the plasmid pEMBL8 is able to synthesise a toxin which is functionally identical to the pertussis toxin produced by B.pertussis BP165 and the pertussis toxin can be neutralised by antibodies for the toxin itself.

Example 10

Expression and purification of the 5 subunits of the pertussis toxin

a) Expression of the 5 subunits

The hybrid plasmids PTE255 (S1), PTE211 (S2), PTE221 (S3), PTE240 (S4) and PTE230 (S5) constructed as described in the examples 4 to 8 were introduced by transformation of the strain of E.coli K12 Hl trp.

Each of the transformed strains was then grown in 10 ml of LB medium at 30°C for one night. At the end of this period, the 10 ml of each culture were added to 400 ml of fresh LB medium in two-litre flasks.

The flasks were kept under agitation at 30°C for two hours and at a temperature of 42°C for 2.5 hours.

The cultures were centrifuged and the cells separated and resuspended in 3 ml of 25% sucrose, 10 ml Tris - HCl (pH 8.0), 1 mM EDTA.

5µl of each of the said cultures then had added to it 80µl of lysis buffer (4% SDS, 125 mM Tris (pH 6.8), 10% B-mercaptoethanol, 10% glycerol and 0.02% bromophenol blue, they were brought to boiling point for five minutes and then loaded onto a 15% polyacrylamide gel.

The proteins were then subjected to electrophoresis at 25 milliamps for five hours and the gel coloured and decoloured as reported by Laemli (Nature, 227, 680-85, 1970).

Figure 10A shows the electrophoresis of the total lysate of the strains which produce the polymerase of MS2 (A) and the 5 unpurified subunits (S1 - S5) fused to this.

b) Purification of the 5 subunits

The cells of each of the said cultures were resuspended in 3.2 ml of 2.5% sucrose solution, 0.1 ml of lysozyme (40 mg/ml) and 0.8 ml of 0.5M EDTA was added and they were incubated at 37°C for 30 minutes.

At the end of this period, to each of the suspensions were added 8 ml of lysis buffer (1% Triton X 100, 50 mM Tris pH 6.00, 63 mM EDTA) and then they were kept at 0°C for 15 minutes and at 37°C for 30 minutes.

Subsequently the cells were subjected to sonic disruption and centrifuged at 10000 revolutions for 10 minutes.

The precipitate thus separated was resuspended in 5 ml of 1M urea, kept at 37°C for 30 minutes, centrifuged and, after separation of the supernatant liquor, resuspended in 5ml of 7M urea. Thus partial purification of the subunits produced was obtained as given in Figure 10B.

The partially-purified proteins were resuspended again in 5ml of 7M urea, loaded on to a preparative polyacrylamide gel (3mm x 50cm) and subjected to electrophoresis at 50 milliamps for 8 hours.

After colourating, the band containing the fusion protein was cut and electroeluted at 200 volts for 48 hours in a dialysis bag.

The electroeluted protein was then dialysed against distilled water and precipitated by the addition of 9 volumes of acetone.

The protein was then recovered by centrifuging and resuspended in 0.1M $NaHCO_3$.

Figure 10C shows the results obtained for the individual purified proteins.

Preparation of sera against the 5 subunits

The purified, fused proteins (S1, S2, S3, S4 and S5) obtained as indicated in Example 10 above were used to immunise rabbits in accordance with the following scheme:

Day 1: 1 ml of solution containing about 1 mg of the fused protein was mixed with 1 ml of whole Freund adjuvant and injected subcutaneously into a rabbit.

Day 18: The treatment of Day 1 was repeated with the use of incomplete adjuvant.

Day 27: 1 ml of a solution with a protein content of about 1 mg was injected intravenously.

Day 37: The rabbits were bled and the serum recovered.

The anti-sera to the 5 subunits thus prepared were then tested by the Western Blot technique to check whether they recognised the five natural proteins.

About 100 mg of the purified pertussis toxin indicated in Example 1 was loaded on to a 15% polyacrylamide gel and subjected to electrophoresis.

The subunits thus separated were then transferred on to nitrocellulose by electro-blotting.

The sheet of nitrocellulose containing the subunits was cut vertically into a number of identical strips each of which was subsequently analysed by the Western blot technique.

In practice, the strips of nitrocellulose were suspended in PBS 0.15 M NaCl, 10 mM phosphates pH 7.4 containing 1 X Denhart(0.03% bovine albumin, 0.02% FiColl 70 and 0.02% polyvinyl pyrrolidone) and 0.05% Tween for two hours and were washed two times, for 3 minutes each time, with PBS containing 0.05% of Tween 20.

They were subsequently incubated for one night at ambient temperature with a 1/100 dilution of the desired serum in PBS with the addition of 0.05% Tween 20.

They were then washed three times for 15 minutes each time, with a solution of 10 mM Tris, 0.9% NaCl and 0.1% Tween 20 (TBS), incubated with a conjugate of gammaglobulin anti-globulin of goat-peroxidase or globulin anti-globulin of rabbit peroxidase (Miles) diluted 1/100 in TBS and finally washed 3 times in TBS and once in Tris 0.01 M (pH 6.8) for 10 minutes. To each of the solutions was then added the substrate for the peroxidase: 20 ml Tris 0.05M pH 6.8, 5 ml 0.3% 4-chloro 1 naphthol in methanol and 7µl of $H_2O_2$.

The reaction was stopped by washing the filters in distilled water.

The results given in Figure 11 show that the fused proteins obtained with the use of the genes which code for the five subunits of PT, when injected into rabbits, induce the formation of specific antibodies capable of recognising each of the subunits of the natural toxin.

Example 12

Analysis of the enzymatic activity of the fused protein S1

10µl of fused protein S1 and 10µl of PT preincubated with 25 mM of dithiothreitol at ambient temperature for 30 minutes were added to a solution containing 10µl of homogenised ox retina (ROS), 80µl of $H_2O$, 5µl of Tris 2M (pH7.5), 1µl of ATP 100 mM, 1µl of GTP 10 mM, 10 µl of thymidine and 1µl (1 µCi) of $^{32}P$ NAD.

The mixture was kept at ambient temperature for 30 minutes, centrifuged, the supernat nt liquor separated and the precipitate containing the ROS was dissolved in sodium dodecylsulphate loading buffer, loaded on to 15% polyacrylamide gel and subjected to a potential difference of 125 volts for 5 hours.

At the end of this period, the gel was dried and subjected to autoradiography.

The results (Figure 12) show that: 1) the pertussis toxin (PT) ADP ribosyles the transducin, 2) in the absence of the pertussis toxin this is not marked and 3) the fused electroeluted S1 has the same ADP ribosylating activity as PT.

Example 13

Cloning, sequence and expression of the genes of Bordetella bronchiseptica and Bordetella parapertussis

Although B. bronchiseptica and B. pertussis do not produce active pertussis toxin, we have found that they contain the coding genes for this. By operating as described in Examples 2, 3 and 4, we have cloned and sequenced the genes of Bordetella bronchiseptica (ATCC 4617) and Bordetella parapertussis (ATCC 9305) which code for the five subunits of the pertussis toxin.

The nucleotide sequence obtained, given in Figure 13, shows that there are small differences between the three strains. One of these lacks the EcoRI site at 4696 and hence the genes of B. bronchiseptica and B. parapertussis are contained in EcoRI fragments with 4935 bp instead of 4696. This difference in dimensions may be used as a diagnostic criterion for distinguishing B. pertussis from B. parapertussis and B. bronchiseptica, in the following manner: Bordetella chromosomal DNA was digested with EcoRI on an agarose gel, transferred on to nitrocellulose and hybridised by the techniques described for the plasmid PPT 101 and its fragments of cloned DNA.

The results of the autoradiography enable the B. pertussis to be distinguished from the B. parapertussis and bronchiseptica which hybridise in a higher molecular weight band (Figure 14).

Figure 15 gives the amino acid sequences deduced from the five subunits in the three species of Bordetella. As may be seen, there are several changes of amino acids. To check whether these changes alter the function and immunogenicity of the subunits, operating as described in Example 4, we have expressed the genes which code for the subunit S1 of B. bronchiseptica and parapertussis. The fused proteins obtained were immunogenically similar to those of B. pertussis and in fact were recognised in Western blot by antitoxin antibodies of pertussis.

Moreover, by operating as described in Example 12, we found that both the proteins had the same enzymatic activity as the subunit S1 of B. pertussis (Figure 16). This example shows that the proteins with the sequence given in Figure 15 although containing several variations, may be used as a vaccine against pertussis.

## Claims

**Claims for the following Contracting States : AT, BE, CH, DE, FR, GB, LI, LU, NL, SE**

1. An EcoRI fragment of Bordetella pertussis chromosomal DNA with 4696 base pairs containing the genes which code for the five subunits of the pertussis toxin or a further fragment thereof, where the further fragment contains at least one gene which codes for at least one subunit of the pertussis toxin.

2. A DNA fragment or further fragment according to claim 1 having the sequence given in Figure 3.

3. A DNA fragment or further fragment according to claim 1 or 2 containing genes encoding subunits S1, S2, S3, S4 and S5 of the pertussis toxin and at least one of open reading frames (ORFs) A,B, C and D.

4. A hybrid plasmid containing the DNA fragment or further fragment thereof as defined in claim 1 or 2 capable of expressing the DNA fragment or further fragment when carried by a microorganism, to synthesise pertussis toxin or one or more subunits of pertussis toxin.

5. A hybrid plasmid according to claim 4, where the DNA fragment or further fragment thereof is placed under the control of region which regulates expression.

6. Hybrid plasmid pT101 according to claim 4 or 5, in which the 4696 base-pair EcoRI fragment is inserted in the EcoRI restriction site of pEMBL8.

7. Hybrid plasmid PTE255 according to claim 4 or 5 in which the Sau3A-Xba I fragment which codes for the subunit S1 is inserted in the BamHI-XbaI site of the plasmid 31B.

8. Hybrid plasmid PTE211 according to claim 4 or 5, in which the Sau96-SmaI fragment which codes for the subunit S2 is inserted in the BamHI site in the plasmid 31A.

9. Hybrid plasmid PTE221 according to claim 4 or 5, in which the SpH1-DDE1 fragment which codes for the subunit S3 is inserted in the BamHI site in the plasmid 31C.

10. Hybrid plasmid PTE240 according to claim 4 or 5, in which the BstN1-BstN1 fragment which codes for the subunit S4 is inserted in the BamHI site of the plasmid 31B.

11. Hybrid plasmid PTE230 according to claim 4 or 5, in which the fragment Aat2-SnaBI which codes for the subunit S5 is inserted in the site BamHI of the plasmid 31A.

12. A micro-organism transformed by an hybrid plasmid as defined in any one of claim 4 to 11.

13. The micro-organism Escherichia coli transformed with a hybrid plasmid according to claim 7.

14. The transformed microorganism Escherichia coli ATCC 53212.

15. A method for the preparation of the pertussis toxin or one or more of the subunits of the pertussis toxin which comprises the step of culturing a transformed microorganism as defined in any one of claims 12 to 14 in a suitable culture medium.

16. A method for the cloning and sequencing of the DNA fragment according to claim 1 or 2, including:
    a) isolating and cloning, in an E. coli lambda phage EMBL4, fragments of chromosomal DNA with 15,000-20,000 base pairs, obtained by the digestion of the chromosomal DNA of Bordetella pertussis BP 356, a mutant strain which does not produce the pertussis toxin, which contains a transposon Tn5 in its own chromosomal DNA;

Moreover, by operating as described in Example 12, we found that both the proteins had the same enzymatic activity as the subunit S1 of B. pertussis (Figure 16). This example shows that the proteins with the sequence given in Figure 15 although containing several variations, may be used as a vaccine against pertussis.

## Claims

**Claims for the following Contracting States : AT, BE, CH, DE, FR, GB, LI, LU, NL, SE**

1. An EcoRI fragment of Bordetella pertussis chromosomal DNA with 4696 base pairs containing the genes which code for the five subunits of the pertussis toxin or a further fragment thereof, where the further fragment contains at least one gene which codes for at least one subunit of the pertussis toxin.

2. A DNA fragment or further fragment according to claim 1 having the sequence given in Figure 3.

3. A DNA fragment or further fragment according to claim 1 or 2 containing genes encoding subunits S1, S2, S3, S4 and S5 of the pertussis toxin and at least one of open reading frames (ORFs) A,B, C and D.

4. A hybrid plasmid containing the DNA fragment or further fragment thereof as defined in claim 1 or 2 capable of expressing the DNA fragment or further fragment when carried by a microorganism, to synthesise pertussis toxin or one or more subunits of pertussis toxin.

5. A hybrid plasmid according to claim 4, where the DNA fragment or further fragment thereof is placed under the control of region which regulates expression.

6. Hybrid plasmid pT101 according to claim 4 or 5, in which the 4696 base-pair EcoRI fragment is inserted in the EcoRI restriction site of pEMBL8.

7. Hybrid plasmid PTE255 according to claim 4 or 5 in which the Sau3A-Xba I fragment which codes for the subunit S1 is inserted in the BamHI-XbaI site of the plasmid 31B.

8. Hybrid plasmid PTE211 according to claim 4 or 5, in which the Sau96-SmaI fragment which codes for the subunit S2 is inserted in the BamHI site in the plasmid 31A.

9. Hybrid plasmid PTE221 according to claim 4 or 5, in which the SpH1-DDE1 fragment which codes for the subunit S3 is inserted in the BamHI site in the plasmid 31C.

10. Hybrid plasmid PTE240 according to claim 4 or 5, in which the BstN1-BstN1 fragment which codes for the subunit S4 is inserted in the BamHI site of the plasmid 31B.

11. Hybrid plasmid PTE230 according to claim 4 or 5, in which the fragment Aat2-SnaBI which codes for the subunit S5 is inserted in the site BamHI of the plasmid 31A.

12. A micro-organism transformed by an hybrid plasmid as defined in any one of claim 4 to 11.

13. The micro-organism Escherichia coli transformed with a hybrid plasmid according to claim 7.

14. The transformed microorganism Escherichia coli ATCC 53212.

15. A method for the preparation of the pertussis toxin or one or more of the subunits of the pertussis toxin which comprises the step of culturing a transformed microorganism as defined in any one of claims 12 to 14 in a suitable culture medium.

16. A method for the cloning and sequencing of the DNA fragment according to claim 1 or 2, including:
    a) isolating and cloning, in an E. coli lambda phage EMBL4, fragments of chromosomal DNA with 15,000-20,000 base pairs, obtained by the digestion of the chromosomal DNA of Bordetella pertussis BP 356, a mutant strain which does not produce the pertussis toxin, which contains a transposon Tn5 in its own chromosomal DNA;



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b) isolating a fragment of recombinant phage DNA which hybridises with a probe marked for Tn5 DNA from the positive recombinant phages obtained;
c) cutting the fragment of recombinant phage DNA with the restriction enzyme HincII and isolating a fragment with 1900 bp containing a nucleotide sequence which codes for the subunit S3 by hybridisation with a probe for Tn5 DNA;
d) isolating and cloning in an E. coli lambda phage EMBL4 fragments of chromosomal DNA with 15,000-20,000 base pairs obtained by the digestion of the chromosomal DNA of Bordetella pertussis BP165;
e) isolating a fragment of recombinant phage DNA which hybridises with a probe for S3 as obtained at point c) from the positive recombinant phages so obtained;
f) cutting the recombinant phage DNA fragment with the restriction enzyme EcoRI and isolating an EcoRI fragment of chromosomal DNA with 4696 base pairs by hybridisation with a probe for S3 DNA;
g) inserting the fragment obtained into the plasmid vector pPEML8 in the EcoRI restriction site, determining the nucleotide sequence of the DNA fragment and identifying genes which code for the five subunits of the pertussis toxin.

17. Use of the pertussis toxin or one or more subunits of the pertussis toxin obtained as in claim 15 for the preparation of an anti-pertussis vaccine wherein the pertussis toxin is free from other B. pertussis material.

18. Composition for the simultaneous stimulation of protection against infections of Bordetella pertussis and Vibrio colerae including at least an effective quantity of the peptide S1 obtained by chemical synthesis or from microorganisms transformed with molecules of recombinant DNA containing the gene which codes for S1.

19. Use of the fragment of chromosomal DNA according to claim 1 for the preparation of diagnostic kits for the determination of pertussis infections.

20. Use of the fragment of chromosomal DNA according to claim 1 for distinguishing B. pertussis from B. parapertussis and bronchiseptica.

**Claims for the following Contracting State : ES**

1. A method for the preparation of the pertussis toxin or one or more of the subunits of the pertussis toxin which comprises the step of culturing a transformed microorganism in a suitable culture medium wherein the transformed microorganism is a microorganism transformed with a hybrid plasmid containing an EcoRI fragment of Bordetella pertussis chromosomal DNA with 4696 base pairs containing the genes which code for the five subunits of the pertussis toxin or a further fragment thereof, where the further fragment contains at least one gene which codes for at least one subunit of the pertussis toxin.

2. A method for the preparation of a hybrid plasmid comprising inserting into a plasmid an EcoRI fragment of Bordetella pertussis chromosomal DNA with 4696 base pairs containing the genes which code for the five subunits of the pertussis toxin or a further fragment thereof, where the further fragment contains at least one gene which codes for at least one subunit of the pertussis toxin.

3. A method for the preparation of a transformed microorganism comprising transforming a microorganism with a hybrid plasmid containing an EcoRI fragment of Bordetella pertussis chromosomal DNA with 4696 base pairs containing the genes which code for the five subunits of the pertussis toxin or a further fragment thereof, where the further fragment contains at least one gene which codes for at least one subunit of the pertussis toxin.

4. A method according to claim 3 wherein the microorganism is Escherichia coli.

5. A method according to claim 4 wherein the transformed microorganism is ATCC 53212.

6. A method according to any one of claims 1 to 5 wherein the DNA fragment or further fragment thereof is placed under the control of region which regulates expression.



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7. A method according to any one of claims 1 to 6 wherein the DNA fragment or further fragment contains genes encoding subunits S1, S2, S3, S4 and S5 of the pertussis toxin and at least one of open reading frames (ORFs) A,B, C and D.

8. A method according to any one of claims 1 to 6 wherein the hybrid plasmid is pT101 in which the 4696 base-pair EcoRI fragment is inserted in the EcoRI restriction site of pEMBL8.

9. A method according to any one of claims 1 to 6 wherein the hybrid plasmid is PTE255 in which the Sau3A-Xba I fragment which codes for the subunit S1 is inserted in the BamHI-XbaI site of the plasmid 31B.

10. A method according to any one of claims 1 to 6 wherein the hybrid plasmid is PTE211 according to claim 4 or 5, in which the Sau96-SmaI fragment which codes for the subunit S2 is inserted in the BamHI site in the plasmid 31A.

11. A method according to any one of claims 1 to 6 wherein the hybrid plasmid is PTE221 in which the SpH1-DDE1 fragment which codes for the subunit S3 is inserted in the BamHI site in the plasmid 31C.

12. A method according to any one of claims 1 to 6 wherein the hybrid plasmid is PTE240 in which the BstN1-BstN1 fragment which codes for the subunit S4 is inserted in the BamHI site of the plasmid 31B.

13. A method according to any one of claims 1 to 6 wherein the hybrid plasmid is PTE230 in which the fragment Aat2-SnaBI which codes for the subunit S5 is inserted in the site BamHI of the plasmid 31A.

14. A method according to any one of claims 1 to 13 wherein the DNA fragment or further fragment has the sequence given in Figure 3.

15. A method for the cloning and sequencing of an EcoRI fragment of Bordetella pertussis chromosomal DNA with 4696 base pairs containing the genes which code for the five subunits of the pertussis toxin or a further fragment thereof, where the further fragment contains at least one gene which codes for at least one subunit of the pertussis toxin including:

a) isolating and cloning, in an E. coli lambda phage EMBL4, fragments of chromosomal DNA with 15,000-20,000 base pairs, obtained by the digestion of the chromosomal DNA of Bordetella pertussis BP 356, a mutant strain which does not produce the pertussis toxin, which contains a transposon Tn5 in its own chromosomal DNA;

b) isolating a fragment of recombinant phage DNA which hybridises with a probe marked for Tn5 DNA from the positive recombinant phages obtained;

c) cutting the fragment of recombinant phage DNA with the restriction enzyme HincII and isolating a fragment with 1900 bp containing a nucleotide sequence which codes for the subunit S3 by hybridisation with a probe for Tn5 DNA;

d) isolating and cloning in an E. coli lambda phage EMBL4 fragments of chromosomal DNA with 15,000-20,000 base pairs obtained by the digestion of the chromosomal DNA of Bordetella pertussis BP165;

e) isolating a fragment of recombinant phage DNA which hybridises with a probe for S3 as obtained at point c) from the positive recombinant phages so obtained;

f) cutting the recombinant phage DNA fragment with the restriction enzyme EcoRI and isolating an EcoRI fragment of chromosomal DNA with 4696 base pairs by hybridisation with a probe for S3 DNA;

g) inserting the fragment obtained into the plasmid vector pPEML8 in the EcoRI restriction site, determining the nucleotide sequence of the DNA fragment and identifying genes which code for the five subunits of the pertussis toxin.

16. Use of the pertussis toxin or one or more subunits of the pertussis toxin obtained as in claim 15 for the preparation of an anti-pertussis vaccine wherein the pertussis toxin is free from other B. pertussis material.

17. Use of the fragment of chromosomal DNA according to claim 1 for the preparation of diagnostic kits for the determination of pertussis infections.



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18. Use of the fragment of chromosomal DNA according to claim 1 for distinguishing B. pertussis from B. parapertussis and bronchiseptica.

**Claims for the following Contracting State : GR**

1. An EcoRI fragment of Bordetella pertussis chromosomal DNA with 4696 base pairs containing the genes which code for the five subunits of the pertussis toxin or a further fragment thereof, where the further fragment contains at least one gene which codes for at least one subunit of the pertussis toxin.

2. A DNA fragment or further fragment according to claim 1 or 2 having the sequence given in Figure 3.

3. A DNA fragment or further fragment according to claim 1 containing genes encoding subunits S1, S2, S3, S4 and S5 of the pertussis toxin and at least one of open reading frames (ORFs) A,B, C and D.

4. A hybrid plasmid containing the DNA fragment or further fragment thereof as defined in claim 1 or 2 capable of expressing the DNA fragment or further fragment when carried by a microorganism, to synthesise pertussis toxin or one or more subunits of pertussis toxin.

5. A hybrid plasmid according to claim 4, where the DNA fragment or further fragment thereof is placed under the control of region which regulates expression.

6. Hybrid plasmid pT101 according to claim 4 or 5, in which the 4696 base-pair EcoRI fragment is inserted in the EcoRI restriction site of pEMBL8.

7. Hybrid plasmid PTE255 according to claim 4 or 5 in which the Sau3A-Xba I fragment which codes for the subunit S1 is inserted in the BamHI-XbaI site of the plasmid 31B.

8. Hybrid plasmid PTE211 according to claim 4 or 5, in which the Sau96-SmaI fragment which codes for the subunit S2 is inserted in the BamHI site in the plasmid 31A.

9. Hybrid plasmid PTE221 according to claim 4 or 5, in which the SpH1-DDE1 fragment which codes for the subunit S3 is inserted in the BamHI site in the plasmid 31C.

10. Hybrid plasmid PTE240 according to claim 4 or 5, in which the BstN1-BstN1 fragment which codes for the subunit S4 is inserted in the BamHI site of the plasmid 31B.

11. Hybrid plasmid PTE230 according to claim 4 or 5, in which the fragment Aat2-SnaBI which codes for the subunit S5 is inserted in the site BamHI of the plasmid 31A.

12. A micro-organism transformed by an hybrid plasmid as defined in any one of claim 4 to 11.

13. The micro-organism Escherichia coli transformed with a hybrid plasmid according to claim 7.

14. The transformed microorganism Escherichia coli ATCC 53212.

15. A method for the preparation of the pertussis toxin or one or more of the subunits of the pertussis toxin which comprises the step of culturing a transformed microorganism as defined in any one of claims 12 to 14 in a suitable culture medium.

16. A method for the cloning and sequencing of the DNA fragment according to claim 1 or 2, including:
a) isolating and cloning, in an E. coli lambda phage EMBL4, fragments of chromosomal DNA with 15,000-20,000 base pairs, obtained by the digestion of the chromosomal DNA of Bordetella pertussis BP 356, a mutant strain which does not produce the pertussis toxin, which contains a transposon Tn5 in its own chromosomal DNA;
b) isolating a fragment of recombinant phage DNA which hybridises with a probe marked for Tn5 DNA from the positive recombinant phages obtained;
c) cutting the fragment of recombinant phage DNA with the restriction enzyme HincII and isolating a fragment with 1900 bp containing a nucleotide sequence which codes for the subunit S3 by



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hybridisation with a probe for Tn5 DNA;
d) isolating and cloning in an E. coli lambda phage EMBL4 fragments of chromosomal DNA with 15,000-20,000 base pairs obtained by the digestion of the chromosomal DNA of Bordetella pertussis BP165;
e) isolating a fragment of recombinant phage DNA which hybridises with a probe for S3 as obtained at point c) from the positive recombinant phages so obtained;
f) cutting the recombinant phage DNA fragment with the restriction enzyme EcoRI and isolating an EcoRI fragment of chromosomal DNA with 4696 base pairs by hybridisation with a probe for S3 DNA;
g) inserting the fragment obtained into the plasmid vector pPEML8 in the EcoRI restriction site, determining the nucleotide sequence of the DNA fragment and identifying genes which code for the five subunits of the pertussis toxin.

17. Use of the pertussis toxin or one or more subunits of the pertussis toxin obtained as in claim 15 for the preparation of an anti-pertussis vaccine wherein the pertussis toxin is free from other B. pertussis material.

18. A method for the preparation of a composition for the simultaneous stimulation of protection against infections of Bordetella pertussis and Vibrio colerae comprising producing the peptide S1 by chemical synthesis or by micro-organisms transformed with molecules of recombinant DNA containing the gene which codes for S1.

19. Use of the fragment of chromosomal DNA according to claim 1 for the preparation of diagnostic kits for the determination of pertussis infections.

20. Use of the fragment of chromosomal DNA according to claim 1 for distinguishing B. pertussis from B. parapertussis and bronchiseptica.

**Patentansprüche**

**Patentansprüche für folgende Vertragsstaaten : AT, BE, CH, DE, FR, GB, LI, LU, NL, SE**

1. EcoRI-Fragment aus chromosomaler DNA von Bordetella pertussis mit 4696 Basenpaaren, das die Gene enthält, die die fünf Untereinheiten des Pertussistoxins codieren, oder ein weiteres Fragment davon, wobei das weitere Fragment mindestens ein Gen enthält, das mindestens eine Untereinheit des Pertussistoxins codiert.

2. DNA-Fragment oder weiteres Fragment nach Anspruch 1 mit der in Figur 3 gezeigten Sequenz.

3. DNA-Fragment oder weiteres Fragment nach Anspruch 1 oder 2, das Gene enthält, die die Untereinheiten S1, S2, S3, S4 und S5 des Pertussistoxins codieren, und mindestens eines der offenen Leseraster (ORF) A, B, C und D.

4. Hybridplasmid, enthaltend das DNA-Fragment oder das weitere Fragment davon gemäß der Definition in Anspruch 1 oder 2, das das DNA-Fragment oder das weitere Fragment exprimieren kann, wenn es in einem Mikroorganismus enthalten ist, wobei das Pertussistoxin oder eine oder mehrere Untereinheiten des Pertussistoxins synthetisiert werden.

5. Hybridplasmid nach Anspruch 4, wobei das DNA-Fragment oder das weitere Fragment davon unter der Kontrolle eines Bereiches ist, der die Expression reguliert.

6. Hybridplasmid pT101 nach Anspruch 4 oder 5, in dem das 4696 Basenpaare umfassende EcoRI-Fragment in die EcoRI-Restriktionsstelle von pEMBL8 inseriert ist.

7. Hybridplasmid PTE255 nach Anspruch 4 oder 5, in dem das Sau3A-XbaI-Fragment, das die Untereinheit S1 codiert, in die BamHI-XbaI-Stelle des Plasmids 31B inseriert ist.

8. Hybridplasmid PTE211 nach Anspruch 4 oder 5, in dem das Sau96-SmaI-Fragment, das die Untereinheit S2 codiert, in die BamHI-Stelle des Plasmids 31A inseriert ist.



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9. Hybridplasmid PTE221 nach Anspruch 4 oder 5, in dem das SpH1-DDE1-Fragment, das die Untereinheit S3 codiert, in die BamHI-Stelle des Plasmids 31C inseriert ist.

10. Hybridplasmid PTE240 nach Anspruch 4 oder 5, in dem das BstN1-BstN1-Fragment, das die Untereinheit S4 codiert, in die BamHI-Stelle des Plasmids 31B inseriert ist.

11. Hybridplasmid PTE230 nach Anspruch 4 oder 5, in dem das Aat2-SnaBI-Fragment, das die Untereinheit S5 codiert, in die BamHI-Stelle des Plasmids 31A inseriert ist.

12. Mikroorganismus, der mit einem Hybridplasmid gemäß der Definition in einem der Ansprüche 4 bis 11 transformiert ist.

13. Mikroorganismus Escherichia coli, der mit einem Hybridplasmid nach Anspruch 7 transformiert ist.

14. Transformierter Mikroorganismus Escherichia coli ATCC 53212.

15. Verfahren zur Herstellung des Pertussistoxins oder einer oder mehrerer der Untereinheiten des Pertussistoxins, umfassend die Züchtung eines transformierten Mikroorganismus gemäß der Definition in einem der Ansprüche 12 bis 14 in einem geeigneten Kulturmedium.

16. Verfahren zur Clonierung und Sequenzierung des DNA-Fragments nach Anspruch 1 oder 2, umfassend:
a) In einem E. coli lambda-Phagen EMBL4 stattfindende Isolierung und Clonierung von Fragmenten der chromosomalen DNA mit 15 000 bis 20 000 Basenpaaren, erhalten durch die Verdauung der chromosomalen DNA von Bordetella pertussis BP 356, einem Mutantenstamm, der Pertussistoxin nicht produziert, der ein Transposon Tn5 in seiner eigenen chromosomalen DNA enthält;
b) Isolierung eines Fragments von rekombinanter Phagen-DNA, das mit einer Sonde hybridisiert, die für Tn5-DNA markiert ist, aus den erhaltenen positiven rekombinanten Phagen;
c) Spalten des Fragments von rekombinanter Phagen-DNA mit dem Restriktionsenzym HincII und Isolierung eines Fragments mit 1900 Basenpaaren, das eine Nucleotidsequenz enthält, die die Untereinheit S3 codiert, durch Hybridisierung mit einer Sonde für Tn5-DNA;
d) In einem E. coli lambda-Phagen EMBL4 stattfindende Isolierung und Clonierung von Fragmenten von chromosomaler DNA mit 15 000 bis 20 000 Basenpaaren, erhalten durch die Verdauung der chromosomalen DNA von Bordetella pertussis BP165;
e) Isolierung eines Fragments von rekombinanter Phagen-DNA, das mit einer gemäß Schritt c) erhaltenen Sonde für S3 hybridisiert, aus den dabei erhaltenen positiven rekombinanten Phagen;
f) Spalten des rekombinanten Phagen-DNA-Fragments mit dem Restriktionsenzym EcoRI und Isolierung eines EcoRI-Fragments aus chromosomaler DNA mit 4696 Basenpaaren durch Hybridisierung mit einer Sonde für S3-DNA;
g) Insertion des erhaltenen Fragments in den Plasmidvektor pPEML8 in die EcoRI-Restriktionsstelle, Bestimmung der Nucleotidsequenz des DNA-Fragments und Identifizierung der Gene, die die fünf Untereinheiten des Pertussistoxins codieren.

17. Verwendung des Pertussistoxins oder einer oder mehrerer Untereinheiten des Pertussistoxins, erhalten gemäß Anspruch 15, zur Herstellung eines anti-Pertussisimpfstoffes, in dem das Pertussistoxin frei ist von anderem B. pertussis-Material.

18. Mittel für die gleichzeitige Stimulierung des Schutzes gegen Infektionen durch Bordetella pertussis und Vibrio cholerae, enthaltend mindestens eine wirksame Menge des Peptids S1, erhalten durch chemische Synthese oder von Mikroorganismen, die mit rekombinanten DNA-Molekülen transformiert wurden, die das S1 codierende Gen enthalten.

19. Verwendung des Fragments von chromosomaler DNA nach Anspruch 1 zur Herstellung von Diagnosekits zur Bestimmung von Pertussisinfektionen.

20. Verwendung des Fragments von chromosomaler DNA nach Anspruch 1 zur Unterscheidung zwischen B. pertussis und B.parapertussis bzw. bronchiseptica.



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**Patentansprüche für folgenden Vertragsstaat : ES**

1. Verfahren zur Herstellung des Pertussistoxins oder einer oder mehrerer der Untereinheiten des Pertussistoxins, umfassend die Züchtung eines transformierten Mikroorganismus in einem geeigneten Kulturmedium, wobei der transformierte Mikroorganismus ein Mikroorganismus ist, der mit einem Hybridplasmid transformiert ist, das ein EcoRI-Fragment aus chromosomaler DNA von Bordetella pertussis mit 4696 Basenpaaren enthält, das die Gene enthält, die die fünf Untereinheiten des Pertussistoxins codieren, oder ein weiteres Fragment davon, wobei das weitere Fragment mindestens ein Gen enthält, das mindestens eine Untereinheit des Pertussistoxins codiert.

2. Verfahren zur Herstellung eines Hybridplasmids, umfassend die Insertion eines EcoRI-Fragments aus chromosomaler DNA von Bordetella pertussis mit 4696 Basenpaaren, das die Gene enthält, die die fünf Untereinheiten des Pertussistoxins codieren, oder eines weiteren Fragments davon, in ein Plasmid, wobei das weitere Fragment mindestens ein Gen enthält, das mindestens eine Untereinheit des Pertussistoxins codiert.

3. Verfahren zur Herstellung eines transformierten Mikroorganismus, umfassend die Transformation eines Mikroorganismus mit einem Hybridplasmid, das ein EcoRI-Fragment aus chromosomaler DNA von Bordetella pertussis mit 4696 Basenpaaren enthält, das die Gene enthält, die die fünf Untereinheiten des Pertussistoxins codieren, oder ein weiteres Fragment davon, wobei das weitere Fragment mindestens ein Gen enthält, das mindestens eine Untereinheit des Pertussistoxins codiert.

4. Verfahren nach Anspruch 3, wobei der Mikroorganismus Escherichia coli ist.

5. Verfahren nach Anspruch 4, wobei der transformierte Mikroorganismus ATCC 53212 ist.

6. Verfahren nach einem der Ansprüche 1 bis 5, wobei das DNA-Fragment oder das weitere Fragment davon unter der Kontrolle eines Bereiches ist, der die Expression reguliert.

7. Verfahren nach einem der Ansprüche 1 bis 6, wobei das DNA-Fragment oder das weitere Fragment Gene enthält, die die Untereinheiten S1, S2, S3, S4 und S5 des Pertussistoxins codieren, und mindestens eines der offenen Leseraster (ORF) A, B, C und D.

8. Verfahren nach einem der Ansprüche 1 bis 6, wobei das Hybridplasmid pT101 ist, in dem das 4696 Basenpaare umfassende EcoRI-Fragment in die EcoRI-Restriktionsstelle von pEMBL8 inseriert ist.

9. Verfahren nach einem der Ansprüche 1 bis 6, wobei das Hybridplasmid PTE255 ist, in dem das Sau3A-XbaI-Fragment, das die Untereinheit S1 codiert, in die BamHI-XbaI-Stelle des Plasmids 31B inseriert ist.

10. Verfahren nach einem der Ansprüche 1 bis 6, wobei das Hybridplasmid PTE211 ist, in dem das Sau96-SmaI-Fragment, das die Untereinheit S2 codiert, in die BamHI-Stelle des Plasmids 31A inseriert ist.

11. Verfahren nach einem der Ansprüche 1 bis 6, wobei das Hybridplasmid PTE221 ist, in dem das SpH1-DDE1-Fragment, das die Untereinheit S3 codiert, in die BamHI-Stelle des Plasmids 31C inseriert ist.

12. Verfahren nach einem der Ansprüche 1 bis 6, wobei das Hybridplasmid PTE240 ist, in dem das BstN1-BstN1-Fragment, das die Untereinheit S4 codiert, in die BamHI-Stelle des Plasmids 31B inseriert ist.

13. Verfahren nach einem der Ansprüche 1 bis 6, wobei das Hybridplasmid PTE230 ist, in dem das Aat2-SnaBI-Fragment, das die Untereinheit S5 codiert, in die BamHI-Stelle des Plasmids 31A inseriert ist.

14. Verfahren nach einem der Ansprüche 1 bis 13, wobei das DNA-Fragment oder das weitere Fragment die in Figur 3 gezeigte Sequenz hat.

15. Verfahren zur Clonierung und Sequenzierung eines EcoRI-Fragments aus chromosomaler DNA von Bordetella pertussis mit 4696 Basenpaaren, das die Gene enthält, die die fünf Untereinheiten des Pertussistoxins codieren, oder eines weiteren Fragments davon, wobei das weitere Fragment minde-



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stens ein Gen enthält, das mindestens eine Untereinheit des Pertussistoxins codiert, umfassend:

a) In einem E. coli lambda-Phagen EMBL4 stattfindende Isolierung und Clonierung von Fragmenten der chromosomalen DNA mit 15 000 bis 20 000 Basenpaaren, erhalten durch die Verdauung der chromosomalen DNA von Bordetella pertussis BP 356, einem Mutantenstamm, der Pertussistoxin nicht produziert, der ein Transposon Tn5 in seiner eigenen chromosomalen DNA enthält;

b) Isolierung eines Fragments von rekombinanter Phagen-DNA, das mit einer Sonde hybridisiert, die für Tn5-DNA markiert ist, aus den erhaltenen positiven rekombinanten Phagen;

c) Spalten des Fragments von rekombinanter Phagen-DNA mit dem Restriktionsenzym HincII und Isolierung eines Fragments mit 1900 Basenpaaren, das eine Nucleotidsequenz enthält, die die Untereinheit S3 codiert, durch Hybridisierung mit einer Sonde für Tn5-DNA;

d) In einem E. coli lambda-Phagen EMBL4 stattfindende Isolierung und Clonierung von Fragmenten von chromosomaler DNA mit 15 000 bis 20 000 Basenpaaren, erhalten durch die Verdauung der chromosomalen DNA von Bordetella pertussis BP165;

e) Isolierung eines Fragments von rekombinanter Phagen-DNA, das mit einer gemäß Schritt c) erhaltenen Sonde für S3 hybridisiert, aus den dabei erhaltenen positiven rekombinanten Phagen;

f) Spalten des rekombinanten Phagen-DNA-Fragments mit dem Restriktionsenzym EcoRI und Isolierung eines EcoRI-Fragments aus chromosomaler DNA mit 4696 Basenpaaren durch Hybridisierung mit einer Sonde für S3-DNA;

g) Insertion des erhaltenen Fragments in den Plasmidvektor pPEML8 in die EcoRI-Restriktionsstelle, Bestimmung der Nucleotidsequenz des DNA-Fragments und Identifizierung der Gene, die die fünf Untereinheiten des Pertussistoxins codieren.

16. Verwendung des Pertussistoxins oder einer oder mehrerer Untereinheiten des Pertussistoxins, erhalten gemäß Anspruch 15, zur Herstellung eines anti-Pertussisimpfstoffes, in dem das Pertussistoxin frei ist von anderem B. pertussis-Material.

17. Verwendung des Fragments von chromosomaler DNA nach Anspruch 1 zur Herstellung von Diagnosekits zur Bestimmung von Pertussisinfektionen.

18. Verwendung des Fragments von chromosomaler DNA nach Anspruch 1 zur Unterscheidung zwischen B. pertussis und B.parapertussis bzw. bronchiseptica.

**Patentansprüche für folgenden Vertragsstaat : GR**

1. EcoRI-Fragment aus chromosomaler DNA von Bordetella pertussis mit 4696 Basenpaaren, das die Gene enthält, die die fünf Untereinheiten des Pertussistoxins codieren, oder ein weiteres Fragment davon, wobei das weitere Fragment mindestens ein Gen enthält, das mindestens eine Untereinheit des Pertussistoxins codiert.

2. DNA-Fragment oder weiteres Fragment nach Anspruch 1 mit der in Figur 3 gezeigten Sequenz.

3. DNA-Fragment oder weiteres Fragment nach Anspruch 1, das Gene enthält, die die Untereinheiten S1, S2, S3, S4 und S5 des Pertussistoxins codieren, und mindestens eines der offenen Leseraster (ORF) A, B, C und D.

4. Hybridplasmid, enthaltend das DNA-Fragment oder das weitere Fragment davon gemäß der Definition in Anspruch 1 oder 2, das das DNA-Fragment oder das weitere Fragment exprimieren kann, wenn es in einem Mikroorganismus enthalten ist, wobei das Pertussistoxin oder eine oder mehrere Untereinheiten des Pertussistoxins synthetisiert werden.

5. Hybridplasmid nach Anspruch 4, wobei das DNA-Fragment oder das weitere Fragment davon unter der Kontrolle eines Bereiches ist, der die Expression reguliert.

6. Hybridplasmid pT101 nach Anspruch 4 oder 5, in dem das 4696 Basenpaare umfassende EcoRI-Fragment in die EcoRI-Restriktionsstelle von pEMBL8 inseriert ist.

7. Hybridplasmid PTE255 nach Anspruch 4 oder 5, in dem das Sau3A-XbaI-Fragment, das die Untereinheit S1 codiert, in die BamHI-XbaI-Stelle des Plasmids 31B inseriert ist.



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8. Hybridplasmid PTE211 nach Anspruch 4 oder 5, in dem das Sau96-SmaI-Fragment, das die Untereinheit S2 codiert, in die BamHI-Stelle des Plasmids 31A inseriert ist.

9. Hybridplasmid PTE221 nach Anspruch 4 oder 5, in dem das SpH1-DDE1-Fragment, das die Untereinheit S3 codiert, in die BamHI-Stelle des Plasmids 31C inseriert ist.

10. Hybridplasmid PTE240 nach Anspruch 4 oder 5, in dem das BstN1-BstN1-Fragment, das die Untereinheit S4 codiert, in die BamHI-Stelle des Plasmids 31B inseriert ist.

11. Hybridplasmid PTE230 nach Anspruch 4 oder 5, in dem das Aat2-SnaBI-Fragment, das die Untereinheit S5 codiert, in die BamHI-Stelle des Plasmids 31A inseriert ist.

12. Mikroorganismus, der mit einem Hybridplasmid gemäß der Definition in einem der Ansprüche 4 bis 11 transformiert ist.

13. Mikroorganismus Escherichia coli, der mit einem Hybridplasmid nach Anspruch 7 transformiert ist.

14. Transformierter Mikroorganismus Escherichia coli ATCC 53212.

15. Verfahren zur Herstellung des Pertussistoxins oder einer oder mehrerer der Untereinheiten des Pertussistoxins, umfassend die Züchtung eines transformierten Mikroorganismus gemäß der Definition in einem der Ansprüche 12 bis 14 in einem geeigneten Kulturmedium.

16. Verfahren zur Clonierung und Sequenzierung des DNA-Fragments nach Anspruch 1 oder 2, umfassend:
a) In einem E. coli lambda-Phagen EMBL4 stattfindende Isolierung und Clonierung von Fragmenten der chromosomalen DNA mit 15 000 bis 20 000 Basenpaaren, erhalten durch die Verdauung der chromosomalen DNA von Bordetella pertussis BP 356, einem Mutantenstamm, der Pertussistoxin nicht produziert, der ein Transposon Tn5 in seiner eigenen chromosomalen DNA enthält;
b) Isolierung eines Fragments von rekombinanter Phagen-DNA, das mit einer Sonde hybridisiert, die für Tn5-DNA markiert ist, aus den erhaltenen positiven rekombinanten Phagen;
c) Spalten des Fragments von rekombinanter Phagen-DNA mit dem Restriktionsenzym HincII und Isolierung eines Fragments mit 1900 Basenpaaren, das eine Nucleotidsequenz enthält, die die Untereinheit S3 codiert, durch Hybridisierung mit einer Sonde für Tn5-DNA;
d) In einem E. coli lambda-Phagen EMBL4 stattfindende Isolierung und Clonierung von Fragmenten von chromosomaler DNA mit 15 000 bis 20 000 Basenpaaren, erhalten durch die Verdauung der chromosomalen DNA von Bordetella pertussis BP165;
e) Isolierung eines Fragments von rekombinanter Phagen-DNA, das mit einer gemäß Schritt c) erhaltenen Sonde für S3 hybridisiert, aus den dabei erhaltenen positiven rekombinanten Phagen;
f) Spalten des rekombinanten Phagen-DNA-Fragments mit dem Restriktionsenzym EcoRI und Isolierung eines EcoRI-Fragments aus chromosomaler DNA mit 4696 Basenpaaren durch Hybridisierung mit einer Sonde für S3-DNA;
g) Insertion des erhaltenen Fragments in den Plasmidvektor pPEML8 in die EcoRI-Restriktionsstelle, Bestimmung der Nucleotidsequenz des DNA-Fragments und Identifizierung der Gene, die die fünf Untereinheiten des Pertussistoxins codieren.

17. Verwendung des Pertussistoxins oder einer oder mehrerer Untereinheiten des Pertussistoxins, erhalten gemäß Anspruch 15, zur Herstellung eines anti-Pertussisimpfstoffes, in dem das Pertussistoxin frei ist von anderem B. pertussis-Material.

18. Verfahren zur Herstellung eines Mittels für die gleichzeitige Stimulierung des Schutzes gegen Infektionen durch Bordetella pertussis und Vibrio cholerae, umfassend die Produktion des Peptids S1 durch chemische Synthese oder durch Mikroorganismen, die mit rekombinanten DNA-Molekülen transformiert wurden, die das S1 codierende Gen enthalten.

19. Verwendung des Fragments von chromosomaler DNA nach Anspruch 1 zur Herstellung von Diagnosekits zur Bestimmung von Pertussisinfektionen.



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20. Verwendung des Fragments von chromosomaler DNA nach Anspruch 1 zur Unterscheidung zwischen B. pertussis und B.parapertussis bzw. bronchiseptica.

**Revendications**

**Revendications pour les Etats contractants suivants : AT, BE CH, DE, FR, GB, LI, LU, NL, SE**

1. Fragment EcoRI d'ADN chromosomique de Bordetella pertussis avec 4696 paires de bases contenant les gènes qui codent pour les cinq sous-unités de la toxine de pertussis ou un autre de ses fragments, où l'autre fragment contient au moins un gène qui code pour au moins une sous-unité de la toxine de pertussis.

2. Fragment d'ADN ou autre fragment selon la revendication 1 ayant la séquence donnée dans la figure 3.

3. Fragment d'ADN ou autre fragment selon la revendication 1 ou 2 contenant les gènes codant pour les sous-unités S1, S2, S3, S4 et S5 de la toxine de pertussis et au moins un des cadres ouverts de lecture (ORF) A, B, C et D.

4. Plasmide hybride contenant le fragment d'ADN ou autre de ses fragments tel que défini dans le revendication 1 ou 2 capable d'exprimer le fragment d'ADN ou autre fragment lorsqu'il est porté par un microorganisme, pour synthétiser la toxine de pertussis ou une ou plusieurs sous-unités de toxine de pertussis.

5. Plasmide hybride selon la revendication 4, dans lequel le fragment d'ADN ou autre de ses fragments est placé sous la commande d'une région qui régule l'expression.

6. Plasmide hybride pT101 selon la revendication 4 ou 5, dans lequel le fragment EcoRI a 4696 paires de bases est inséré dans le site de restriction EcoRI de pEMBL8.

7. Plasmide hybride PTE255 selon la revendication 4 ou 5 dans lequel le fragment Sau3A-Xba I qui code pour la sous-unité S1 est inséré dans le site BamHI-XbaI du plasmide 31B.

8. Plasmide hybride PTE211 selon la revendication 4 ou 5, dans lequel le fragment Sau96-SmaI qui code pour la sous-unité S2 est inséré dans le site BamHI dans le plasmide 31A.

9. Plasmide hybride PTE221 selon la revendication 4 ou 5, dans lequel le fragment SpH1-DDE1 qui code pour la sous-unité S3 est inséré dans le site BamHI dans le plasmide 31C.

10. Plasmide hybride PTE240 selon la revendication 4 ou 5, dans lequel le fragment BstN1-BstN1 qui code pour la sous-unité S4 est inséré dans le site BamHI du plasmide 31B.

11. Plasmide hybride PTE230 selon la revendication 4 ou 5, dans lequel le fragment Aat2-SnaBI qui code pour la sous-unité S5 est inséré dans le site BamHI du plasmide 31A.

12. Micro-organisme transformé par un plasmide hybride tel que défini dans l'une quelconque des revendications 4 à 11.

13. Micro-organisme Escherichia coli transformé avec un plasmide hybride selon la revendication 7.

14. Le micro-organisme transformé Escherichia coli ATCC 53212.

15. Procédé de préparation de la toxine de pertussis ou d'une ou plusieurs des sous-unités de la toxine de pertussis qui comprend l'étape de mise en culture d'un micro-organisme transformé tel que défini dans l'une quelconque des revendications 12 à 14 dans un milieu de culture approprié.

16. Procédé de clonage et de séquençage du fragment d'ADN selon la revendication 1 ou 2, dans lequel:
    a) on isole et on clone, dans un phage lambda EMBL4 d'E. coli, des fragments d'ADN chromosomique à 15 000 - 20 000 paires de bases, obtenus par digestion de l'ADN chromosomique de Bordetella pertussis BP 356, une souche mutante qui ne produit pas la toxine de pertussis, qui



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contient un transposon Tn5 dans son propre ADN chromosomique;
b) on isole un fragment d'ADN de phage recombinant qui s'hybride avec une sonde marquée pour l'ADN de Tn5 provenant des phages recombinants positifs obtenus;
c) on découpe le fragment d'ADN de phage recombinant avec l'enzyme de restriction HincII et on isole un fragment avec 1900 pb contenant une séquence de nucléotide qui code pour la sous-unité S3 par hybridation avec une sonde pour l'ADN de Tn5;
d) on isole et on clone dans un phage lambda EMBL4 d'E. coli des fragments d'ADN chromosomique à 15 000 - 20 000 paires de bases obtenus par digestion de l'ADN chromosomique de Bordetella pertussis BP165;
e) on isole un fragment d'ADN de phage recombinant qui s'hybride avec une sonde pour S3 telle qu'obtenue au point c) à partir des phages recombinants positifs ainsi obtenus;
f) on découpe le fragment d'ADN de phage recombinant avec l'enzyme de restriction EcoRI et on isole un fragment EcoRI d'ADN chromosomique à 4696 paires de bases par hybridation avec une sonde pour l'ADN de S3;
g) on insère le fragment obtenu dans le vecteur plasmidique pPEML8 dans le site de restriction EcoRI, on détermine la séquence nucléotidique du fragment d'ADN et on identifie les gènes qui codent pour les cinq sous-unités de la toxine de pertussis.

17. Application de la toxine de pertussis ou d'une ou plusieurs sous-unités de la toxine de pertussis obtenue comme dans la revendication 15, à la préparation d'un vaccin anti-pertussis dans lequel la toxine de pertussis est dépourvue d'autre matière de B. pertussis.

18. Composition pour la stimulation simultanée de la protection contre les infections par Bordetella pertussis et Vibrio colerae incluant au moins une quantité efficace du peptide S1 obtenu par synthèse chimique ou à partir de micro-organismes transformés avec des molécules d'ADN recombinant contenant le gène qui code pour S1.

19. Application du fragment d'ADN chromosomique selon la revendication 1 à la préparation de trousses de diagnostic pour la détermination d'infection à pertussis.

20. Application du fragment d'ADN chromosomique selon la revendication I pour distinguer B. pertussis de B. parapertussis et bronchiseptica.

**Revendications pour l'Etat contractant suivant : ES**

1. Procédé de préparation de la toxine de pertussis ou d'une ou plusieurs des sous-unités de la toxine de pertussis comprenant l'étape de mise en culture d'un micor-organisme transformé dans un milieu de culture approprié, où le micro-organisme transformé est un micro-organisme transformé avec un plasmide hybride contenant un fragment EcoRI d'ADN chromosomique de Bordetella pertussis à 4696 paires de bases contenant les gènes qui codent pour les cinq sous-unités de la toxine de pertussis ou un autre de ses fragments, où l'autre fragment contient au moins un gène qui code pour au moins une sous-unité de la toxine de pertussis.

2. Procédé de préparation d'un plasmide hybride dans lequel on insère dans un plasmide un fragment EcoRI d'ADN chromosomique de Bordetella pertussis à 4696 paires de bases contenant les gènes qui codent pour les cinq sous-unités de la toxine de pertussis ou un autre de ses fragments, où l'autre fragment contient au moins un gène qui code pour au moins une sous-unité de la toxine de pertussis.

3. Procédé de préparation d'un micro-organisme transformé dans lequel on transforme un micro-organisme avec un plasmide hybride contenant un fragment EcoRI d'ADN chromosomique de Bordetella pertussis à 4696 paires de bases contenant les gènes qui codent pour les cinq sous-unités de la toxine de pertussis ou un autre de ses fragments, où l'autre fragment contient au moins un gène qui code pour au moins une sous-unité de la toxine de pertussis.

4. Procédé selon la revendication 3 dans lequel le micro-organisme est Escherichia coli.

5. Procédé selon la revendication 4 dans lequel le micro-organisme transformé est ATCC 53212.



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6. Procédé selon l'une quelconque des revendications 1 à 5 dans lequel le fragment d'ADN ou autre fragment est placé sous la commande d'une région qui régule l'expression.

7. Procédé selon l'une quelconque des revendications 1 à 6 dans lequel le fragment d'ADN ou autre fragment contient des gènes codant pour les sous-unités S1, S2, S3, S4 et S5 de la toxine de pertussis et au moins un des cadres ouverts de lecture (ORF) A, B, C et D.

8. Procédé selon l'une quelconque des revendications 1 à 6 dans lequel le plasmide hybride est pT101 dans lequel le fragment EcoRI à 4696 paires de bases est inséré dans le site de restriction EcoRI de pEMBL8.

9. Procédé selon l'une quelconque des revendications 1 à 6 dans lequel le plasmide hybride est PTE255 où le fragment Sau3A-Xba I qui code pour la sous-unité S1 est inséré dans le site BamHI-XbaI du plasmide 31B.

10. Procédé selon l'une quelconque des revendications 1 à 6 dans lequel le plasmide hybride est PTE211 selon la revendication 4 ou 5, où le fragment Sau96-SmaI qui code pour la sous-unité S2 est inséré dans le site BamHI dans le plasmide 31A.

11. Procédé selon l'une quelconque des revendications 1 à 6 dans lequel le plasmide hybride est PTE221 où le fragment SpH1-DDE1 qui code pour la sous-unité S3 est inséré dans le site BamHI dans le plasmide 31C.

12. Procédé selon l'une quelconque des revendications 1 à 6 dans lequel le plasmide hybride est PTE240 où le fragment BstN1-BstN1 qui code pour la sous-unité S4 est inséré dans le site BamHI du plasmide 31B.

13. Procédé selon l'une quelconque des revendications 1 à 6 dans lequel le plasmide hyride est PTE230 où le fragment Aat2-SnaBI qui code pour la sous-unité S5 est inséré dans le site BamHI du plasmide 31A.

14. Procédé selon l'une quelconque des revendications 1 à 13 dans lequel le fragment d'ADN ou autre fragment a la séquence donnée dans la figure 3.

15. Procédé de clonage et de séquençage d'un fragment EcoRI d'ADN chromosomique de Bordetella pertussis à 4696 paires de bases contenant les gènes qui codent pour les cinq sous-unités de la toxine de pertussis ou un autre de ses fragments, ou l'autre fragment contient au moins un gène qui code pour au moins une sous-unité de la toxine de pertussis, dans lequel:
a) on isole et on clone, dans un phage lambda EMBL4 d'E. coli, des fragments d'ADN chromosomique à 15 000 - 20 000 paires de bases, obtenu par digestion de l'ADN chromosomique de Bordetella pertussis BP 356, une souche mutante qui ne produit pas la toxine de pertussis, qui contient un transposon Tn5 dans son propre ADN chromosomique;
b) on isole un fragment d'ADN de phage recombinant qui s'hybride avec une sonde marquée pour l'ADN de Tn5 à partir des phages recombinants positifs obtenus;
c) on découpe le fragment d'ADN de phage recombinant avec l'enzyme de restriction HincII et on isole un fragment à 1900 pb contenant une séquence de nucléotide qui code pour la sous-unité S3 par hybridation avec une sonde pour l'ADN de Tn5;
d) on isole et on clone dans un phage lambda EMBL4 d'E. coli des fragments d'ADN chromosomique à 15 000 - 20 000 paires de bases obtenus par la digestion de l'ADN chromosomique de Bordetella pertussis BP165;
e) on isole un fragment d'ADN de phage recombinant qui s'hybride avec une sonde pour S3 telle qu'obtenue au point c) à partir des phages recombinants positifs ainsi obtenus;
f) on découpe le fragment d'ADN de phage recombinant avec l'enzymle de restriction EcoRI et on isole un fragment EcoRI d'ADN chromosomique à 4696 paires de bases par hybridation avec une sonde pour l'ADN de S3;
g) on insère le fragment obtenu dans le vecteur plasmidique pPEML8 dans le site de rectriction EcoRI, on détermine la séquence nucléotidique du fragment d'ADN et on identifie les gènes qui codent pour les cinq sous-unités de la toxine de pertussis.



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16. Application de la toxine de pertussis ou d'une ou plusieurs sous-unités de la toxine de pertussis obtenue selon la revendication 15 à la préparation d'un vaccin anti-pertussis dans lequel la toxine de pertussis est dépourvue de toute autre matière de B. pertussis.

17. Application du fragment d'ADN chromosomique selon la revendication 1 à la préparation de trousses de diagnostic pour la détermination des infections à pertussis.

18. Application du fragment d'ADN chromosomique selon la revendication 1 pour distinguer B. pertussis d'avec B. parapertussis et bronchiseptica.

**Revendications pour l'Etat contractant suivant : GR**

1. Fragment EcoRI d'ADN chromosomique de Bordetella pertussis à 4696 paires de bases contenant les gènes qui codent pour les cinq sous-unités de la toxine de pertussis ou un autre de ses fragments, où l'autre fragment contient au moins un gène qui code pour au moins une sous-unité de la toxine de pertussis.

2. Fragment d'ADN ou autre fragment selon la revendication 1 ou 2 ayant la séquence donnée dans la figure 3.

3. Fragment d'ADN ou autre fragment selon la revendication 1 contenant des gènes codant pour des sous-unités S1, S2, S3, S4 et S5 de la toxine de pertussis et au moins un des cadres ouverts de lecture (ORF) A, B, C et D.

4. Plasmide hybride contenant le fragment d'ADN ou autre fragment tel que défini dans la revendication 1 ou 2 capable d'exprimer le fragment d'ADN ou autre fragment lorsqu'il est porté par un micro-organisme, pour synthétiser la toxine de pertussis ou une ou plusieurs sous-unités de toxine de pertussis.

5. Plasmide hybride selon la revendication 4, dans lequel le fragment d'ADN ou autre fragment est placé sous la commande d'une région qui régule l'expression.

6. Plasmide hybride pT101 selon la revendication 4 ou 5, dans lequel le fragment EcoRI à 4696 paires de bases est inséré dans le site de restriction EcoRI de pEMBL8.

7. Plasmide hybride PTE255 selon la revendication 4 ou 5 dans lequel le fragment Sau3A-Xba I qui code pour la sous-unité S1 est inséré dans le site BamHI-XbaI du plasmide 31B.

8. Plasmide hybride PTE211 selon la revendication 4 ou 5, dans lequel le fragment Sau96-SmaI qui code pour la sous-unité S2 est inséré dans le site BamHI dans le plasmide 31A.

9. Plasmide hybride PTE 221 selon la revendication 4 ou 5, dans lequel le fragment SpH1-DDE1 qui code pour la sous-unité S3 est inséré dans le site BamHI dans le plasmide 31C.

10. Plasmide hybride PTE240 selon la revendication 4 ou 5, dans lequel le fragment BstN1-BstN1 qui code pour la sous-unité S4 est inséré dans le site BamHI du plasmide 31B.

11. Plasmide hybride PTE230 selon la revendication 4 ou 5, dans lequel le fragment Aat2-SnaBI qui code pur la sous-unité S5 est inséré dans le site BamHI du plasmide 31A.

12. Micro-organisme transformé par un plasmide hybride tel que défini dans l'une quelconque des revendications 4 à 11.

13. Le micro-organisme Escherichia coli transformé avec un plasmide hybride selon la revendication 7.

14. Le micro-organisme transformé Escherichia coli ATCC 53212.



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15. Procédé de préparation de la toxine de pertussis ou d'une ou plusieurs des sous-unités de la toxine de pertussis comprenant l'étape de mise en culture d'un micro-organisme transformé tel que défini dans l'une quelconque des revendications 12 à 14 dans un milieu de culture approprié.

16. Procédé de clonage et de séquençage du fragment d'ADN selon la revendication 1 ou 2, dans lequel:
a) on isole et on clone, dans un phage lambda EMBL4 d'E. coli, des fragments d'ADN chromosomique à 15 000 - 20 000 paires de bases, obtenus par digestion de l'ADN chromosomique de Bordetella pertussis BP 356, souche mutante qui ne produit pas la toxine de pertussis, qui contient un transposon Tn5 dans son propre ADN chromosomique;
b) on isole un fragment d'ADN de phage recombinant qui s'hybride avec une sonde marquée pour l'ADN Tn5 provenant des phages recombinants positifs obtenus;
c) on découpe le fragment d'ADN de phage recombinant avec l'enzyme de restriction HincII et on isole un fragment à 1900 pb contenant une séquence nucléotidique qui code pour la sous-unité S3 par hybridation avec une sonde pour l'ADN de Tn5;
d) on isole et on clone dans un phage lambda EMBL4 d'E. coli des fragments d'ADN chromosomique à 15 000 - 20 000 paires de bases obtenus par digestion de l'ADN chromosomique de Bordetella pertussis BP165;
e) on isole un fragment d'ADN de phage recombinant qui s'hybride avec une sonde pour S3 telle qu'obtenue au point c) à partir des phages recombinants positifs ainsi obtenus;
f) on découpe le fragment d'ADN de phage recombinant avec l'enzyme de restriction EcoRI et on isole un fragment EcoRI d'ADN chromosomique à 4696 paires de bases par hybridation avec une sonde pour l'ADN de S3;
g) on insère le fragment obtenu dans le vecteur plasmidique pPEML8 dans le site de restriction EcoRI, on détermine la séquence nucléotidique du fragment d'ADN et on identifie les gènes qui codent pour les cinq sous-unités de la toxine de pertussis.

17. Application de la toxine de pertussis ou d'fune ou plusieurs sous-unités de la toxine de pertussis obtenues comme dans la revendication 15 à la préparation d'un vaccin anti-pertussis dans lequel la toxine de pertussis est dépourvue d'autre matière de B. pertussis.

18. Procédé de préparation d'une composition pour la stimulation simultanée de la protection contre les infections à Bordetella pertussis et Vibrio colerae dans lequel on prépare le peptide S1 par synthèse chimique ou par des micro-organismes transformés avec des molécules d'ADN recombinant contenant le gène qui code pour S1.

19. Application du fragment d'ADN chromosomique selon la revendication 1 à la préparation de trousses de diagnostic pour la détermination des infections à pertussis.

20. Application du fragment d'ADN chromosomique selon la revendication 1 pour distinguer B. pertussis de B. parapertussis et bronchiseptica.



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