

Description

The present invention relates to the cloning and sequencing of the gene which codes for a new proteinaceous subunit of the pili of *Bordetella pertussis*.

The invention also concerns a recombinant plasmid which includes the gene or fragments thereof and a host microorganism transformed by the recombinant plasmid.

The present invention also relates to immunologically-active synthetic peptides which have an aminoacid sequence identical to that of the proteinaceous subunit.

Pertussis is a disease of the respiratory tract caused by *Bordetella pertussis* (*B. pertussis*), a microorganism which is transmitted from a sick person to a susceptible healthy individual during the catarrhal and convulsive stage.

Pertussis may cause convulsions, brain damage and sometimes death, particularly in infants and in newborn babies without maternal anti-pertussis antibodies. An effective vaccine against the disease is therefore particularly desirable.

A cellular vaccine against pertussis is currently used which is constituted by virulent bacteria killed with merthiolate and treated at 56°C and which, whilst providing permanent protection, may induce undesirable side effects. There is therefore a need to develop new acellular vaccines against infection by *B. pertussis* which do not have the disadvantages described above.

An essential step in the pathogenesis of pertussis is represented by the adhesion of the bacteria to the epithelial cells of the upper respiratory tract which thus enables the microorganism to elude the defensive system of the host.

Although it is not yet completely clear which components of the cell surface of the bacterium are involved in this process, it seems, however, that this adhesion takes place by means of extracellular proteins constituted by polymerised subunits, known as fimbriae or pili present on the surface of the bacterium.

Ashworth et al. (1982) (*Infect. Immun.* 37: 1278-1281) first suggested that the fimbriae of *B. pertussis* were serotype-specific agglutinogens, that is superficial antigens which stimulate the production of antibodies which agglutinate the bacterial cells.

Irons and collaborators (*Dev. Biol. Standard.* 61, 153-163 (1985)) isolated and purified *B. pertussis* fimbriae which were classified as serotype 2 and 3 agglutinogens. The fimbriae appeared to be constituted by subunits with molecular weights of 21,000, 22,000 and 24,000 daltons.

As well as the above agglutinogens, fimbriae of serotypes 1, 4, 5 and 6 have now been isolated and purified. Numerous studies have been carried out concerning their role in adhesion to the epithelial cells (Urisu, T.H. et al (1986) *Infect. Immun.* 52 : 695-701), their immunogenic activity (Zhang J.M. et al. *Dev. Biol. Stand.* 61: 173-185 (1985) and their structure (Zhang J.M. *Infect. Immun.* 48 : 422-427 (1985)), with a view to the use of the fimbriae or subunits thereof for the development of an acellular vaccine which is protective against pertussis. Although it has been observed that mice inoculated with purified *B. pertussis* pili were protected against subsequent intranasal infection with the virulent bacterium (Robinson et al (1985) *Dev. Biol. Stand.* 61 : 165-172), the design of an acellular vaccine based on the purified pili or subunits thereof isolated from pili (pilins) must take into account the antigenic variations observed in different strains of *B. pertussis*.

In fact, immunisation with fimbriae of a particular serotype does not always confer protective immunity against infections caused by a strain containing a different serotype.

Thus, for example, anti-serotype 2 antibodies agglutinate only *B. pertussis* cells containing type 2 agglutinin and the same applies to anti-serotype 6 antibodies (Cowell J.L. et al. (1987) *Inf. and Immun.* Vol. 55, N.4, 916-922).

Moreover, the observation that anti-serotype 2 and 3 monoclonal antibodies inhibit the binding of *B. pertussis* to VIRO cells in a serotype-specific manner *in vitro* (Gorringe et al. (1985) *FEMS Microbiol. Sect.* 26: 5-9) suggests that the fimbriae are antigenically different. It therefore seems to be of fundamental importance to acquire further knowledge concerning the number of different antigenic types of fimbriae which can be expressed by *B. pertussis* in order to be able to develop a completely satisfactory polyvalent acellular vaccine.

The gene which codes for the larger pilin subunit which corresponds to serotype 2 has recently been cloned and sequenced (Livey J. et al. (1987) *Molecular Microbiol.* 1 (2) 203-209).

A study was recently started to identify by molecular cloning a third previously unknown fimbriae subunit gene which was supposed to be present in *Bordetella pertussis* (Mool et al., *Microbiol. Pathog.* 2 (1987), 437-484).

A new gene which codes for a new pilin subunit of *B. pertussis* has now been isolated and sequenced. The aminoterminal sequence of the mature portion of the subunit is similar, but not identical to that of pilins 2, 3 and 6.

A subject of the present invention is therefore the cloning and sequencing of the gene which codes for a new pilin subunit of *Bordetella pertussis*.

Another subject of the present invention is constituted by a recombinant plasmid, characterised in that it contains the gene which codes for the subunit or fragments thereof, and host microorganisms transformed by the recombinant plasmid.

A further subject of the present invention is constituted by immunologically-active peptides with an aminoacid sequence identical to that of the pilin subunit or to regions thereof.

Another subject of the present invention is constituted by the use of the peptides for the preparation of an acellular vaccine against pertussis. Further subjects of the present invention will become clear from a reading of the text and from the examples which follow.

For a better understanding of the present invention, a brief description of the terms used is given below.
 15 Gene Library or genome bank - this term means the of clones of a host microorganism each of which carries a DNA fragment derived from the donor organism whose bank is to be produced.

A genome bank is defined as representative when the group of individual fragments contained in each clone reconstitute the whole chromosomal DNA of the organism.

Expression - indicates the mechanism by which a host organism can synthesise the protein for which a particular gene codes.

It includes a process of transcription, that is the transmission of the genetic information from the DNA to the messenger RNA (mRNA), and of translation, that is the transmission of the information from the mRNA to the protein according to the rules of the genetic code, described by J.D. Watson (Molecular Biology of the Gene, W.A. Benjamin Inc. N.Y., 3rd ed. 1976), in which the codons, that is the triplets of nucleotide
 25 bases, which code for a particular aminoacid are given. Various codons may code for the same aminoacid but, for each aminoacid, there are only certain codons and no others.

Restriction enzymes - are hydrolytic enzymes which can cut a DNA molecule at specific sites, the recognition sites of the restriction enzymes.

Cloning vectors - are DNA molecules which contain all the genetic information which enable their replication when transferred into a host microorganism. Examples of cloning vectors are plasmids, the DNA of some bacteriophages and cosmids.

Plasmid DNA, which is circular in shape, can be cut by appropriate techniques and a fragment of heterologous DNA can be inserted and the ring reclosed to form a larger molecule: - a recombinant DNA molecule or hybrid plasmid. The vectors are used to insert the heterologous DNA fragments, that is
 35 fragments of DNA which code for a protein not generally produced by the organism transformed by the vector.

Primer: is an oligonucleotide of 15-50 bases which is complementary to the region of the single strand which is to be sequenced by an enzymatic method.

40 Brief description of the appended drawings:

Figure 1: shows the nucleotide sequence of the gene which codes for STX pilin obtained from the SA1 strain of *B. pertussis* and the corresponding aminoacid sequence.

The synthetic oligonucleotides used for identifying and sequencing the gene are indicated by the
 45 arrows (→ ←).

The initial methionine, the first aminoacid of the mature protein and its stop codon are shown.

The cutting site between the mature portion of STX pilin and the signal sequence is indicated by the arrow (↓). The Bam HI and Eco RI restriction sites are underlined (-----).

Figure 2: shows the comparison between the NH₂ terminal sequences of the mature portions of the
 50 STX, ST2, ST3 and ST6 pilins.

The identical aminoacid residues are grouped in boxes.

Figure 3: homology between STX pilin (top row) and ST2 pilin (bottom row).

Recent progress in the cloning of genes associated with the virulence of *B. pertussis* indicates that the most suitable methods for their identification are those which are based, not on the direct expression of the genes in *E.coli* (Locht et al. (1986) Nucleic Acid Res. 14 : 3251-3261; Nicosia et al (1986) P.N.A.S. USA 83 :
 55 4631-4635), but on the identification, with the use of specific probes, of the cloned DNA fragments which contain the genes.

According to the present invention, therefore, the aminoterminal region of the principal proteinaceous subunit of the fimbriae of *B. pertussis* strain 165 (serotype 1, 2, 3) has been sequenced by means of Edmans degradation and purified by the method described by Zhang, J.M. et al (1985); Dev. Biol. Stand. 61 : 173-185).

The aminoacid sequence shown below:

1 2 3 4 5 6 7 8 9 10 11 12
NH-Asp-Asp Gly-Thr Ile Val Ile Thr Gly Thr Ile Thr

which was subsequently confirmed as being identical to that of the major subunit of the fimbriae of serotype 2 (Livey J et al. Molecular Microbiol. (1987) 1 (2) 203-209), was used to synthesise oligonucleotides, account being taken of the relative degeneracy of the genetic code.

The region which corresponds to aminoacids 7-12 was selected for this purpose and a family of oligonucleotides, representative of all the possible combinations of triplets, with the third base degenerate was synthesised by means of an automatic system.

This family was designated probe A, and is shown below:

7	8	9	10	11	12
Ile	Thr	Gly	Thr	Ile	Thr
ATC	ACC	GGC	ACC	ATC	AC
T	T	T	T	T	
A	G	G	G	A	
		A			

The probe A, marked by the method of Arrand J.E. (Nucleic Acid Hybridisation: A Practical Approach, Edited by B.D. Hames - S. Higgins, Press Washington DC 1985, P. 34) was then used to identify within a *B. pertussis* gene library those clones which carry the chromosomal DNA fragment which hybridises with the probe.

According to the present invention, a gene library representative of the genome of *B. pertussis*, was constructed by the digestion of the chromosomal DNA of the bacterium with suitable restriction enzymes.

Various strains of *B. pertussis* may be used for this purpose.

In particular the commercially available *B. pertussis* strain 165 (serotype 1, 2, 3) (SCLAVO) was used.

The chromosomal DNA of this strain was extracted from the lysed cells, digested with Sau3A restriction enzyme in the manner suggested by the suppliers and recovered by precipitation with ethanol and centrifuging. The chromosomal DNA fragments thus obtained were then introduced into a cloning vector and, after hybridisation with the probe A, were sequenced by the method of Sanger F. et al (1977) (P.N.A.S. 74, 5463).

This method is based on an enzymatic sequencing system and is particularly suitable for the determination of sequences of long and little-known DNA fragments.

It requires the availability of the fragment which is to be sequenced in single stranded form, and of a small primer oligonucleotide (15-50 bases) which is complementary to the initial region of the single strand. The DNA fragments were accordingly cloned in an M13 phage vector. The preferred phage vectors used were M13mp8 (Messing J. et al. (1981) Nucleic Acid Reser. 9, 309) and M13mpg (Messing J. et al. (1982), Gene, 19 263) in which the position of the cloning sites is arranged in a reverse manner to the annealing site of the primer enabling the sequence of an insert to be defined starting from the two opposite ends. In practice, the phages, digested beforehand with BamHI enzyme, were ligated to the DNA fragments in the presence of T4 DNA ligase enzyme by generally known techniques.

The ligation mixture was then used to transform *E.coli* cells made competent with CaCl_2 by the technique described by Mandel M. and Higa (1970) (J. Mol. Biol. 53, 154).

The transformants were then selected on a suitable culture medium at a temperature of from 35° to 40° C over night.

The positive plaques (white) thus obtained were then transferred to nitrocellulose filters and hybridised with the probe A marked according to J.E. Arrand's method.

Of the plaques which gave a positive hybridisation signal, two were then used for the sequencing of the single stranded DNA by Sanger's method, with the use of the successive primers strategy (Strauss E.C. et al. 1986, Anal.Biochem. 154, 353).

It was thus found that the sequence of the single strand of one plaque contained a nucleotide region which is identical to that deduced from the N-terminal aminoacid sequence used for the preparation of the probe and corresponds to the N-terminal region of the serotype 2 pilin subunit for which the ST2 gene codes.

The single strand of the other plaque however contained a region with a nucleotide sequence which is similar but not identical to that of the ST2 gene, which suggested the presence of a different gene.

Since this clone contained only a portion of the gene, designated STX, in order to obtain the whole chromosomal DNA fragment containing the gene, a gene library of *B. pertussis* was constructed with the use of a cosmid as the cloning vector.

Cosmids are certain plasmids in which the cos end of the lambda phage has been inserted. They enable the recombinant molecule to be packed with the viral proteins so as to form viral particles. The DNA of the cosmids is then injected into the bacteria by the viral particle as if it were lambda DNA.

Since lambda DNA is very large whilst the cosmid is relatively small, long fragments of DNA can be inserted in the vectors.

In practice the cosmid pCH79 (Hohn B. et al. (1980) Gene 11: 291-298) and the *B. pertussis* strain SA1 (Arico, B. and Rappuoli R. (1987) J. Bacteriol. 169: 2847-2853), were used.

The chromosomal DNA of *B. pertussis* SA1 was separated from the lysed cells and partially digested with SmaI enzyme.

After precipitation by ethanol and separation by centrifuging, the chromosomal DNA fragments of 35-45 kbases were isolated and ligated in the presence of T4DNA ligase, with the cosmid which had previously been digested with Bam HI enzyme. This ligation mixture was then used to transform *E.coli* cells and the transformants were selected on a selective medium to which ampicillin had been added.

The positive colonies were then analysed by the hybridisation technique with the use of two pairs of probes: 1-2 and 3-4, which are complementary to different portions of the previously-determined region of STX and ST2.

In particular, the probes, which are synthesised by means of an automatic system, have the following sequences:

Probe 1 (STX) C C G C C C A T G C C G A A G A C

Probe 2 (STX) G C C G G T A A T G A C A A T G G

Probe 3 (ST2) C C T T C A G C T T G A T G A T

Probe 4 (ST2) G T G A T G A C G A T G G T G

The colonies were transferred to nitrocellulose, filters, lysed and then hybridised in parallel with the two pairs of probes.

The prehybridisation process was carried out for the two pairs of probes at 65 °C for 6 hours, whilst the hybridisation process was effected at different temperatures for each of the four probes. Some positive clones were thus identified which hybridised with both the probes of a pair.

In order to identify the cosmid and/or cosmids containing the chromosomal DNA fragment including the STX gene, the cosmids were extracted from the positive clones by the rapid extraction method (Maniatis et al. (1982) "Molecular Cloning: A Laboratory Manual, Cold Spring Harbor N.Y.) and analysed by digestion with Eco RI.

It was thus found that one of them, indicated below as pSM 280, contained a sequence identical to the previously identified portion of the STX gene. According to the present invention and in order to sequence the gene, a fragment of the chromosomal DNA of *B. pertussis* SA1 which hybridises with the specific probe for STX, was extracted from the cosmid pSM 280 and subcloned in a vector of *E.coli*.

Vectors suitable for the purposes of the present invention may be selected from plasmids, viruses, and bacteriophages generally used in recombinant DNA techniques.

In particular, the plasmid pUC12 (2730 bp) described by Messing J. et al. (1982) (Gene, 19, 259) which enables the quick and easy identification of the clones which have incorporated an extraneous DNA fragment, was used.

In fact the plasmid is a carrier of the genes for β -lactamase and for β -galactoxidase and thus enables the *E.coli* strain which hosts it to grow on a medium containing ampicillin. When an extraneous fragment is inserted between the restriction sites present in the gene for β -galactoxidase, which are particularly suitable for cloning, the gene is interrupted and is no longer able to produce the enzyme.

It is thus possible, with the use of substances similar to galactose, which can produce molecules which are chromogenic even if degraded, to distinguish the recombinant clones (not coloured) from those containing the plasmid.

According to the present invention, the pSM 280 cosmid was digested with Eco RI restriction enzyme and the digestion mixture was loaded in duplicate on to agarose gel.

After electrophoresis, the DNA bands were transferred to nitrocellulose filters and hybridised with the probes 1 and 2, suitably marked.

The pre-hybridisation treatment and the hybridisation treatment were carried out under different operative conditions for each probe. The filters were then washed appropriately and subjected to radiography.

The results obtained showed a single positive band of 1.2 Kilobases (Kb) for both probes.

The chromosomal DNA fragment corresponding to the band was electroeluted and ligated to the plasmid pUC 12 which had previously been digested with Eco RI.

The ligation reaction was carried out in a buffer mixture in the presence of T4 DNA ligase with the use of a plasmid/DNA fragment ratio in the favour of the fragment, preferably 1:3.

Upon completion of the ligation reaction, the mixture was used to transform competent *E.coli* cells and the transformants were selected on a culture medium to which ampicillin had been added.

From the positive clones thus obtained, some which contained the required recombinant plasmids were isolated.

One of these, designated pSM 281, was used to isolate and sequence the 1.2kb chromosomal DNA fragment.

The sequencing was carried out by Sanger's method with the use of the successive primers strategy.

The sequencing reactions were carried out on the denatured plasmids according to the normal Boehringer protocol with the use of a "pUC Sequencing" kit and with the use of ATP as the α [P^{32}] tracer.

By means of the operation described above, it was found that the 1.2 Kb fragment is constituted by a 375-base region of the cosmid pH79 and by a 844-base region of the STX gene, whose nucleotide and aminoacid sequences are given in Figure 1.

An open reading frame was identified in the sequence which extends from the 5' terminal of the 844-base region to the stop codon situated 628 bases downstream. Moreover, the nucleotide and the aminoacid sequences of the mature protein for which the STX gene codes were identified, on the basis of the similarity with the aminoterminal sequence of the ST2 subunit.

This sequence, represented by the N-terminal aminoacids NH-Glu-Asp-Gly-Thr-Ile-Val-Ile-Thr-Gly, is preceded by the Ala-Ala-His-Ala tetrapeptide which is identical to that present between the mature protein of the pilin subunit ST2 and its secretion signal (leader sequence). The tetrapeptide, which represents the specific cutting site for a membrane leader-peptidase, is preceded by an aminoacid sequence of 29 aminoacids which contains a central hydrophobic domain (aminoacids -6 to -16), which is characteristic of leader sequences, and a methionine in position -21.

The ATG which codes for the Met in position -21 is, very probably, the translation codon. In fact the sequence downstream of the codon up to the start of the mature protein (GAA), codes for a polypeptide which has the length and characteristics typical of a signal sequence (Perlman, D. et al. (1983), J. Mol. Biol. 167, 391-409).

The aminoacid sequence of the STX gene product, deduced from its nucleotide sequence, shows a considerable similarity to the ST2 gene product. A great similarity between the two proteins was observed in the aminoterminal region and in the carboxyterminal region of 12 aminoacids which, as shown for other pilin proteins, are those which have the highest degree of conservation (Livey et al (1987) Molecular Microbiol., 1 (2), 203-209).

The overall degree of similarity of the mature proteins is estimated as 66% at the aminoacid level and 61% at the nucleotide level.

The presumed signal sequence of STX has a slightly lower similarity (52%) to that of the subunit ST2 than the rest of the protein. Moreover, the hydrophobicity/hydrophilicity model predicted by the sequences of ST2 and STX is very similar.

In conclusion, the STX gene of *B. pertussis* codes for a pilin-type mature protein with a molecular weight of approximately 20 kd, which could correspond to a serotypically-different pilin 2, 3 or 6 or to a new pilin.

According to the present invention and in order to identify the relationships existing in the chromosomal DNA of *B. pertussis* between the two genes ST2 and STX, a genome analysis was carried out.

In particular, the chromosomal DNA of *B. pertussis* 165 was digested with various restriction enzymes and the digestion mixtures were hybridised with probes specific for the STX and ST2 genes.

In no case was a band identified which could hybridise with both probes.

This indicated that these two genes were not situated in a single operon, or near each other, but that they must be situated at a minimum distance of several kilobases.

This observation was also confirmed by the absence of a cosmid containing both the genes from the gene library of *B. pertussis* SA1.

According to the present invention, peptides with an amino acid sequence identical to that of the mature protein for which the STX gene codes, or to immunologically-active regions thereof, are particularly useful for the preparation of acellular polyvalent vaccines against pertussis. The plasmid pSM 281 was deposited on 7-12-1987 as *E.coli* JM 103 (pSM 281) at the American Type Culture Center as ATCC 67572.

The following experimental examples are illustrative of the invention and not limiting.

Example 1

Extraction of the chromosomal DNA from *B. pertussis* 165

100 ml of fermentation medium having the following composition:

Beta casamino acids (DIFCO)	14 g
KCl	0.2 g
MgCl ₂ .6H ₂ O	0.1 g
K ₂ PO ₄	0.5 g
nicotinic acid	0.02 g
glutathione	0.01 g
starch	1.00 g
H ₂ O	1 l
pH 6.8	

previously sterilised at 120 °C for 15 minutes, was inoculated with *B. pertussis* strain 165 (serotype 1, 2, 3) and kept under agitation (200 revolutions per minute, r.p.m.) at 37 °C for 3 days.

At the end of this period, the cells were separated from the supernatant liquid by centrifuging (10 minutes, 5000 rpm) in a Sorvall RC5B model SS34 rotor at 4 °C and washed (2x120 ml) with a solution containing 100 mM NaCl, 50 mM Tris-HCl pH 7.5.

The suspension thus obtained was centrifuged again as described above and the cells were recovered and resuspended in 10 ml of a buffer solution (100 mM EDTA, 50 mM NaCl, 2.5% sucrose, pH 6.9) containing 1 mg/ml of lysozyme (SIGMA). The suspension was agitated and kept at 37 °C for 30 minutes and SDS (sodium dodecyl sulphate) was then added up to a final concentration of 1% and kept at 60 °C for 30 minutes.

1mg/ml of proteinase K, previously incubated at 37 °C for 30 minutes in 1xSSC (1xSSC = 0.15 M NaCl, 15 mM sodium citrate), was then added to the solution and the resulting mixture was reacted at 37 °C for 2 hours. After the addition of NaCl to a final concentration of 1M, the mixture was kept in ice for 30 minutes and then centrifuged. The DNA contained in the supernatant liquid was precipitated with 2-3 volumes of cold ethanol (-20 °C), collected with a glass rod and resuspended in 10 ml of 0.1xSSC. The suspension was kept at ambient temperature (20-25 °C) with agitation for one night and, after the addition of RNase (10 γ/ml), at 37 °C for 30 minutes.

The saline concentration of the solution was then brought to 1xSSC, extracted with phenol (1 volume), and the DNA precipitated by the addition of isopropanol dropwise to the solution which was kept at ambient temperature with gentle agitation.

The DNA was then recovered by centrifuging and resuspended in 1 ml of 0.1xSSC.

The quantity of chromosomal DNA, evaluated by spectrophotometric reading at OD260 with the use of a Perkin-Elmer spectrophotometer mod. 515, was 0.645 mg/ml.

Example 2

Isolation and sequencing of the gene which codes for a pilin subunit.5 A) Preparation of the gene library of *B. pertussis* 165 in Sau3A.

10 $10\ \mu\text{g}$ of chromosomal DNA obtained as described in Example 1 were digested in $200\ \mu\text{l}$ of a reaction buffer $6\ \text{mM}$ Tris-HCl pH 7.5, $50\ \text{mM}$ NaCl, $5\ \text{mM}$ MgCl_2 with 100 units (U) of Sau3A restriction enzyme (Boehringer) at 37°C for 1.5 hours.

15 The DNA thus digested was precipitated by the addition of 3M sodium acetate and ethanol to the mixture, separated by centrifuging at 4°C for 15 minutes at 12,000 rpm in an Eppendorf centrifuge and then resuspended in $100\ \mu\text{l}$ of TE buffer ($10\ \text{mM}$ Tris-HCl pH 8.0, $1\ \text{mM}$ EDTA). Simultaneously, the phages M13mp8 ($5\ \mu\text{g}$) and M13mp9 ($5\ \mu\text{g}$) were digested separately with 20 U of Bam HI (Boehringer) enzyme in $50\ \mu\text{l}$ of reaction buffer ($10\ \text{mM}$ Tris-HCl pH 7.8, $10\ \text{mM}$ MgCl_2 , $50\ \text{mM}$ NaCl) at 37°C for 1.5 hours.

The phage DNA was then precipitated from the digestion mixture, separated by centrifuging and resuspended in TE buffer as given above.

20 $15\ \mu\text{l}$ of the solution containing the chromosomal DNA fragments ($1.5\ \mu\text{g}$) were then ligated with $12.5\ \mu\text{l}$ of the phage DNA solution ($1.25\ \mu\text{g}$) in $1.25\ \text{ml}$ of ligation buffer ($66\ \text{mM}$ Tris-HCl pH 7.6, $1\ \text{mM}$ ATP, $10\ \text{mM}$ MgCl_2 , $10\ \text{mM}$ dithiothreitol) in the presence of 1 U of T4 DNA ligase at 14°C for 18 hours.

25 $125\ \mu\text{l}$ portions of the ligation mixture were then used to transform *E. coli* 71/18 cells (Miller, J.H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Lab. New York) made competent by treatment with $50\ \text{mM}$ CaCl_2 (Mandel M and Higa (1970) J. Mol. Biol. 53, 154). The selection of the transformants was then carried out by the placing of the cells on $1 \times \text{YT}$ medium plates ($8\ \text{g/l}$ Bacto Triptone (DIFCO), $5\ \text{g/l}$ Bacto-yeast extract (DIFCO) and $5\ \text{g/l}$ NaCl) made selective by the addition of $50\ \mu\text{g/ml}$ of ampicillin, $0.03\ \text{mM}$ IPTG (isopropyl- β -D-thiogalactopyranoside), 0.05% X-Gal (5-bromo-4-chloro-3-indolyl-D-galactopyranoside) and incubated for 12 hours at 37°C in a thermostatically-controlled chamber.

$14,000$ positive plaques (white) were thus obtained.

30 B) Construction of the specific probe

The N-terminal portion of the larger proteinaceous subunit (2) of the pili of *B. pertussis* 165 was analysed by Edman degradation and had the following sequence:

35

1	2	3	4	5	6	7	8	9	10	11	12
NH-Asp-Asp	Gly-Thr	Ile	Val	Ile	Thr	Gly	Thr	Ile	Thr		

40 The nucleotide sequence deduced from the amino acid sequence was then used to synthesise oligonucleotides for use as probes, account being taken of the relative degeneracy of the genetic code. In particular, a System 1 Plus DNA synthesiser (Beckman) automatic system was used to synthesise a family of oligonucleotides with the following sequence:

45

	7	8	9	10	11	12
	Ile	Thr	Gly	Thr	Ile	Thr
	ATC	ACC	GGC	ACC	ATC	AC
50	T	T	T	T	T	
	A	G	G	G	A	
			A			

55 $0.9\ \mu\text{g}$ of the oligonucleotides were marked at the $5'$ OH end with $500\ \mu\text{Ci}$ of γ (P^{32}) ATP ($3000\ \text{Ci/mmol}$) according to the method of Arrand J.E. (Nucleic Acid Hybridisation: A Practical Approach, edited by B.D. Hames - S. Higgings, Press Washington DC 1985, p 34). The calculated specific activity (LS 7500 Beckman

scintillator) was 8.3×10^8 cpm/ μ g of DNA.

C) Screening by means of the specific probe

5 The plaques of the gene library in M13 were transferred to nitrocellulose filters (Schleicher & Schnell 0.45 μ m) and the filters were then hybridised with the specific probe by the method of P.J. Mason and J. Williams (Nucleic Acid Hybridisation: A practical Approach p. 123). The prehybridisation treatment was carried out at 39°C for 6 hours whilst the hybridisation was carried out at 39°C for 18 hours. Finally the filters were washed at 25°C with 0.1% sodium dodecyl sulphate (SDS), 6xSSC (0.9 M NaCl, 0.09 M Na citrate) for 1 hour and put in contact with Kodak X-Omat AR radiographic plates.

10 5 plaques which gave the positive signal were thus identified. Two of these were then characterised.

D) Sequencing of the single strands obtained from the positive plaques.

15 The single strands of the two positive plaques were extracted by the technique of Messing J. (1983) (Methods of Enzymol, V.101) and sequenced by means of the successive primers strategy described by Strauss E.C. et al. (1986) (Analytical Biochem. 154, 353). The sequence of one of the single strands contained a region identical to that of the ST2 gene whilst the other contained a similar, but not identical sequence which suggested the presence of a different gene, hereinafter termed STX.

Example 3

Sequencing of the gene STX

25 A. Construction of a genome bank of B. pertussis SA1

In order to obtain the entire coding sequence of the gene designated STX, a genome bank of B. pertussis SA1 was constructed with the use of the cosmid pH79.

30 500 μ g of the chromosomal DNA of B. pertussis SA1 extracted as described in Example 1 were partially digested with 5 U of Sau 3A restriction enzyme at 37°C for 15 minutes.

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

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

35 The gradient was then centrifuged at 26,000 rpm for 16 hours in a Beckman SW 28 rotor.

Fractions, each of 1 ml, were then collected and the molecular weight of the DNA contained in each fraction was determined by electrophoresis on agarose (Maniatis T. et al. "Molecular Cloning A Laboratory Manual. Cold Spring Harbor N.Y. (1982)).

40 The fractions containing DNA fragments of 35-45 kb were then dialysed and the DNA precipitated with ethanol as described above.

The DNA was then separated by centrifuging and resuspended in a 10 mM Tris, 1 mM EDTA buffer (pH 7.5) at a concentration of 1 μ g/ μ l of DNA.

The chromosomal DNA fragments were then cloned.

45 20 μ g of the cosmid pH79 were digested with 40 U of Bam HI enzyme at 37°C for 1 hour in 200 μ l of digestion mixture.

2 μ g of chromosomal DNA were then ligated with 0.5 μ g of the DNA of the cosmid in 10 μ l of ligation mixture in the presence of 1 U of T4 DNA ligase at 14°C for 18 hours.

At the end of this period, 2.5 μ l of the ligation mixture were used in vitro with a Stratagene Packagene Kit.

50 The recombinant cosmids thus obtained were used to infect the JM109 strain of E.coli and the transformants were selected on LB medium (Bacto Triptone 10 g, Bacto Y.E. 5 g, NaCl 10 g, H₂O 1 l, pH 7.5).

Approximately 15,000 positive colonies were produced.

1500 of the colonies were then screened with two pairs of probes having the following sequences:

55 Probe 1 (STX) C C G C C C A T G C C G A A G A C

Probe 2 (STX) G C C G G T A A T G A C A A T G G

Probe 3 (ST2) C C T T C A G C T T G A T G A T

Probe 4 (ST2) G T G A T G A C G A T G G T G

The oligonucleotides were synthesised by the Beckman automatic system and marked at the 5' OH end with 105 μ Ci of γ (P^{32})ATP by the following method.

200 ng of oligonucleotides were suspended in 30 μ l of an aqueous solution constituted by 3 μ l of Kinase buffer (Boehringer), 21 μ l ATP, T4 polynucleotide kinase 10 U μ l (1 μ l).

The mixture was kept at 37 °C for 45 minutes and the enzyme deactivated at 65 °C for 10 minutes.

The marked probes were then purified in a Sephadex G-50 column in pH8 TE buffer to eliminate the marker which had not been incorporated.

13 fractions of 150 μ l each were collected.

2 μ l of each fraction were put in 4 ml of scintillating liquid and measured in the scintillator.

The colonies of the bank, in cosmids, were transferred to nitrocellulose filters (Schleicher and Schuell 0.45 μ m) and after lysis with NaOH, their DNA was immobilised by Southern's technique (Maniatis 1982); The filters were hybridised in parallel with the two pairs of probes marked as described above.

The prehybridisation treatment was carried out at 65 °C for 6 hours whilst the hybridisation was carried out at:

53 °C for probe 1

47 °C for probe 2

41 °C for probe 3

45 °C for probe 4

for 18 hours.

The filters were then washed and put in contact with radiographic plates as described in point C) of Example 2.

5 clones which hybridised with the four probes were isolated by the method described above.

The cosmids extracted from the clones were analysed by digestion with Eco RI and it was found that: three of them contained sequences similar but not identical to probes 3 and 4, one contained a sequence identical to the previously-isolated region of the STX gene and the last, which hybridised with probes 1 and 3, contained a sequence which was not identical to that of the STX gene.

The clone containing the cosmid with the STX gene was designated pSM 280.

B) Subcloning of the DNA fragment containing the STX gene

The cosmid pSM280 was extracted by rapid extraction and 250 ng were then digested in 10 μ l of 100 mM Tris-HCl pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$ digestion mixture with 5 U of Eco RI (Boehringer) at 37 °C for 1 hour.

The enzymatic reaction was then stopped at 65 °C for 10 minutes and the mixture was loaded in parallel on to two 0.8% agarose gels and, after electrophoresis at 80 volts for 3 hours, the bands of DNA were transferred to 2 nitrocellulose filters (Micro Filtration System 0.45 μ m) and then hybridised with the probes 1 and 2 by the technique described in point C).

The prehybridisation treatment was carried out in 10 ml of 6xSSC (1xSSC = 150 mM NaCl, 25 mM Na citrate), 10 x Denhardt's (1 x Denhardt's = 1% Ficoll, 1% PVP, 1% BSA Pentax fraction V) and 50 μ g/ml of denatured salmon sperm, at 53 °C for 4 hours for filter No. 1 and at 47 °C for 4 hours for filter No. 2.

The hybridisation was carried out in 10 ml of the mixture described above with the addition of 600 μ l of probe 1 (6×10^8 cpm/ γ DNA) for the filter No. 1 and 600 μ l of probe 2 (8.4×10^8 cpm/ γ DNA) for filter No. 2.

The filters 1 and 2 were then incubated at 53 °C and 47 °C respectively for one night. Finally the filters were washed four times for 15 minutes each with 100 ml of 6xSSC containing 0.1% of sodium dodecyl sulphate (SDS) and placed in contact with Kodak X-Omat AR radiographic plates at -80 °C for 1 night.

The results showed a single positive band of 1.2 kbases with both probes.

B) Subcloning of the 1.2 kbase Eco RI fragment in PUC12.

1 μ g of the vector pUC₁₂ was digested in 10 μ l of digestion mixture with 5U of Eco RI at 37 °C for 1 hour.

The enzymatic reaction was deactivated at 65 °C for 10 minutes and the DNA precipitated with ethanol and separated by centrifuging.

50 ng of the digested plasmid DNA were then ligated with 66 ng of the 1.2 kbase Eco RI fragment of DNA in 25 μ l of ligation mixture in the presence of 1 U of T4 DNA ligase at 12 °C for 1 night.

20 μ l of the ligation mixture were used to transform 200 μ l of competent *E.coli* JM 103 cells (Maniatis, 1982). The selection of the transformants was then carried out on LB agar medium plates with the addition of 50 γ /ml of ampicillin, 40 γ /ml X gel and 125 γ /ml of IPTG at 37 °C for 18 hours.



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24 positive clones were obtained, of which 17 contained the required recombinant plasmids.

One of these plasmids, designated pSM 281, was then extracted from the positive clones by means of rapid extraction.

5 C) Sequencing of pSM 281

The recombinant plasmid pSM281 was sequenced by the method of Sanger et al. (1977) (P.N.A.S. 74, 5463) by means of the successive primers strategy described by Strauss et al (Anal. Biochem. 154, 553 (1986). The oligonucleotides used as primers were synthesised by means of an automatic 1 Plus DNA
10 Synthesiser system (Beckman).

The sequencing reactions were carried out according to the normal Boehringer protocol on the denatured plasmids with the use of a "pUC Sequencing" kit, and with α [P^{32}] dATP as the tracer.

The apparatus used for the electrophoretic separation was a MacroPhor sequencing System (LKB).

The entire 1.2 kbase fragment thus sequenced consisted of 375 bases of the cosmid pHCT9 and 844
15 bases of the STX gene (Figure 1).

As shown in Figure 3, the aminoacid sequence of the STX gene product has a considerable similarity to the product of the ST2 gene.

Genome Analysis

The chromosomal DNA of B. pertussis 165 was extracted as described in Example 1 and the portions of the DNA were digested separately with various enzymes.

In practice, 1.5 ug of chromosomal DNA were treated with 15 U of each of the following enzymes Stu I, Sph I, Sma I, Pvu II, Pst I, Eco RI and Bam HI at 37° C for 3 hours in 20 ml. of digestion mixture.

25 The mixtures were then loaded onto 0.8% agarose gel and run at 100 V for 3-4 hours.

The bands of chromosomal DNA were then transferred to nitrocellulose filters and hybridised separately with the probes 1 and 4 as described in Example 2.

In no case was a single band identified which was capable of hybridising the two probes.

This indicates that these two genes are not situated in a single operon or near each other, but that they
30 must be at a minimum distance of several kbases.

This observation is confirmed by the absence from the gene library of B. pertussis of a cosmid which contains both the genes.

Claims

- 35 1. A gene isolated from the chromosomal DNA of a strain of Bordetella pertussis which codes for a new pilin subunit, where the gene is characterised by the nucleotide sequence (I):

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55

24 positive clones were obtained, of which 17 contained the required recombinant plasmids.

One of these plasmids, designated pSM 281, was then extracted from the positive clones by means of rapid extraction.

5 C) Sequencing of pSM 281

The recombinant plasmid pSM281 was sequenced by the method of Sanger et al. (1977) (P.N.A.S. 74, 5463) by means of the successive primers strategy described by Strauss et al (Anal. Biochem. 154, 553 (1986). The oligonucleotides used as primers were synthesised by means of an automatic 1 Plus DNA
10 Synthesiser system (Beckman).

The sequencing reactions were carried out according to the normal Boehringer protocol on the denatured plasmids with the use of a "pUC Sequencing" kit, and with α [P^{32}] dATP as the tracer.

The apparatus used for the electrophoretic separation was a Macrophor sequencing System (LKB).

The entire 1.2 kbase fragment thus sequenced consisted of 375 bases of the cosmid pH79 and 844
15 bases of the STX gene (Figure 1).

As shown in Figure 3, the aminoacid sequence of the STX gene product has a considerable similarity to the product of the ST2 gene.

Genome Analysis

The chromosomal DNA of *B. pertussis* 165 was extracted as described in Example 1 and the portions of the DNA were digested separately with various enzymes.

In practice, 1.5 ug of chromosomal DNA were treated with 15 U of each of the following enzymes Stu I, Sph I, Sma I, Pvu II, Pst I, Eco RI and Bam HI at 37° C for 3 hours in 20 ml. of digestion mixture.

25 The mixtures were then loaded onto 0.8% agarose gel and run at 100 V for 3-4 hours.

The bands of chromosomal DNA were then transferred to nitrocellulose filters and hybridised separately with the probes 1 and 4 as described in Example 2.

In no case was a single band identified which was capable of hybridising the two probes.

This indicates that these two genes are not situated in a single operon or near each other, but that they
30 must be at a minimum distance of several kbases.

This observation is confirmed by the absence from the gene library of *B. pertussis* of a cosmid which contains both the genes.

Claims

- 35 1. A gene isolated from the chromosomal DNA of a strain of *Bordetella pertussis* which codes for a new pilin subunit, where the gene is characterised by the nucleotide sequence (I):

40

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50

55



30
 .ATG CAA ECC AAA ACC TTC CTC CTS ECC ECG ECG CTC
 5 60 90
 ECC GGC GTC ECG CTC GCC GCC CAT GCC EAA GAC GGC ACC ATT ETC ATT ACC GGC ACC ATC
 120 150
 ACC GAC CAG ACC TGC ACC ATC GAG GAC CCG ACC CCC GGT TAC ATC AAG GTC GTG CAC CTC
 10 180 210
 CCC ACC ATC TCC AAG AGC GCG CTC AAG AAC ECC GGC GAC GTG ECG GGG CCG ACT CEC TTC
 240 270
 GAT ATC AAG CTC AAG GAC TGC CCG ACC ACC GTC AAC ACT CTC AAG CTC TAC TTC GAG CCC
 15 300 330
 GGC CCC ACC ACC GAT TAC GGC ACC AAG GAT CTC AAA ECC TAT AAG CAG GCT TGG TAC GTC
 360 390
 GAC GCC GCA ACC CTC CTC AAA TCG CCG CCC AGT GTG ACC GAA GCC AAG GGG GTG CAG ATC
 20 420 450
 CCG CTC ATG AAC CTC AAC GGC AAG CAG ATT CCC ATG GGC GAG ACC GAG CCC AAC CAG CAT
 480 510
 25 ECC GCG GCA TTT TCC GGC ACC ATG CAA ECC GGC CAG GGA CAG AAA TCG TTC ACC TTG CAC
 540 570
 TAC CTC GCC GGC TAC GTG AAG AAG GCC AGT GGA GAG ETC EAG GCG ACC ATG CTG ACC ACC
 600
 30 TAC GTG GGC TTT TCG ETC GTC TAC CCC TGA

2. A gene according to Claim 1, in which the pilin subunit is characterised by the aminoacid sequence (II):



10 20
 Met Gln Ala Lys Thr Phe Leu Leu Gly Ala Ala Leu Ala Gly Val Ala Leu Ala Ala His
 30 40
 5 Ala Glu Asp Gly Thr Ile Val Ile Thr Gly Thr Ile Thr Asp Gln Thr Cys Thr Ile Glu
 50 60
 Asp Pro Ser Pro Gly Tyr Ile Lys Val Val His Leu Pro Thr Ile Ser Lys Ser Ala Leu
 70 80
 Lys Asn Ala Gly Asp Val Ala Gly Arg Thr Arg Phe Asp Ile Lys Leu Lys Asp Cys Pro
 10 90 100
 Thr Thr Val Asn Thr Leu Lys Leu Tyr Phe Glu Pro Gly Pro Thr Thr Asp Tyr Gly Thr
 110 120
 Lys Asp Leu Lys Ala Tyr Lys Gln Ala Trp Tyr Val Asp Ala Ala Thr Leu Leu Lys Ser
 15 130 140
 Pro Pro Ser Val Thr Glu Ala Lys Gly Val Gln Ile Arg Leu Met Asn Leu Asn Gly Lys
 150 160
 Gln Ile Pro Met Gly Glu Thr Glu Pro Asn Gln His Ala Ala Ala Phe Ser Gly Thr Met
 170 180
 Gln Ala Gly Gln Gly Gln Lys Ser Phe Thr Leu His Tyr Leu Ala Gly Tyr Val Lys Lys
 190 200
 Ala Ser Gly Glu Val Glu Ala Thr Met Leu Thr Thr Tyr Val Gly Phe Ser Val Val Tyr
 Pro End

3. A structural gene which codes for the mature pilinic subunit contained in the gene according to Claim 1, characterised by the nucleotide sequence (III):

30
 10 20 30 40 50 60
 GAAGACGGCA CCATTGTCAT TACGGGCAGC ATCACCAGAC AGACCTGCAC GATCGAGGAC
 70 80 90 100 110 120
 CCGAGCCCCG GTTACATCAA GGTCTGTCAC CTGCCACCA TCTCCAAGAG CECCTGAAG
 35 130 140 150 160 170 180
 AACGCCGGCG ACGTGGCGGG GCGCACTCGC TTCGATATCA AGCTGAAGGA CTGCCCGACC
 190 200 210 220 230 240
 ACCGTCAACA CTCTCAAGCT GTACTTCGAG CCCGCCCCCA CCACGGATTA CCGCACCAAG
 40 250 260 270 280 290 300
 GATCTGAAGG CCTATAAGCA GGCTTGATAC GTCGACGCCG CAACGCTGCT CAAATCGCCG
 310 320 330 340 350 360
 CCCAGTGTGA CCGAAGCCAA GGGGGTGCAG ATCCGGCTGA TGAACCTGAA CCGCAAGCAG
 45 370 380 390 400 410 420
 ATTCCCATGG GCGAGACCGA GCCCAACCA CATGCCGCGG CATTTTCCGG CACCATGCRA
 430 440 450 460 470 480
 GCCGGCCAGG GACAGAAATC GTTCACCTTG CACTACCTGG CCGGCTACGT GAAGAAGGCC ..
 490 500 510 520 530 540
 AGTGGAGAGG TCGAGGCGAC CATGCTGACC ACCTACGTGG GCTTTTCCGT CGTCTACCCC

TGA

- 55 4. A structural gene according to Claim 3, in which the mature pilinic subunit is characterised by the aminoacid sequence (IV):



10
 Glu Asp Gly Thr Ile Val Ile Thr Gly Thr Ile Thr Asp Gln Thr Cys Thr Ile Glu Asp
 20
 30
 5 Pro Ser Pro Gly Tyr Ile Lys Val Val His Leu Pro Thr Ile Ser Lys Ser Ala Leu Lys
 40
 50
 Asn Ala Gly Asp Val Ala Gly Arg Thr Arg Phe Asp Ile Lys Leu Lys Asp Cys Pro Thr
 60
 70
 Thr Val Asn Thr Leu Lys Leu Tyr Phe Gly Pro Gly Pro Thr Thr Asp Tyr Gly Thr Lys
 80
 10 90
 Asp Leu Lys Ala Tyr Lys Gln Ala Trp Tyr Val Asp Ala Ala Thr Leu Leu Lys Ser Pro
 100
 110
 Pro Ser Val Thr Glu Ala Lys Gly Val Gln Ile Arg Leu Met Asn Leu Asn Gly Lys Gln
 120
 130
 15 Ile Pro Met Gly Glu Thr Glu Pro Asn Gln His Ala Ala Ala Phe Ser Gly Thr Met Gln
 140
 150
 Ala Gly Gln Gly Gln Lys Ser Phe Thr Leu His Tyr Leu Ala Gly Tyr Val Lys Lys Ala
 160
 170
 20 Ser Gly Gly Val Gly Ala Thr Met Leu Thr Thr Tyr Val Gly Phe Ser Val Val Tyr Pro
 180
 End

- 25 5. A chromosomal DNA fragment isolated from a strain of Bordetella pertussis, characterised in that it contains the gene according to Claim 1.
6. A chromosomal DNA fragment according to Claim 5, characterised by the nucleotide sequence shown in Figure 1.
- 30 7. A recombinant DNA molecule produced by the joining of a cloning vector to the gene according to Claim 1 and Claim 2.
8. A recombinant DNA molecule produced by the joining of a cloning vector to the structural gene
35 according to Claim 3 and Claim 4.
9. A recombinant DNA molecule produced by the joining of a cloning vector to the chromosomal DNA fragment according to Claim 5 and Claim 6.
- 40 10. A recombinant DNA molecule according to Claims 7 to 9 in which the vector is a plasmid for expression in bacteria or yeasts.
11. A recombinant DNA molecule according to Claim 10, in which the plasmid is pUC12.
- 45 12. A pSM 281 recombinant DNA molecule according to Claim 11, in which the plasmid is bound to the chromosomal DNA fragment according to Claim 5 and Claim 6.
13. A microorganism transformed by a recombinant DNA molecule according to Claims 7 to 12, where the microorganism is selected from E.coli, Bacillus and yeasts.
- 50 14. A microorganism according to Claim 13, Escherichia coli JM 103 (pSM 281) ATCC 67572.
15. Use of immunologically-active synthetic peptides with an amino acid sequence identical to that of the pilinic subunit of Bordetella pertussis encoded by the gene claimed in Claim 2 for the preparation of an
55 acellular vaccine against infections caused by Bordetella pertussis in humans.



Patentansprüche

- Ein aus der chromosomalen DNA eines Stammes von *Bordetella pertussis* isoliertes und für eine neue Pili-Untereinheit codierendes Gen mit der nachstehenden Nukleotidsequenz (I):

```

5
                                     30
      .ATG CAA GCC AAA ACG TTC CTC CTC GGC GCG GCG CTC
                                     60
10  GCC GGC GTC GCG CTC GCC GCC CAT GCC GAA GAC GGC ACC ATT CTC ATT ACC GGC ACG ATC
                                     90
      ACC GAC CAG ACC TGC ACG ATC GAG GAC CCG AGC CCC GGT TAC ATC AAG GTC GTG CAC CTC
                                     120
15  CCC ACG ATC TCC AAG AGC GCG CTC AAG AAC GCC GGC GAC GTG GCG GGC CCG ACT CCG TTC
                                     150
      GAT ATC AAG CTC AAG GAC TGC CCG ACC ACC GTC AAC ACT CTC AAG CTC TAC TTC GAG CCC
                                     180
20  GTC CCC ACC ACG GAT TAC GGC ACC AAG GAT CTC AAA GGC TAT AAG CAG GCT TGT TAC GTC
                                     210
      GAC GCC GCA ACG CTC CTC AAA TCG CCG CCC AGT GTG ACC GAA GCC AAG GGT GTG CAG ATC
                                     240
25  CCG CTC ATG AAC CTC AAC GGC AAG CAG ATT CCC ATG GGC GAG ACC GAG CCC AAC CAG CAT
                                     270
      GCC GCG GCA TTT TCC GGC ACC ATG CAA GCC GGC CAG GGA CAG AAA TCG TTC ACC TTG CAG
                                     300
30  TAC CTC GCC GGC TAC GTG AAG AAG GCC AGT GGA GAG GTC GAG GCG ACC ATG CTC ACC ACC
                                     330
      TAC GTG GGC TTT TCG CTC GTC TAC CCC TGA
                                     360
                                     390
                                     420
                                     450
                                     480
                                     510
                                     540
                                     570
                                     600

```

- Gen nach Anspruch 1, worin die Pili-Untereinheit durch die nachfolgende Aminosäuresequenz (II) codiert ist:

40

45

50

55



10
 Met Gln Ala Lys Thr Phe Leu Leu Gly Ala Ala Leu Ala Gly Val Ala Leu Ala Ala His
 20
 30
 40
 5
 Ala Glu Asn Gly Thr Ile Val Ile Thr Gly Thr Ile Thr Asn Gln Thr Cys Thr Ile Glu
 50
 60
 Asn Pro Ser Pro Gly Tyr Ile Lys Val Val His Leu Pro Thr Ile Ser Lys Ser Ala Leu
 70
 80
 10
 Lys Asn Ala Gly Asp Val Ala Gly Arg Thr Arg Phe Asn Ile Lys Leu Lys Asn Cys Pro
 90
 100
 Thr Thr Val Asn Thr Leu Lys Leu Tyr Phe Glu Pro Gly Pro Thr Thr Asn Tyr Gly Thr
 110
 120
 Lys Asn Leu Lys Ala Tyr Lys Gln Ala Trp Tyr Val Asp Ala Ala Thr Leu Leu Lys Ser
 130
 140
 15
 Pro Pro Ser Val Thr Glu Ala Lys Gly Val Gln Ile Arg Leu Met Asn Leu Asn Gly Lys
 150
 160
 Gln Ile Pro Met Gly Glu Thr Glu Pro Asn Gln His Ala Ala Ala Phe Ser Gly Thr Met
 170
 180
 20
 Gln Ala Gly Gln Gly Gln Lys Ser Phe Thr Leu His Tyr Leu Ala Gly Tyr Val Lys Lys
 190
 200
 Ala Ser Gly Glu Val Glu Ala Thr Met Leu Thr Thr Tyr Val Gly Phe Ser Val Val Tyr
 210
 220
 230
 240
 25
 Pro End

3. Strukturgen, das für die reife, in dem Gen nach Anspruch 1 enthaltene Pili-Untereinheit codiert, mit nachstehender Nukleotidsequenz (III):

30
 10 20 30 40 50 60
 GAGGACGGCA CCATTGTCAT TACCGGCACG ATCACCAGCC AGACCTGCAC GATCGAGGAC
 70 80 90 100 110 120
 CCGAGCCCCG GTTACATCAA GGTCTGTGCAC CTGCCCCACGA TCTCCAAGAG CECCTGAAG
 35
 130 140 150 160 170 180
 AACCCCGCCG ACGTGCGCGG GCGCACTCGC TTCGATATCA AGCTGAAGGA CTGCCCCGAC
 190 200 210 220 230 240
 ACCGTCAACA CTCTCAAGCT GTACTTCGAG CCCGCCCCCA CCACGGATTA CGGCACCAAG
 40
 250 260 270 280 290 300
 SATCTGAAGG CCTATAAGCA GCTTGTGTAC GTCSACGCCG CAACGCTGCT CAAATCGCCG
 310 320 330 340 350 360
 CCCAGTGTGA CCGAAGCCAA GGGGGTGCAG ATCCGGCTGA TGAACCTGAA CCGCAAGCAG
 370 380 390 400 410 420
 45
 ATTECCATGG GCGAGACCGA GCGCAACCGA CATGCCGCGG CATTTCGGG CACCATGCAA
 430 440 450 460 470 480
 GCGGCGCCAG GACAGAAATC GTTCACCTTG CACTACCTGG CCGGCTACGT GAAGAAGGCC
 490 500 510 520 530 540
 50
 AGTGGAGAGG TCGAGCGCAC CATGCTGACC ACCTACGTTG GCTTTTCGGT CGTCTACCCG
 TGA

- 65 4. Strukturgen nach Anspruch 3, worin die reife Pili-Untereinheit die nachfolgende Aminosäuresequenz (IV) aufweist:



10
 Glu Asp Gly Thr Ile Val Ile Thr Gly Thr Ile Thr Asp Gln Thr Cys Thr Ile Glu Asp
 20
 30
 40
 5
 Pro Ser Pro Gly Tyr Ile Lys Val Val His Leu Pro Thr Ile Ser Lys Ser Ala Leu Lys
 50
 60
 Asn Ala Gly Asp Val Ala Gly Arg Thr Arg Phe Asp Ile Lys Leu Lys Asp Cys Pro Thr
 70
 80
 Thr Val Asn Thr Leu Lys Leu Tyr Phe Glu Pro Gly Pro Thr Thr Asp Tyr Gly Thr Lys
 10
 90
 100
 Asp Leu Lys Ala Tyr Lys Gln Ala Trp Tyr Val Asp Ala Ala Thr Leu Leu Lys Ser Pro
 110
 120
 Pro Ser Val Thr Glu Ala Lys Gly Val Gln Ile Arg Leu Met Asn Leu Asn Gly Lys Gln
 130
 140
 15
 Ile Pro Met Gly Gly Thr Gly Pro Asn Gln His Ala Ala Ala Phe Ser Gly Thr Met Gln
 150
 160
 Ala Gly Gln Gly Gln Lys Ser Phe Thr Leu His Tyr Leu Ala Gly Tyr Val Lys Lys Ala
 170
 180
 Ser Gly Gly Val Gly Ala Thr Met Leu Thr Thr Tyr Val Gly Phe Ser Val Val Tyr Pro
 End .

- 25 5. Chromosomales, aus einem Stamm von *Bordetella pertussis* isoliertes, das Gen nach Anspruch 1 enthaltendes DNA-Fragment.
6. Chromosomales DNA-Fragment nach Anspruch 5 mit der in Figur 1 gezeigten Nukleotidsequenz.
- 30 7. Rekombinantes, durch Verbindung eines Klonierungsvektors mit dem Gen nach Anspruch 1 oder 2 produziertes DNA-Molekül.
8. Rekombinantes, durch Verbindung eines Klonierungsvektors mit dem Strukturgen nach Anspruch 3 oder 4 produziertes DNA-Molekül.
- 35 9. Rekombinantes, durch Verbindung eines Klonierungsvektors mit dem chromosomalen DNA-Fragment nach Anspruch 5 oder 6 produziertes DNA-Molekül.
- 40 10. Rekombinantes DNA-Molekül nach einem der Ansprüche 7-9, worin der Vektor ein Expressionsplasmid für Bakterien oder Hefe ist.
11. Rekombinantes DNA-Molekül nach Anspruch 10, worin das Plasmid pUC12 ist.
- 45 12. Rekombinantes DNA-Molekül pSM 281 nach Anspruch 11, worin das Plasmid an das chromosomale DNA-Fragment nach Anspruch 5 oder 6 gebunden ist.
13. Ein durch ein rekombinantes DNA-Molekül nach einem der Ansprüche 7-12 transformierter, aus *E.coli*, *Bacillus* und Hefen ausgewählter Mikroorganismus.
- 50 14. Mikroorganismus *Escherichia coli* JM 103 (pSM 281) ATCC 67572 nach Anspruch 13.
- 55 15. Verwendung von immunologisch-aktiven, synthetischen Peptiden mit einer Aminosäuresequenz, die mit jener der durch das Gen nach Anspruch 2 codierten Pili-Untereinheit von *Bordetella pertussis* identisch ist, zur Herstellung einer zellfreien Vakzine gegen durch *Bordetella pertussis* beim Menschen verursachte Infektionen.



Revendications

1. Un gène isolé à partir de l'ADN chromosomique d'une souche de *Bordetella pertussis* qui code pour une nouvelle sous-unité pilinique où le gène est caractérisé par la séquence nucléotidique (I) :

5

30
 .ATG CAA GCC AAA ACC TTC CTC GTC GGC GCG GCG CTC
 60
 10 GGC GGC GTC GCG CTC GGC GGC CAT GCC GAA GAC GGC ACC ATT CTC ATT ACC GGC ACC ATC
 90
 120
 ACC GAC CAG ACC TGC ACC ATC GAG GAC CCG AGC CCC GGT TAC ATC AAG GTC GTG CAC CTC
 150
 180
 15 CCC ACC ATC TCC AAG AGC GCG CTC AAG AAC GCC GGC GAC GTG GCG GGC CCG ACT CEC TTC
 210
 240
 20 GAT ATC AAG CTC AAG GAC TGC CCG ACC ACC GTC AAC ACT CTC AAG CTC TAC TTC GAG CCC
 270
 300
 25 GGC CCC ACC ACC GAT TAC GTC ACC AAG GAT CTC AAA GCG TAT AAG CAG GCT TGG TAC GTC
 330
 360
 30 GAC GCG GCA ACC CTC CTC AAA TCG CCG CCC AGT GTC ACC GAA GCG AAG GGT GTG CAG ATC
 390
 420
 25 CCG CTC ATG AAC CTC AAC GGC AAG CAG ATT CCC ATG GGC GAG ACC GAG CCC AAC CAG CAT
 450
 480
 30 GCG GCG GCA TTT TCC GGC ACC ATG CAA GCG GGC CAG GGA CAG AAA TCG TTC ACC TTG CAG
 510
 540
 35 TAC CTC GCG GGC TAC GTG AAG AAG GCC AGT GGA GAG GTC GAG GCG ACC ATG CTG ACC ACC
 570
 600
 TAC GTG GGC TTT TCG CTC CTC TAC CCC TGA

2. Un gène selon la revendication 1 dans lequel la sous-unité pilinique est caractérisée par la séquence d'acidesaminés (II) :

40

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10
 Met Gln Ala Lys Thr Phe Leu Leu Gly Ala Ala Leu Ala Gly Val Ala Leu Ala Ala His
 20
 30
 40
 5
 Ala Glu Asn Gly Thr Ile Val Ile Thr Gly Thr Ile Thr Asn Gln Thr Cys Thr Ile Glu
 60
 70
 80
 Asn Pro Ser Pro Gly Tyr Ile Lys Val Val His Leu Pro Thr Ile Ser Lys Ser Ala Leu
 90
 100
 10
 Lys Asn Ala Gly Asn Val Ala Gly Arg Thr Arg Phe Asn Ile Lys Leu Lys Asn Cys Pro
 110
 120
 Thr Thr Val Asn Thr Leu Lys Leu Tyr Phe Glu Pro Gly Pro Thr Thr Asn Tyr Gly Thr
 130
 140
 15
 Lys Asn Leu Lys Ala Tyr Lys Gln Ala Trp Tyr Val Asn Ala Ala Thr Leu Leu Lys Ser
 160
 170
 180
 190
 200
 25
 Pro End

3. Un gène de structure qui code pour la sous-unité pilinique mature contenue dans le gène selon la revendication 1, caractérisé par la séquence nucléotidique (III) :

30
 40
 50
 60
 70
 80
 90
 100
 110
 120
 130
 140
 150
 160
 170
 180
 190
 200
 210
 220
 230
 240
 250
 260
 270
 280
 290
 300
 310
 320
 330
 340
 350
 360
 370
 380
 390
 400
 410
 420
 430
 440
 450
 460
 470
 480
 490
 500
 510
 520
 530
 540
 TGA

4. Un gène de structure selon la revendication 3, dans lequel la sous-unité pilinique mature est caractérisée par la séquence d'acides aminés (IV) :



10
 Glu Asp Gly Thr Ile Val Ile Thr Gly Thr Ile Thr Asp Gln Thr Cys Thr Ile Glu Asp 20
 30
 Pro Ser Pro Gly Tyr Ile Lys Val Val His Leu Pro Thr Ile Ser Lys Ser Ala Leu Lys 40
 50
 Asn Ala Gly Asp Val Ala Gly Arg Thr Arg Phe Asp Ile Lys Leu Lys Asp Cys Pro Thr 60
 70
 Thr Val Asn Thr Leu Lys Leu Tyr Phe Glu Pro Gly Pro Thr Thr Asp Tyr Gly Thr Lys 80
 90
 Asp Leu Lys Ala Tyr Lys Gln Ala Trp Tyr Val Asp Ala Ala Thr Leu Leu Lys Ser Pro 100
 110
 Pro Ser Val Thr Gly Ala Lys Gly Val Gln Ile Arg Leu Met Asn Leu Asn Gly Lys Gln 120
 130
 Ile Pro Met Gly Gly Thr Gly Pro Asn Gln His Ala Ala Ala Phe Ser Gly Thr Met Gln 140
 150
 Ala Gly Gln Gly Gln Lys Ser Phe Thr Leu His Tyr Leu Ala Gly Tyr Val Lys Lys Ala 160
 170
 Ser Gly Gly Val Gly Ala Thr Met Leu Thr Thr Tyr Val Gly Phe Ser Val Val Tyr Pro 180
 End

- 25 5. Un fragment d'ADN chromosomique isolé d'une souche de Bordetella pertussis, caractérisé en ce qu'il contient le gène selon la revendication 1.
6. Un fragment d'ADN chromosomique selon la revendication 5, caractérisé par la séquence nucléotidique représentée sur la Figure 1.
- 30 7. Une molécule d'ADN recombinante produite par l'association d'un vecteur de clonage au gène selon la revendication 1 et la revendication 2.
8. Une molécule d'ADN recombinante produite par l'association d'un vecteur de clonage au gène de structure selon la revendication 3 et la revendication 4.
- 35 9. Une molécule d'ADN recombinante produite par l'association d'un vecteur de clonage au fragment d'ADN chromosomique selon la revendication 5 et la revendication 6.
- 40 10. Une molécule d'ADN recombinante selon l'une des revendications 7 à 9, dans laquelle le vecteur est un plasmide pour expression dans les bactéries ou levures.
11. Une molécule d'ADN recombinante selon la revendication 10, dans laquelle le plasmide est pUC12.
- 45 12. Une molécule d'ADN recombinante pSM 281 selon la revendication 11, dans laquelle le plasmide est lié au fragment d'ADN chromosomique selon la revendication 5 et la revendication 6.
13. Un microorganisme transformé par une molécule d'ADN recombinante selon l'une des revendications 7 à 12, où le microorganisme est choisi à partir de E.coli, Bacillus et les levures.
- 50 14. Un microorganisme selon la revendication 13, Escherichia coli JM 103 (pSM 281) ATCC 67572.
15. Utilisation de peptides synthétiques immunologiquement actifs avec une séquence d'acides aminés identique à celle de la sous-unité pilinique de Bordetella pertussis codé par le gène revendiqué dans la revendication 2, pour la préparation d'un vaccin acellulaire contre les infections provoquées par Bordetella pertussis chez les humains.
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