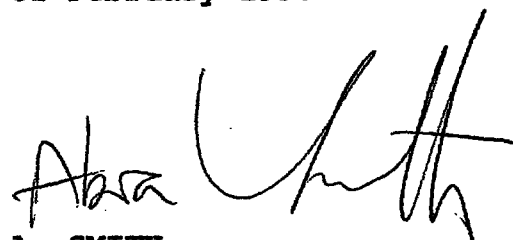


I, Abraham SMITH DipIng DipDoc,
translator to RWS Translations Ltd., of Europa House, Marsham
Way, Gerrards Cross, Buckinghamshire, England, hereby declare
that I am conversant with the English and French languages and
am a competent translator thereof. I declare further that to the
best of my knowledge and belief the following is a true and
correct translation of the accompanying documents in the French
language.

Signed this 20th day of February 1996

A handwritten signature in black ink, appearing to read 'Abraham Smith', written in a cursive style.

A. SMITH

For and on behalf of RWS Translations Ltd.

REPLACED SHEETS		SG
☒	Description : paged1..... to paged ...31.....	
☒	Claim(s) : paged ...31... to paged ...33...	
☒	Drawing(s) : paged ...VI... to paged7.....	
☒	Abstract : paged1..... to paged7.....	
DATE OF RECEIPT		

NUCLEOTIDE SEQUENCES CODING FOR A PROTEIN WITH UREASE
ACTIVITY

The present invention concerns nucleotide
5 sequences coding for a urease such as is expressed
naturally in Campylobacter pylori and its biological
applications, notably for the detection of C. pylori in
humans or in the environment.

C. pylori is a Gram-negative bacterium
10 heretofore found exclusively at the mucosa surface of
the antral region of the human stomach, and more
particularly around the lesions and craters of gastric
and duodenal ulcers. 25% of the population are C.
pylori carriers: 8% present an ulcerous illness, 9%
15 suffer from nonulcerous dyspepsia and 8% are
asymptomatic carriers.

All the C. pylori isolated and described
heretofore possess the following three properties:

The bacteria produce an extremely active
20 urease.

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They adhere very strongly to the epithelial cells of the gastric mucosa, this property being manifested in vitro by an extremely strong adhesion to the Hela cells.

5 The C. pylori produce and release an enzyme which exhibits a proteolytic activity leading to the degradation of the mucus and thus to a weakening of the natural barrier protecting the gastric mucosa.

 However, the detection of C. pylori in situ in
10 humans and in the environment, and the study of its pathogenic power - it is considered responsible for active gastritis in humans - is complicated by difficulties in culturing this organism.

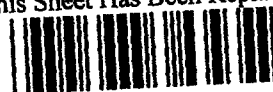
 Its growth is very slow (6 to 7 days on blood-
15 agar medium) and must take place in microaerophilic conditions, the oxygen in the air being toxic.

 The search for means of detecting this bacterium has led the inventors to identify the genes in C. pylori responsible for producing urease.

20 The determination of the nucleotide sequence of an intragenic fragment has made it possible to obtain a tool which can be used as a detection probe for the specific identification of the C. pylori.

 The invention is thus intended to supply new
25 sequences of nucleotides which are capable of coding for proteins with urease activity such as those expressed in C. pylori.

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It is also intended to supply fragments of this sequence which may be used as detection probes for C. pylori.

In addition, the invention aims to supply a
5 protein with urease activity, such as is expressed in C. pylori, of a high degree of purity, and fragments of the said protein.

According to the invention, the sequence of nucleotides is characterized in that it comprises at
10 least one part of a sequence coding for a protein with urease activity such as is expressed in C. pylori.

The invention more particularly concerns a sequence of nucleotides which is capable of hybridizing with genes coding for a protein with urease activity
15 such as is expressed in C. pylori, under the following conditions: 68°C, 6 x SSC (1 x SSC is made up of 0.15M of NaCl and 0.015M of sodium citrate, pH7), in Denhardt medium (1% Ficoll, 1% polyvinylpyrrolidone, 1% bovine serum albumin).

20 According to another aspect of the invention, the sequence of nucleotides is characterized in that it carries the information required for the production of a protein with urease activity or of its fragments, capable of forming an immunological complex with
25 antibodies directed respectively against a protein with urease activity such as is expressed in C. pylori or against fragments of the said protein.

The sequence according to the invention is also characterized in that it comprises at least one part of



a fragment of about 8 kb corresponding to the restriction fragment EcoRI (ClaI, BamHI) - PstI (HindIII).

The enzymatic restriction map of this fragment
5 is represented in Figure 2.

A more specifically preferred sequence comprises at least one part of a fragment of about 4.2 kb ($\pm$ 5%) delimited by the end nucleotides respectively situated at a distance of approximately
10 0.55 kb upstream of site H2, as represented in Figure 2, and of approximately 0.7 kb downstream of site B1, as represented in Figure 2.

The sequences constituted by this fragment carry the information necessary for the expression of a
15 protein with urease activity as expressed naturally in C. pylori.

The expression of these sequences may occur in any other micro-organism capable of exploiting to its advantage the information carried by the said
20 sequences, notably in a strain of Campylobacter which is naturally deficient in genes coding for the production of a protein with urease activity (or in a strain of urease- campylobacter).

According to yet another aspect, the invention
25 concerns a recombinant sequence comprising one of the above-defined sequences, possibly in association with a promoter capable of controlling the transcription of the sequence and a DNA sequence coding for termination signals of the transcription.

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The nucleotide sequences of the invention are characterized in that they are capable of hybridizing with a probe formed from the sequence exhibiting:

- the following chain (I) of nucleotides:

5 AAGCTTTTAGGGGIGITAGGGGTTTATCAAAAATCTAAAAAGOOCTTTCMTCTCAAGCA
ATTGTGCTACAAACATGAGCAATTTAGOOCTTAAAGAATACTTAAAATOOCAAGATTTA
GAATTGAAGCATTGOGOGATTGGGGATAAGTTTGTGAGOGAATGCATGOGATTGAACAAA
GCCAATTTTGGAGGOGAGCAAGOGGGCATATCATTTTLAGOGATTAGCTAAAACOGGC
GATGGCTTGGTGTGUGCTTTCAGTGAGOGOGTTAGTGTAGAAAGTAAGCTT

10 - or the following chain (II) of nucleotides:

AAOGCATGATTGATTGCAGAAGGAGTGGTGOOOCAGTCTTOGTGAATTTTAAAGOCAATC
GCAOOGGCTTCAATTTGATOGGCTAAGCTOGCATOGTTAGAAGOGTTAOCCTTAGOCAAG
AAOCTAAATTCATAGAATATTCTTCAGOOGCTCTGAGCATOCATTTTAAATTTCTTCTG
OCTGGAGTGATAGTAGTOGCATTAGTGCATCAGCAGGAOOGGTTCAOCCAOCAATCATG
15 GTTGTACAOOGCTTOGAAAAGCTGTAGGGATTGTGTGGGGTGAATGAAGTGGATGTGT
GTGTCAATAOCCAGOOGTTAOGATCAAAOCTTCAOOGGCTAAGGCTTCAGTAGCAGGA
OCTAOGCTAAGATTGTTTTAAOGOCATCTTGCATGTCTTGTAAOOGCTTTAOCATG
CCAGOGATTTTGOCATCTTTAATAOCCATATCOGCCTTATAAATAOOGGTTAATOCAOG
ATTAAAGOGTTAGTGATAATTAAATOCAACCTTCTTTGCTAGGGTTGTAGATTGGCTC
ATGOCTTCTCTTAGGGGTTTAAOOGOCAOOGAATTTAAGCTCTTOGOCATAAATGGTGTA
GTCATGTCTTACTTCAGOGATCAAGTCTGATOGOOCAATCTCACCTTATOGOGCTGTAGTA
20 GGAOCATACATAGAAACATATCTTTCTGCTAACTCTTTTCAATTTCTTACTOCTTAATT
GTTTACATAGTTGTTCATOGCTTTTAGG

- or the following chain (II bis) of nucleotides:

25

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OCTAAAGOGATGCAACTATGTAACAAACATTAAAGGAGTAAGAAATGAAAAGATTAGC
AGAAAAGAATATGTTTCTATGTATGGTCTACTACAGGOGATAAAGTGAGATTGGGGGAT
CAGACTTGATOGCTGAAGTAGAACATGACTACACCATTATGGOGAAGAGCTTAAATTG
GTGGGGGTAAAACCOCTAAGAGAAGGCATGAGOCAATCTAACAAOCTAGCAAAGAAGAG
TTGGATTTAATTATCACTAAOGCTTTAATOGTGGATTACAOOGGTATTATAAAGOGGAT
ATTGGTATTAAAGATGGCAAAATOGCTGGCATTGGTAAAGGOGGTAAACAAAGCATGCAA
5 GATGGOGTTAAAACAATCTTAGOGTAGGTCTGCTACTGAAGCCTTAGOOGGTGAAGGT
TTGATOGTAAGGCTGGTGGTATTGACACACACATOCACCTTCATTTCACOOCAACAATC
OCTACAGCTTTTGAAGOGGTGTAAACAACCATGATTGGTGGTGGAAOOGGTCTGCTGAT
GGCACEAATGOGACTACTATCACTOCAGGCAGAAGAAATTTAAATGGATGCTCAGAGOG
GCTGAAGAATATTCTATGAATTAGGTTTCTTGGCTAAAGGTAAOGCTTCTAAOGATGOG
AGCTTAGOOGATCAAATTGAAGOOGGTGGGATTGGCTTTAAATTCAGAAGACTGGGGC
10 AOCACCTCTCTGCAATCAATCATGOGTT

- or the chains of nucleotides which are complementary respectively to the above-mentioned chains (I), (II) and (II bis).

Sequences of nucleotides according to the invention are characterized in that they comprise the
15 above-defined nucleotide chains or are constituted by at least a part of the said chains.

It is self-evident that the bases of the nucleotide sequences may be in a different order from
20 that found in the genes, and/or that the said bases may possibly be substituted, when a probe elaborated from such sequences gives a characteristic and unequivocal response with respect to the ability to recognize the presence of genes coding for the protein with urease
25 activity of C. pylori.

The invention also covers any sequence of nucleotides which is hybridizable with that of the above-mentioned chains of nucleotides, such as that



obtained by reverse enzymatic transcription of the corresponding RNA, or else by chemical synthesis.

The invention also concerns a sequence of nucleotides which corresponds, according to the
5 universal genetic code, to at least one part of the following amino acid sequence (III):

**KLGLGVYQKSKNALSSQAIVATNMSNALKEYLKSQLELKHCAIGDKFVSEOMRNLK
ANFGGEQSGHIIIESDYAKTGDGLVCALQVSALVLESKL**

The invention also relates to a sequence of
10 nucleotides which corresponds, according to the universal genetic code, to at least one part of the following amino acid sequence (IV) (encoded by the nucleotide sequence (II bis):

**NMTTFMAKSLNSVAVKPLREGMSQSNNPSKEFLDLITNALIVDYTGTYKADIGIKDGK
15 IAGIGKGGNKMDQGVKNLSVGPATEALAGEGLIVTAGGIDTHIHFIISPOQIPTAFRSG
VTIMIGGGTGPADGINATTITPGRNLKWLRAAEYSNGLFLAKGNASNDASLADQIE
AGAIGFKIHEDWGTIPSAINHA**

The invention also concerns recombinant expression and cloning vectors capable of transforming
20 an appropriate host cell, the said vectors containing at least one part of a sequence of nucleotides as defined above under the control of regulatory elements permitting its expression.

Preferred recombinant vectors include at least
25 one part of the above-mentioned sequence of approximately 4.2 kb.

The strains of transformed microorganisms are also covered by the invention. These strains contain



one of the above-defined sequences of nucleotides or a recombinant vector as defined previously.

Strain E. coli S17-1, deposited on 16 August 1988 under number I-795 at the Collection Nationale de
5 Culture de Microorganismes (C.N.C.M.) of the Institut Pasteur in PARIS (France), carries plasmid pILL590 of approximately 16.3 kb comprising the above-mentioned restriction fragment EcoRI(ClaI, BamHI)-PstI (HindIII) of approximately 8 kb.

10 The invention also concerns a protein exhibiting a urease activity of the type expressed naturally in C. pylori, as well as the peptide fragments of this protein.

The protein of the invention, and its
15 fragments, correspond, according to the universal genetic code, to the above-defined sequences of nucleotides, in particular to at least one part of the sequence of approximately 4.2 kb.

The protein of the invention is also
20 characterized in that it is a protein such as is obtained by the transformation of host cells by means of a recombinant vector as defined above, culturing, in an appropriate medium, of the transformed host cells and recovery of the protein from these cells or
25 directly from the culture medium. The invention also concerns the production of urease or of fragments of the latter by this process.

The protein of the invention, and its fragments, which may also be obtained by chemical



synthesis, exhibit advantageously a high degree of purity and are used, according to standard techniques, to form polyclonal antibodies.

Such polyclonal antibodies, and the monoclonal
5 antibodies capable of recognizing specifically the above protein and its fragments, are also covered by the invention.

The above-defined sequences of nucleotides are obtained, according to the standard techniques of
10 genetic engineering, by cloning and identification of the genes responsible for the synthesis of a protein with urease activity in C. pylori.

The invention also concerns the biological applications of the sequences of nucleotides, of the
15 corresponding proteins, and of the monoclonal or polyclonal antibodies.

These applications include the elaboration, from intragenic fragments, of probes for the detection of C. pylori. This elaboration comprises, in
20 particular, the denaturation of the double-stranded sequences in order to obtain a single-stranded sequence which may be used as a probe.

The tests performed with such fragments with a view to detecting the possible presence of
25 complementary sequences in various Campylobacteria and in microorganisms belonging to different genera have revealed the high degree of specificity of these fragments.

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The invention therefore concerns detection probes characterized in that they comprise at least one part of an above-defined sequence of nucleotides.

Any probe which, with regard to its sequence of
5 nucleotides, is distinguished from the previously mentioned probe only in respect of substitutions or alterations of nucleotides not causing modification of its hybridization properties with the genome of C. pylori is covered by the invention.

10 The DNA fragment used as a probe contains a sufficient number of nucleotides to obtain the required specificity and the formation of a stable hybrid.

It is possible to use fragments of up to several kb, but high-specificity results are also
15 obtained with shorter fragments of approximately 25 to 40 nucleotides.

Appropriate probes for this type of detection are advantageously labelled with a radioactive element or any other group allowing its recognition in the
20 hybridized state with the preparation comprising the DNA to be studied.

According to the standard techniques, these probes are brought into contact with a biological sample containing bacteria, or directly with these
25 bacteria or their nucleic acids, in conditions permitting the possible hybridization of the nucleotide sequence of the probe with a complementary sequence possibly contained in the tested product.

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The blot hybridization method may, for example, be used. This method involves, after denaturation of the DNA obtained beforehand from bacteria originating from antral biopsies, the deposition of an aliquot
5 quantity of this DNA on nitrocellulose membranes, the hybridization of each membrane under the usual conditions with the probe, and the detection, in standard fashion, of the hybrid formed.

A replica hybridization method may also be
10 used, according to the Southern technique. This method involves the electrophoretic separation in agarose gel of the fragments of DNAs engendered after treatment of the DNA by restriction enzymes, transfer after alkaline denaturation onto appropriate membranes, and their
15 hybridization with the probe under the usual conditions. It is not always necessary to carry out the preliminary expression of the DNA; it is enough that the DNA should be rendered accessible to the probe.

Detection for the specific identification of
20 the C. pylori may also be carried out by DNA amplification techniques (PCR). These techniques are in particular described in US patents 4683202 and 4683195 to Cetus Corporation.

To implement these techniques, two primers are
25 used of 10 to 40 nucleotides, advantageously of approximately twenty nucleotides, these primers being included in one of the above-defined sequences of nucleotides or able to hybridize with a part of one of the above-defined nucleotidic sequences, or of their



complementary sequences. When they are hybridized (or linked), these primers are advantageously situated at approximately 200 to 250 nucleotides from the DNA strains to be amplified. one of the sequences is
5 capable of being linked to a nucleotide sequence of one of the DNA fragment strands to be amplified, this sequence being situated at an end of this fragment (for example the 5' end); the other sequence is capable of being linked to a nucleotide sequence of the second DNA
10 fragment strand to be amplified, this latter sequence being situated at the end opposite the above-mentioned end of this fragment (which opposite end is designated by 3' end in the given example).

Preferred primers are included in the
15 nucleotide sequence of restriction fragment H2-H3 in Figure 2, and are approximately 200 to 250 nucleotides apart.

In particular, the fragments concerned are those situated respectively at each end of this
20 restriction sequence HindIII-HindIII.

A method of in vitro detection of the possible presence of C. pylori in a biological sample includes the following steps:

- possibly, the prior amplification of the amount of
25 nucleotide sequences likely to be contained in the sample by bringing this sample into contact with primers as described above, these primers being able to be linked respectively to the 5' end of one strand of the said sequence of nucleotides and to the 3' end of



the other strand of the said sequence of nucleotides,
this contact being followed by a gene amplification
step in the presence of a DNA polymerase and of the
four triphosphate nucleosides dATP, dCTP, dGTP and
5 dTTP, with these primer hybridization and gene
amplification steps being repeated several times;

- the bringing of biological samples in question into
contact with a nucleotide probe according to the
invention, under conditions permitting the production
10 of a hybridization complex formed between the probe and
the sequence of nucleotides,
- the detection of the hybridization complex.

The invention thus provides tools enabling the
rapid detection of C. pylori, both in situ and in the
15 environment, with a high degree of specificity and
without the need for culturing.

Such detection tools are particularly useful in
studying the natural reservoir of these bacteria, as
well as the methods of transmission, circulation and
20 contamination. Furthermore, in the in vitro diagnosis
performed on the biopsies, the use of these probes
makes it possible to save a considerable amount of time
compared to current techniques, which, moreover,
require a technology which can only be carried out in
25 specialized services, i.e. bacteriological techniques
or the use of electron microscopy.

Reproducible results have been obtained using
the above-defined fragment of approximately 8 kb as
intragenic probe.

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Given their specificity with regard to C. pylori, these probes are also tools of considerable interest.

In studying the pathogenic power of C. pylori with a view to preventing infection, it is thus possible to study the role played by C. pylori in the development of ulcerous illnesses and to identify the genetic determinants which seem to be involved in this organism's pathogenic power, with a view to creating, in vitro, isogenic mutants of C. pylori, i.e. bacteria modified genetically in each of the determinants which seem to play a role in the pathogenesis of the infection, which mutants could be tested using animal models.

The invention also concerns the detection of the polymorphism of the genes coding for the urease in C. pylori by means of the above-defined nucleotide probes and appropriate restriction enzymes. The conditions under which this detection is carried out are described in particular in an article by J.F. de Gusella in J. of Clin. - investigations (1986), 77, 1723-1726.

The invention also concerns the immunological applications of the encoded protein, particularly for the elaboration of specific antiserum and of polyclonal and monoclonal antibodies. The polyclonal antibodies are formed, according to standard techniques, by injecting the protein into animals, recovering the



antiserums and then the antibodies from the antiserums, for example by affinity chromatography.

The monoclonal antibodies are produced in the usual manner by fusing myeloma cells with cells of female rats previously immunized by means of the proteins of the invention.

In addition, the invention concerns an in vitro detection method for the possible presence of C. pylori in a biological sample in which it is likely to be contained. This method is characterized in that it comprises:

- the bringing into contact of the sample with an antibody according to the invention, in conditions allowing the production of an immunological complex formed between all or part of the protein with urease activity produced by C. pylori and this antibody, and
- the detection of the immunological complex.

For the implementation of the in vitro detection methods, considered above and based on the use of nucleotide probes, use is advantageously made of equipment or kits comprising:

- if appropriate, at least one pair of primers according to the invention,
- a defined quantity of a nucleotide probe according to the invention,
- advantageously, a medium which is appropriate to the formation of a hybridization reaction between the sequence to be detected and the probe.



- advantageously, reagents allowing the detection of hybridization complexes formed between the nucleotide sequence and the probe during the hybridization reaction.

5 For the implementation of the in vitro detection methods, defined above and based on the use of antibodies, use is advantageously made of equipment or kits comprising:

- a defined quantity of an antibody according to the
10 invention,

- advantageously, an appropriate medium for the formation of an immunological reaction between at least one part of the protein with urease activity produced by a strain of C. pylori and the antibody,

15 - advantageously, reagents permitting the detection of the immunological complexes formed between at least one part of the protein with urease activity and the antibody during the immunological reaction.

 An in-depth study of the above-defined fragment
20 of 4.2 kb has made it possible to locate the structure genes coding for the major polypeptide subunits of the urease of C. pylori.

 This study was carried out by constructing mutants in the recombinant plasmids introduced into E.
25 coli and C. jejuni, which are incapable of synthesizing the urease (A. Baskerville, D. Newell (1988), Gut. 29, 465-472). The system of in vitro transcription-translation of E. coli and a system of expression in minicells were used to identify and locate, in the



fragment of 4.2 kb of C. pylori, all the genes coding for the urease subunits; two major proteins of 26 kDa and 61 kDa were identified. Parallel to this, after various deletions within the fragment of 4.2 kb, a
5 system of transfer by conjugation of a strain of E. coli to C. jejuni (of the type set out in the detailed description which follows) was used to identify the regions indispensable to the expression of urease activity, and to correlate the deletion of a
10 region with the disappearance of a polypeptide and the loss of urease activity. In this way, it was determined that the genes coding for the two polypeptides necessary to the expression of the urease activity are situated in the central region of 03.35 kb, downstream
15 of site H3 in Figure 2, and astride B1. Transcription of the genes coding for the polypeptides of 61 kDa or 26 kDa takes place in the sense EcoRI-PstI. The fragment of 3.35 kb is necessary to, but insufficient for, the expression of the urease activity in
20 C. jejuni. An adjacent region of 0.85 kb, situated upstream of and apparently not associated with the expression of polypeptides in E. coli, is vital to the expression of the urease activity in C. jejuni.

Analysis of the deletion mutants by in vitro
25 transcription-translation made it possible to observe that the two polypeptides of 26 and 61 kDa are encoded by two adjacent DNA fragments.

This invention concerns all or part of the following nucleotide sequence (V):

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1
ATGAACTCAOCCAAAAGAGTTAGATAAGTTGATGCTOACTAOGCTGGAGAATTGGCT
AAAAAAGCAAAGAAAAGGCATTAGCTTAACTATGTAGAAGCAGTAGCTTTGATTAGT
5 GOOCATATTATGGAGAAGOGAGAGCTGGTAAAAAGACTGOGGCTGATTGATGCAAGAA
GGGGCACTCTTTTAAAACAGATGATGTGATGGATGGOGTGGCAAGCATGATOCATGAA
GTGGGTATTGAAGOGATGTTTOCTGATGGGACTAAACTOGTAAOOGTGCATAOOOCTATT
GAGGOCATGGTAAATTAGTTTOCTGGTGAGTTGTTCTTAAAAATGAGACATCACTATC
AAOGAAGGCAAAAAAGOOGTAGOGTGAAGTTAAAAATGTTGGOGACAGAOOGGTTCAA
10 ATOGGCTCACACTTOCATTCTTTGAAGTGAATAGATGCTAGACTTTGACAGAGAAAAA
ACTTTOGGTAAAGCTTAGACATTGOGAGOGGGACAGOGGTANGATTGAGOGCTGGOGAA
GAANAATOOGTAGATTGATTGACATTGGOGGTAAACAGAGAATCTTTGGATTAAOGCA
TTGGTTGATAGACAAGCAGACAAGGAAGCAAAAAATTGCTTTACACAGAGCTAAAGAG
OGTGGTTTTCATGGOGCTAAAGOGATGACAACCTATGTAAAAACAATTAAGGAGTAA

717

15

The invention particularly concerns any nucleotidic sequence corresponding, according to the universal genetic code, to at least one part of the following animated acid sequence (VI) of 26 kDa
20 (encoded by nucleotide sequence (V)):

1
MKLTPEKELDKIMLHYAGELAKKRKEKGDKLNYVEAVALISAHIMEEARAGKCTAAELMOE
GRILLKPDVMDGVASMIHEVGIEAMFPDGTIKLVTVHTPIEANGKLVPGELFLKNEDITI
NEGKAVSVKVNVDGPRPVQIGSHFHFEEVNRCLDFDREKTFGKRLDIASGTAVREEPGE
EKSVELIDIGGNRRIFGENALVDRQADNESKKIALHRAKERGFHGAKSDONYVKTIKE

239

25 239 Amino acids

Molecular weight: 26,522 daltons

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The invention also concerns all or part of the following nucleotide sequence (VII):

1
ATGAAAAGATTAGCAGAAAAGATATGTTTCTATGATGGTOCTACTACAGGOGATAAA
GTGAGATTGGGOGATACAGACTTGATOGCTGAAGTAGACATGACTACAOCATTATGGC
5 GAAGAGCTTAAATTOGGTGGGGTAAAOCTAAGAGAGGCATGAGOCAATCTAACAC
OCTAGCAAAGAGAGTTGGATTTAATTATCATAAOGCTTAAATOGTGGATTACAOGGT
ATTTATAAGOGGATATTGGTATTAAAGATGGCAAATOGCTGGCATTGGTAAAGGOGGT
AACAAAGACATGCAAGATGGOGTTAAAAACAATCTTAGOGTAGGTGCTGCTACTGAGOC
TTAGOOGGTGAAGGTTTGATOGTAOOGGCTGGTGGTATTGACACACACATOCACITCATT
TCAOOOCACAAATOOCTACAGCTTTTGCAAGOGGTGTACAAOCATGATTGGTGGTGA
AOOGGTGCTGCTGATGGCACAATGOGACTACTATCACTOCAGGCAGAGAAATTTAAAA
TGGATGCTCAGAGOGGCTGAAGAATATTCATGAATTTAGGTTTCTTGGCTAAAGGTAC
GCTTCTAAGATGOGAGCTTAGOOGATCAAAATGAAGOOGGTGGGATTGGCTTTAAAT
CAOGAAGACTGGGGCAOCCACTOCTTCTGCAATCAATCATGGGTAGATGTTGOGGACAA
TAOGATGTGCAAGTGGCTATOCACACAGACACTTTGAATGAAGOOGGTGTGTAGAGAC
ACTATGGCTGCTATGCTGGAGGCATATGCACACTTTOCACACTGAGGOGGCTGGOGGC
GGACAOGCTOCTGATATTATTAAAGTAGOOGGTGAACACAACATTCTTGOOGCTTCACT
AAOOOCACCATOOCTTTCAOOGTGAATACAGAGCAAGCACATGGACATGCTTATGGTG
TGOCACCACTTGGATAAAAGCATTAAAGAAGATGTTTCAGTTOGCTGATTCAAGGATOOGC
OCTCAAOCAATTGOGGCTGAAGACACTTTGCATGACATGGGATTTTCTCAATCAOCAGT
TCTGACTCTCAAGOGATGGGOOGTGTGGGTGANGTATCACTAGAACTTGGCAAACAGCT
GCAAAAACAAGAAAGAATTTGGOOGCTTGAAAGAAAGAAAGGOGATAACACAACTTC
AGGATCAAAGCTACTTGTCTAAATACAOCAATTAAOOCAGOGATOGCTCATGGGATTAGC
GAGTATGTAGGTTCAGTAGAGTGGGCAAGTGGCTGACTTGGTATTGTGGAGTCCAGCA
TTCTTTGGOGTGAAAOCAACATGATCATCAAAGOGGATTCATTGOGTTAAGOCAAATG
GGOGATGOGAOGCTTCTATOOCTAOOOCACAAOOGGTTATTACAGAGAAATGTTGGCT
CATCATGGTAAAGCTAAATAOGATGCAACATCACCTTTGTGCTCAAGOGGCTTATGAC
AANGGCATTAAAGAGAATTAGGACTTGAAAGACAAGTGTGGOOGGTAAAAAATTGCAGA
AATATCACTAAAAAGACATGCAATTCAOAGACACTACTGCTCACATTGAAGTCAATCT
GAACTTAOCATGTGTTGGTGGATGGCAAAGAGTAACTTCTAAOOCAGOCAATAAAGTG
AGCTTGGOGCACTCTTTCAGCATTTTCTAG

1710

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The invention particularly concerns any nucleotide sequence corresponding, according to the universal genetic code, to at least one part of the following amino acid sequence (VIII) of 61 kDa (encoded
5 by nucleotide sequence (VII)):

1
MKKSRKEYVSMYGPTTGDKVRLGDTDLAEVEHDYTTYGEELKFGGGKTLREGM 57
SQSNPNPSKEELDLITNALVDYTGMYKADIGIKDGKAGIGKGGNKDMQDGVKNN 107
LSVGPATEALAGEGLIVTAGGIDTHIHFIHQIPTAFASGVTTMIGGGTGPA 165
10 **GTNATTTTPGRRNLKWMRLAAEEYSMNLGFLAKGNASNDASLADQIEAGAIGF** 210
KIHEDWGTTPSAINHALDVADKYDVQVAIHTDTLNEAGCVEDTMAAIAGRTMH 270
TFHTEGAGGGHAPDIIKVAGEHNILPASTNPTIPFTVNTAEHMDMLMVCHLD 320
- **KSIKEDVQFADSRIRPQTIAAEDTLHDMGIFSITSSDSQAMGRVGEVITRTWQT** 370
ADKNKKEFGRLKEEKGDNDNFRIKRYLSKYTINPAIAHGISEYVGSVEVGKVADL 432
15 **VLWSPAFTGVKPNMIKGGFIALSQMGDANASPTPQPVYYREMFAHIGKAKYD** 476
ANITFVSQAAYDKGIKEELGLERQVLPVKNCRNITKKDMQFNDTTAHIEVNPETY 520
HVFVDGKEVTSKPANKVSLAQLFSIF 569

569 Amino acids

20 Molecular weight: 61 729 daltons

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Comparison of amino acid sequence VIII with that of jack bean urease, described in particular in Eur. J. Biochem 175, 151-165 (1988), unexpectedly reveals a high level of amino acid homologies. This structure conservation has enabled the inventors to deduce the active site of the urease subunit of 61 kDa which is comprised, in whole or in part, in the polypeptide sequence limited by the amino acids situated at positions 206 and 338 of the above-described sequence VIII.

The invention also concerns all or part of nucleotide sequence IX consisting successively of sequence V and sequence VII.

The invention also relates to any nucleotide sequence corresponding, according to the universal genetic code, to at least one part of the amino acid sequence (X) of 87 kDa consisting successively of sequence VI and sequence VIII.

The invention also concerns probes for detecting the possible presence of C. pylori in a biological sample, characterized in that they comprise at least one part of a sequence of nucleotides defined above.

It is self-evident that the bases of the sequences of nucleotides under consideration may be in an order differing from that found in the genes, and/or that these bases may if appropriate be substituted, when a probe elaborated from such sequences gives a characteristic and unequivocal response with respect to



the ability to recognize the presence of genes coding for the protein with urease activity of C. pylori.

The invention also covers any sequence of nucleotides which is hybridizable with that of the
5 above-mentioned chains of nucleotides, such as is obtained by reverse enzymatic transcription of the corresponding RNA or by chemical synthesis.

The invention covers any probe which, with regard to its sequence of nucleotides, is distinguished
10 from that mentioned previously only by substitutions or alterations not causing any modification of its hybridization properties with the genome of C. pylori.

The above-mentioned probes may be used in in vitro processes for diagnosing the possible presence of
15 C. pylori in a biological sample, such as are described above.

The invention also concerns nucleotide primers of approximately 10 to approximately 40 nucleotides, these primers being included in one of the above-
20 defined nucleotide sequences V or VII (or in one of the sequences derived from the said sequences V and VII), or being able to hybridize with a part of one of the above-mentioned nucleotide sequences or with their complementary sequences (particularly under the above-
25 defined conditions of hybridization).

The invention also concerns the use of these primers in the above-defined in vitro diagnosis methods, for the preliminary amplification of the quantity of nucleotides of C. pylori likely to be

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contained in the biological sample according to the above-described method.

The invention also relates to the recombinant expression and cloning vectors capable of transforming
5 an appropriate host cell containing all or part of a nucleotide sequence which is identical to, or derived from, the above-mentioned nucleotide sequences V, VII and IX.

The invention also concerns a process for
10 producing proteins corresponding to all or part of the above-mentioned sequences VI, VIII and X, the said process comprising the transformation of appropriate host cells (particularly those described above) by means of the above-mentioned vectors and the recovery
15 of the proteins thus produced followed, if appropriate, by a purification step for these proteins.

The above-defined protein production process may, if appropriate, comprise a preliminary amplification step of the gene coding for the protein
20 whose production is sought, this step being carried out with primer pairs of the invention in accordance with the above-described method.

The invention also concerns the polypeptides (or proteins) encoded by the above-described nucleotide
25 sequences. The invention particularly concerns the above-described amino acid sequence X of 87 kDa, sequence VI of 26 kDa and sequence VIII of 61 kDa, and their fragments.

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The invention also concerns the polyclonal and monoclonal antibodies obtained from all or part of the above-described polypeptides and able to form an immunological complex with them.

5 In particular, the invention concerns the antibodies, notably monoclonal, obtained by means of a peptide sequence of at least approximately 10 to 15 amino acids of sequence VI or of sequence VIII.

Preferred monoclonal antibodies of the
10 invention are directed against all or part of the sequence delimited by the amino acids situated at positions 206 and 338 of sequence VIII, more especially those obtained by means of a peptide sequence of at least approximately 10 to 15 amino acids originating
15 from the sequence delimited by the amino acids situated at positions 206 and 338 of sequence VIII.

The invention also relates to the use of the above-mentioned antibodies to implement the in vitro methods and the in vitro kits, as described above, for
20 diagnosing the infection of an individual by C. pylori.

The invention also concerns immungenic compositions comprising all or part of the above-described polypeptides, and in particular all or part of the polypeptide sequence delimited by the amino
25 acids situated at positions 206 and 338 of sequence VIII, in association with a pharmaceutically acceptable vehicle.

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Such immunogenic compositions may be used as vaccine compositions for preventing the infection of an individual by C. pylori.

The invention also concerns pharmaceutical compositions comprising one (or a number of) antibodies such as are described above, and, in particular, antibodies able to form an immunological complex with all or part of the peptide sequence delimited by the aminos situated at positions 206 and 338 of sequence VIII, in association with a pharmaceutically acceptable vehicle.

Such pharmaceutical compositions according to the invention may be used in the treatment of pathologies linked to the infection of an individual by C. pylori, notably gastritis, and gastric and duodenal ulcers.

Other advantages and characteristics of the invention are detailed in the following description relating to the cloning of genes responsible for the production of the protein with urease activity in C. pylori, the sequencing of the region associated with the expression of the protein with urease activity and the specificity of nucleotide probes for detecting C. pylori.

In this description, reference is made to Figures 1 and 2, of which:

- Figure 1 represents the restriction map of a fragment PstI-EcoRI of the recombinant cosmid IID2 carrying the

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information required for the expression of the urease in C. jejuni, and

- Figure 2 represents the restriction map of a fragment of approximately 8 kb of pILL590.

- 5 1) Construction of a genomic bank in a shuttle cosmid vector; cloning of genes involved in the production of urease in C. pylori.

The chromosomal DNA of the strain C. pylori (85P) is prepared so as to generate DNA fragments of
10 high molecular weight; approximately 300 μ g of this chromosomal DNA are subjected to a controlled partial digestion by the restriction endonuclease Sau3A, and deposited on sucrose gradient in such a way as to isolate selectively digestion fragments comprised
15 between 35 and 45 kilobases. 1.5 μ g of these fragments is ligated in vitro onto the shuttle cosmid vector pILL575 linearized by the endonuclease BamHI and dephosphorylated. pILL575 was constructed from the shuttle cosmid pILL550 described by Labigne-Roussel et
20 al. in J. Bacteriol. 169:5320-5323, 1987, into which was inserted the "cos" site of the lambda phage (fragment Bgl II of 1.7 kb of pERG153 inserted at site Pvu II of pILL550); like this cosmid, it may thus be mobilized by conjugation, codes for a resistance to
25 kanamycin which is expressed in both E. coli and Campylobacter (Km^r) and is replicated in both E. coli and C. jejuni owing to the presence of two replication origins, one of which is functional in E. coli only and the other solely in Campylobacter. The products of this



ligation are packaged in vitro in the lambda particles and then introduced by transfection into a strain of E. coli accommodating a plasmid IncP capable of complementing in trans the transfer functions of the
5 recombinant cosmids. The infected and kanamycin-resistant colonies of E. coli are immediately preserved individually at -80°C; a cosmid genomic bank representing the entire genome of Campylobacter pylori is realized in E. coli by preserving approximately 500
10 independent clones.

Each of the recombinant cosmids is then transferred by conjugation of E. coli to a receptor strain of Campylobacter jejuni C31 which is naturally urease⁻.

15 Each of these kanamycin-resistant transconjugants is checked to see if synthesis of a urease by Campylobacter jejuni occurs.

One cosmid is isolated out of 106 analysed carrying genetic information which gives Campylobacter
20 jejuni C31 the ability to produce a urease. The size of the recombinant cosmid (IID2) is 54 kilobases. Figure 1 shows the relative position of the sites cleaved by the restriction endonucleases PstI, BamHI, SmaI and EcoRI on this sequence.

25 2) Identification of the gene(s) required for the production of urease in Campylobacter pylori.

Subcloning of the urease genes is carried out by performing, from the cosmid DNA IID2 prepared in E. coli, partial digestion by the endonuclease Sau3A so



as to generate partial digestion fragments ranging from 8 to 15 kilobases. The objective is the smallest hybrid plasmid cloned in E. coli which confers the ability to produce a urease after its transfer by conjugation to
5 receptor Campylobacter.

Out of the 37 tested, 4 plasmids carry the information required for the expression of the urease in Campylobacter jejuni: pILL586, pILL589 and pILL590 (cf. Figure 2).

10 A comparison of the restriction maps of these four hybrids reveals the presence of a sequence of nucleotides of 4.2 kilobases which is common to the four hybrids, suggesting that this is the region required for the expression of the urease. A number of
15 deletions, confirming these conclusions, were performed (cf. Figure 2).

Fragment EcoRI-Pst I of pILL590 was used as a probe in hybridizations with the total DNA of C. pylori 85P so as to ensure that the different restriction
20 fragments generated with BamHI and HindIII from pILL590 were present in 85P. The results obtained show that the sequence of 8 kb present in pILL590 was not rearranged in the course of the various cloning steps.

3) Sequence of nucleotides of the region associated
25 with the expression of the urease.

The sequence of the fragment HindIII lying between sites H2 and H3 corresponds to the sequence of 294 nucleotides of the chain (I).

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The following sequence of 610 nucleotides of the chain (II) is internal to the fragment defined by sites H4 and B1 (Figure 2).

4) Specificity of the nucleotidic probes for the
5 detection of Campylobacter pylori.

Fragment EcoRI-PstI of 8 kb originating from pILL590 was used as radioactive probe in hybridization experiments.

32 Campylobacter pylori were tested in
10 hybridization on colonies and gave a positive signal; out of the 35 tested, none of the Campylobacter jejuni, fetus and coli gave a positive signal under standard and stringent hybridization conditions (50% formamide, 37°, 3XSSC).

15 Under the same conditions, the 8 kb probe of C. pylori did not give a positive signal with the list of the following microorganisms, all of which are natural producers of urease:

Klebsiella oxytoca (3 strains), Klebsiella
20 pneumoniae (3 strains), Proteus mirabilis (3 strains)
Proteus morganii (3 strains) Proteus vulgaris (2
strains) Providencia stuartii (3 strains), Pseudomonas
picketti (3 strains), Yersinia enterocolitica (3
strains), 5 strains of Acinetobacter urease⁺, 2 strains
25 of Escherichia coli urease⁺ and 3 strains of Citrobacter
freundii urease⁺.

The absence of false positive reaction with the fragment of 8 kb guarantees the specificity of the proposed nucleotide probes.

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A process for the preparation of antibodies comprises:

- the immunization of an animal by means of a polypeptide as defined above, and
- the recovery of the antibodies formed, using standard techniques.

An appropriate method for preparing the nucleic acids (containing a maximum of 200 nucleotides - or bp in the case of bicatenary nucleic acids) comprises the following steps:

- DNA synthesis using the β -cyanethyl phosphoramidite automated method described in Bioorganic Chemistry 4; 274-325, 1986,
- cloning of the DNAs thus obtained in an appropriate plasmid vector, and recovery of the DNAs by hybridization with an appropriate probe.

A chemical method for the preparation of nucleic acids of a length in excess of 200 nucleotides - or bp (in the case of bicatenary nucleic acids) comprises the following steps:

- assembly of chemically synthesized oligonucleotides equipped with different restriction sites at their ends, whose sequences are compatible with the amino acid chain of the natural polypeptide, according to the principle described in Proc. Nat. Acad. Sci. USA 80; 7461-7465, 1983,



- cloning of the DNAs thus obtained in an appropriate plasmid vector and recovery of the desired nucleic acid by hybridization with an appropriate probe.

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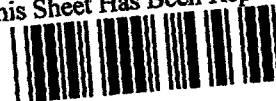




Claims:

1. Immunogenic composition characterized in that it comprises all or part of the sequence between the amino acids situated in positions 206 and 338 of sequence (VIII).
2. Nucleotide sequence corresponding, according to the universal genetic code, to the sequence between the amino acids situated in positions 206 and 338 of sequence (VIII).
3. Nucleotide sequence characterized in that it is delimited by two of the restriction sites of Figure 2 between sites H1 and B2 and including sites H1, H2, H3, H4, B1 and B2.
4. Peptide fragments of a protein with urease activity, characterized in that they are chosen from:
 - a) amino acid sequence (X) of 87 kDa
 - b) amino acid sequence (VI) of 26 kDa
 - c) amino acid sequence (VIII) of 61 kDa
 - d) a fragment of one of the three sequences a), b) and c).
5. Process for producing an immunological complex between the antibodies against the protein with urease activity which is expressed by C. PYLORI or fragments of the said protein, notably those of Claim 4, characterized by the bringing of the said protein or of the said peptide fragments into contact with the said antibodies, notably those which are present in a biological liquid.

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6. Use of the sequences according to Claims 2 and 3, for detecting the presence or absence of these sequences in samples.

7. Use of the fragments according to Claim 4 for
5 inducing the synthesis of specific antibodies against the said fragments.

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