Title: **HELCOBACTER ALIPHATIC AMIDASE AmiE POLYPEPTIDES, AND DNA SEQUENCES ENCODING THOSE POLYPEPTIDES**

### Abstract

This invention relates to *Helicobacter* species aliphatic amidase AmiE polypeptides, the DNA encoding those polypeptides and transformed microorganisms capable of expressing those polypeptides. This invention also relates to the use of *Helicobacter* sp. (particularly *Helicobacter pylori*) amidase AmiE polypeptides and antibodies specific for those polypeptides in immunogenic, therapeutic, and diagnostic applications. The invention additionally relates to processes of producing *Helicobacter* species aliphatic amidase AmiE polypeptides and intermediates useful in the production of those polypeptides.
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**HELICOBACTER ALIPHATIC AMIDASE AmiE POLYPEPTIDES,**
**AND DNA SEQUENCES ENCODING THOSE POLYPEPTIDES**

This invention relates to *Helicobacter* species aliphatic amidase AmiE polypeptides, the DNA encoding those polypeptides, and transformed microorganisms capable of expressing those polypeptides. In addition, this invention relates to the use of *Helicobacter* sp. (particularly *Helicobacter pylori*) amidase AmiE polypeptides and antibodies specific for those polypeptides in immunogenic, therapeutic and diagnostic application.

**BACKGROUND OF THE INVENTION**

An aliphatic amidase is an acylamide amidohydrolase (E.C. 3.5.1.4) (Merck Index). It hydrolyses short-chain aliphatic amides (C1-C4 such as acrylamide, acetamide, propionamide or isobutyramide) to produce ammonia and the corresponding organic acid. In addition, an aliphatic amidase possesses acyl transferase activity, i.e., it is able to transfer the acyl group of amides to hydroxylamine to form an acyl hydroxamate plus ammonia.

Aliphatic amidases have been identified in *Pseudomonas aeruginosa* (Brammar et al., 1987) and *Rhodococcus* sp. R312 (previously named *Brevibacterium* sp. R312; Soubrier et al., 1992). Other aliphatic amidases have been identified in *Methylophilus methylotrophus* (Silman et al., 1991), *Arthrobacter* sp. J-1 (Asano et al., 1982), and *Alcaligenes eutrophus* (Friedrich and Mitrenga, 1981). However, no molecular characterization of these latter three enzymes has been reported.

Aliphatic amidases are cytoplasmic enzymes; they have very similar enzymatic properties and molecular masses (38.4 kDa for *P. aeruginosa*; 38.2 kDa for *Rhodococcus* sp. R312; 37.8 kDa for *M. methylotrophus*; and 39 kDa for *Arthrobacter* sp. J-1), and have either
a tetra-, hexa-, or octameric structure. Some of these amidases have been shown to be inducible by their amide substrate. Database searches with the amino acid sequences of these aliphatic amidases indicates that they are more closely related to nitrilases (which catalyze the direct cleavage of nitriles to ammonia and to the corresponding acid) than to the nitrile hydratases (which hydrolyze nitriles to produce amides) or amidases from other classes (Novo et al., 1995).

The prevailing theory on the physiological role of the aliphatic amidases is that hydrolysis of amides supplies carbon and nitrogen sources to the bacteria. Curiously, Helicobacter sp. possess a very potent urease, which should be sufficient for nitrogen supply in this genus of bacteria. However, Helicobacter sp. are not the only bacteria possessing both urease and amidase, since this is also the case for P. aeruginosa, M. methylotrophus, and A. eutrophus.

Acrylamide, an aliphatic amide, is extensively used in a great number of industrial processes. Global production of acrylamide has been estimated to be over 200,000 tons. Widespread use and indiscriminate discharge of acrylamide have resulted in the contamination of terrestrial and aquatic ecosystems throughout the world. Other aliphatic amides are either active ingredients or metabolites of herbicide degradation (Roberts, 1984). Elimination of acrylamide and other toxic aliphatic amide by-products by an aliphatic amidase would be of great importance because these substances pose serious health hazards for humans and animals (Nawaz et al., 1994, 1996) (Nagasawa and Yamada, 1989).

Helicobacter pylori has become identified as a primary cause of chronic gastroduodenal disorders, such as gastritis, dyspepsia, and peptic ulcers, in humans. H. pylori can be successfully eradicated (80% to 90%) by a treatment combining two antibiotics with a proton pump inhibitor. However, few antibiotics are active against H. pylori, and antibiotic resistant strains (e.g., to metronidazole or clarithromycin) have begun to appear. Like H. pylori, Helicobacter heilmannii has been identified as the cause of gastric ulcers in pigs. Porcine gastric ulcers lead to lower weight pigs and consequently, less food product production. Due to the presence of numerous urea positive bacteria in the porcine gastrointestinal tract, methods that are not based on urease are preferred for detecting, treating
or preventing *Helicobacter* infections in pigs.

Thus, a need exists for an effective method of diagnosing, preventing, and treating gastrointestinal disorders caused by *Helicobacter* sp., particularly *H. pylori* and *H. heilmannii*.

**SUMMARY OF THE INVENTION**

This invention provides polynucleotides corresponding to *Helicobacter* species aliphatic amidase *amiE*. More particularly, this invention provides polynucleotides selected from the group consisting of:

(a) all or part of the DNA sequence encoding *Helicobacter* sp. and particularly, *Helicobacter pylori* aliphatic amidase *amiE* (depicted in Fig.5);

(b) all or part of the DNA sequence depicted in Fig. 4;

(c) a DNA sequence which hybridizes with all or part of DNA sequence (a) or (b) under stringent conditions and encodes a polypeptide having the biological or immunological properties of *Helicobacter* sp. and particularly, *Helicobacter pylori* aliphatic amidase or a fragment thereof; and

(d) an analog of DNA sequence (a), (b), or (c) resulting from the degeneracy of the genetic code.

Other aspects of this invention include polypeptides encoded by the polynucleotides of this invention; antibodies to those polypeptides; immunogenic, pharmaceutical, and therapeutic compositions comprising the polypeptides and antibodies of the invention; methods of using the polypeptides and antibodies of the invention to detect, treat or prevent *Helicobacter* sp. infections in man and animals; detection kits comprising the polypeptides and antibodies of the invention; processes for producing polypeptides according to the invention and intermediates useful in their production.

**BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1a and 1b depict N-terminal amino acid sequences of two internal peptides from an *H. pylori* protein with an apparent molecular mass of 49 kDa that has subsequently been identified as an aliphatic amidase. The sequences of degenerate oligonucleotides, H36
and H37, deduced from the two internal peptides are indicated under each amino acid sequence. H37 corresponds to the deduced anti-parallel sequence. Two residues at the same position in the H36 and H37 sequences (depicted as N/N) indicate that the oligonucleotide preparation contains a mixture of the two types of molecules. ("i" corresponds to inosine residues introduced in the degenerated oligonucleotides.) **Figure 1c** identifies two primers, H46 and H49, used to amplify an internal sequence of the *H. pylori* amidase gene.

**Figure 2** depicts the sequence of the PCR product obtained with oligonucleotides H36 and H37 from chromosomic DNA of *H. pylori* strain 85P. This sequence corresponds to an internal sequence of the *amiE* gene of *H. pylori*. The numbers above the sequences correspond to the nucleic acid number/the amino acid number.

**Figures 3a-3e** depict restriction maps of plasmids pILL400, pILL405, pILL417, pILL835, and pILL836, respectively. The genes are indicated by boxes with an arrow showing the direction of their transcription. Sp, Ap, Km, and Tet correspond to the genes conferring resistance to spectinomycin, ampicillin, kanamycin, and tetracycline, respectively. Ori indicates the origin of replication and OriT the origin of transfer of a conjugative plasmid. *lacZ* corresponds to the gene coding for β-galactosidase, *Plac* to the *lac* promoter, and *amiE* to the gene coding for the *H. pylori* amidase.

**Figures 4a and 4b** depict the sequence and full restriction map of the *H. pylori* DNA insert of plasmid pILL405, respectively.

**Figure 5** depicts the sequence of the *H. pylori* *amiE* gene and deduced amino acid sequence of the AmiE protein. Positions of hybridization with the two degenerate oligonucleotides H36 and H37 and of the two non-degenerate primers H46 and H49 are indicated. The predicted active site of the AmiE protein is underlined (residues 155 to 200) and the predicted active site nucleophile cys\(^{166}\) residue which corresponds to cys\(^{165}\) as described in Skouloubris et al., 1997, is highlighted.

**Figure 6** provides a comparison of the amino acid sequence of the AmiE amidase of *Helicobacter pylori* (amiE-HP) with the two available amidase sequences from (i) *Rhodococcus* sp. R312 (also designated *Brevibacterium* sp. R312, amiE-Brevi) and *Pseudomonas aeruginosa* (amiE-Pseudo).
**Figure 7** depicts a truncated open reading frame situated upstream from the *amiE* gene of *H. pylori*.

**Figure 8**: Restriction map of plasmid pILL839. The shuttle vector comprises two replication origins, CoIE1 and repA active in *E. coli* and in *H. pylori*, respectively. oriT corresponds to an origin of transfer. The catGC gene encodes chloramphenicol acetyltransferase which confers chloramphenicol resistance when expressed in *E. coli* or in *H. pylori*. The large box corresponds to the *H. pylori* DNA insert and comprises a complete *amiE* gene.

**Figure 9**: Construction of plasmid pILL420 expressing a 6His-AmiE fusion protein. The inserted *amiE* gene is shown by a shaded box. The black box corresponds to the 6 His codons of vector pQE30.

**Figure 10**: A) purification of the 6His-AmiE protein fusion. 2 mg of the protein, purified under denaturant conditions (urea 8 M) and dialysed against PBS buffer, are loaded on a 10% SDS-PAGE stained by Coomassie blue. B) crude extracts of N6 and N6-836 were loaded on a 10% SDS-PAGE, transferred onto nitrocellulose membrane and probed with antibodies raised against the 6His-amiE protein fusion (dilution 1:6000).

**Figure 11**: Competitive inhibition of amidase activity by urea. Assays were performed as described in patent DI 97-17, in the absence () or in the presence () of urea (0.5 mM).

**Figure 12**: Nucleotide of the HP1238 open reading frame (Tomb et al., 1997) designated here *amiF*. The stars show the TAA stop codon of *amiF*. The sequence, the hybridization position and the 5'-3' orientation of two primers (H69-H76) used to PCR-amplify the complete *amiF* gene are indicated. The H76 sequence corresponds to the complementary DNA strand. The position and sequence of the 3 oligonucleotides (oligo 1, 2 and 3) deduced from the *amiE* sequence which are supposed to hybridize in Southern blot experimentation to the corresponding *amiF* sequences is also shown.

**DESCRIPTION OF THE INVENTION**

We have identified an aliphatic amidase *amiE* gene of *Helicobacter* species. The amino acid sequence of the *Helicobacter* aliphatic amidase enzyme is closely analogous
(75% identical residues) to the aliphatic amidases from *Pseudomonas aeruginosa* and *Rhodococcus* sp. R312. The *H. pylori amiE* DNA sequence shares 65% identity with *P. aeruginosa* and *Rhodococcus* sp. R312 amidase genes. (There is 81% identity between the *P. aeruginosae* and *Rhodococcus* sp. R312 amidase amino acid sequences and 79% identity between their respective nucleotide sequences.)

The invention includes purified polynucleotides encoding the aliphatic amidase of *Helicobacter* sp. and biologically equivalent variants of *Helicobacter* sp. aliphatic amidase AmiE, expression vectors containing these polynucleotides, and products genetically or immunologically related to *Helicobacter* sp. aliphatic amidase AmiE. The term "products genetically or immunologically related to *Helicobacter* sp. aliphatic amidase amiE" refers to the various products derived from original *Helicobacter* sp. aliphatic amidase amiE DNA whether they be corresponding RNAs, recombinant DNAs containing all or part of the original DNA, DNAs that, as a result of the degeneracy of the genetic code, encode the same polypeptide or fragments thereof as *Helicobacter* sp. aliphatic amidase amiE DNA, or DNAs capable of hybridizing with all or part of the *Helicobacter* sp. aliphatic amidase amiE DNA or other DNAs of this invention under stringent conditions (as defined by Southern, 1975), as well as the "immunological" products resulting from the expression of these DNAs, in competent cell hosts. Thus, the invention includes polypeptides resulting from the transcription and translation of all or part of the different open reading frames of original, recombinant or degenerated *Helicobacter* sp. aliphatic amidase amiE DNA or DNA capable of hybridizing with all or part of any of those DNAs under stringent conditions and antibodies against those polypeptides.

Antibodies according to this invention may be monoclonal or polyclonal and are specific for an isolated or purified *Helicobacter* sp. aliphatic amidase AmiE antigen or antigenic preparation comprising an isolated or purified *Helicobacter* sp. aliphatic amidase AmiE antigen. Such antibodies may be produced by methods well known in the art. The antibodies of this invention may be administered in an immunologically effective amount directly to a patient to confer passive immunity against *Helicobacter* sp. infection or to treat an existing infection. The term "immunologically effective amount" refers to the amount
required to produce, either in a single dose or a series of doses, effective treatment or prevention of *Helicobacter* sp. infection in man or animals.

The purified or isolated DNAs, polypeptides and antibodies of this invention may be used in diagnostic kits and procedures to detect the presence of *Helicobacter* or *Helicobacter* antibodies in a sample from an infected patient or animal. Such diagnostic kits and procedures fall within the ambit of this invention. (The term "purified or isolated" means that the DNAs, polypeptides or antibodies are substantially free (more than 75%) from other products with which those DNAs, polypeptides or antibodies are normally found associated in nature.)

For example, one embodiment of this invention uses a pair of primers (Fig. 1c) which specifically amplify (by PCR) internal sequences of the *H. pylori amiE* gene (Fig. 5) and thus, allow the detection of the bacteria directly on a biological specimen, such as gastric juice, biopsies, stools, or saliva. Another diagnostic procedure according to this invention is the use of aliphatic amidase as a marker to identify *Helicobacter* sp. in a sample. Only a limited number of markers are available, including urease, oxydase, catalase, alkaline phosphatase, and gammaglutamyl transpeptidase activities. Increasing the number of markers will improve the specificity of *Helicobacter* sp. detection. Furthermore, because *Helicobacter* sp. is the only known gastrointestinal bacteria to produce aliphatic amidase, a quick and specific amidase biochemical assay can be developed to detect and identify *Helicobacter* sp. infection. One suitable assay uses acrylamide as a substrate and a colorimetric indicator, such as phenol red, which changes color as a consequence of pH modification. If aliphatic amidase is present in a test sample, it hydrolyzes the acrylamide to ammonia causing a change in pH, and consequently, color.

Additional diagnostic embodiments of this invention include the use of isolated or purified amiE antigenic polypeptide, alone or in combination with other known *Helicobacter* antigen preparations, for serological diagnosis of *Helicobacter* infection, e.g., *H. pylori* infection in humans or *H. heilmanii* in pigs. Such diagnostic immunoassays are well known in the art, and include radioimmunoassays (RIA) and enzyme-linked immunosorbent assays (ELISA).
The invention further relates to the use of the DNA and encoded polypeptides of this invention for immunization and therapeutic purposes. Protocols for the use of isolated polynucleotides to provide an immune response upon in vivo translation of the polynucleotide are described, for example, in WO 90/11092 (Felgner et al.), incorporated herein by reference.

In one embodiment of this invention, isolated native or recombinant AmiE polypeptides or antigenic fragments may be administered to patients suffering from Helicobacter infection or to protect patients from contracting the infection. Prophylactic as well as therapeutic effects of the polypeptides of this invention may be assessed in the H. felis/mouse model or the H. pylori/mouse model using protocols previously described for UreB, and HspA (Ferrero et al., 1995). The immunogenic/therapeutic composition may comprise amidase polypeptides or antigenic fragments alone or in association with a mucosal adjuvant, such as cholera toxin, and/or previously described protective antigens, e.g., UreB and HspA.

As already noted, among the bacteria that colonize the gastrointestinal tract, Helicobacter sp. are the only bacteria known to express an aliphatic amidase. Therefore, using this amidase as a target to eliminate Helicobacter sp. (e.g., by administering a drug or other substance capable of inhibiting amidase activity) will result in a highly specific antibacterial effect. This Helicobacter sp. specific effect is achieved in one aspect of this invention by providing culture conditions where the aliphatic amidase activity becomes an essential function for Helicobacter sp. growth (or the growth of any other organism expressing the Helicobacter amiE gene) -- making it possible to select substances in vitro, which inhibit the amidase activity and thus, are toxic specifically to Helicobacter sp. An example of such culture conditions includes nitrogen starvation with amides as the only nitrogen source.

Another means to achieve a Helicobacter specific effect is to select a non-toxic substance that produces, when hydrolyzed by the aliphatic amidase, a product toxic to Helicobacter growth. For example, glycollamide has been shown to be highly toxic for in vitro bacterial growth when degraded in glycollate by an aliphatic amidase (Brown and Tata, 1987).
Analysis of the amidase catalytic properties (eventual involvement of a metal ion) and determination of the active site is of course of great importance for the design of *Helicobacter* sp. inhibitors. By analogy with nitrilases (Bork et al., 1994, Novo et al., 1995), a region from residue 155 to 200 (Fig. 5) containing a cysteine residue cys$^{166}$ (corresponding to cys$^{165}$ as described by Skouloubris et al., 1997) is proposed to correspond to the *H. pylori* active site. Point mutations in the *P. aeruginosa* amidase have been shown to be sufficient to change its substrate specificity significantly (Clarke, 1984). These same techniques, applied to *Helicobacter* amidase, can be used to unequivocally identify the active site of that enzyme.

Another embodiment of this invention involves the use of *Helicobacter* aliphatic amidase polypeptides and/or recombinant microorganisms capable of overexpressing these polypeptides to eliminate toxic amides from a contaminated environment. In particular, these polypeptides or recombinant microorganisms may be used to degrade and detoxify acrylamide, which appears to be the best substrate of the *Helicobacter* aliphatic amidase. Methods of constructing microorganisms capable of overproducing selected polypeptides are well known in the art. One suitable method is described in Example 14 infra.

The practice of this invention employs conventional techniques of molecular biology, microbiology, and immunology, which are within the skill of the art. These techniques are fully described in the literature. These conventional techniques can be used to prepare the polypeptides of this invention. Thus, this invention includes a process of preparing purified or isolated polypeptides according to this invention by culturing under suitable conditions a procaryotic or eucaryotic host cell transformed or transfected with a polynucleotide of this invention in a manner allowing the host cell to express the desired polypeptide and isolating the polypeptide expression product.

Other information, which may be useful in the practice of this invention, is fully described in WO 94/26901 (Labigne, et al.), incorporated herein by reference.
EXAMPLES

HELICOBACTER PYLORI ALIPHATIC AMIDASE: AmiE POLYPEPTIDES AND DNA SEQUENCE ENCODING AmiE.

1. Bacterial strains and growth conditions

E. coli MC1061 (Casadaban and Cohen, 1980) cells were grown routinely at 37°C on solid or liquid luria medium (Miller, 1992). H. pylori strains (N6, 85P and SS1) were grown on a horse blood agar medium containing an antibiotic mixture and incubated under microaerobic conditions at 37°C (Ferrero et al., 1992). The H. pylori N6-836 and SS1-836 mutant strain was grown on the same supplemented medium with kanamycin (20 µg/ml). Antibiotic concentrations for the selection of recombinant E. coli were as follows: spectinomycin (40 µg/ml), ampicillin (100 µg/ml), kanamycin (50 µg/ml) and tetracyclin (20 µg/ml).

2. Microsequencing

Determination of the N-terminal amino acid sequence of two of the peptides generated by enzymatic proteolysis of a protein with an apparent molecular mass of 49 kDa was performed in the "Laboratoire de Microséquençage des Protéines" at the Institute Pasteur on an Applied Biosystems 473A Sequencer.

3. General molecular biology techniques and electroporation

Standard procedures for endonuclease digestions, ligation, agarose gel electrophoresis and elution of DNA fragments from agarose gels were used (Sambrook et al., 1989). E. coli strains were made competent and transformed with the standard CaCl$_2$ method (Sambrook et al., 1989). Small-scale plasmid preparations were prepared by the alkaline lysis procedure. Preparation of the cosmid or large-scale plasmid preparation was performed with the MAXI and MIDI qiagen columns (QIAGEN), respectively. Approx. 10 µg of plasmid pILL836 of a MAXI qiagen preparation were concentrated by ethanol precipitation without added salts and the pellet was dissolved into 2µl of bidistilled water. This DNA preparation was used directly for electroporation. Strain N6 was used as a recipient strain for the electroporation experiments performed as described in Ferrero et al. (1992).
4. **PCR conditions and direct sequencing of PCR products**

The templates were approximately 10 ng, either from a chromosomic DNA preparation of strain N6 or 85P, or from different plasmid preparations. In order to prepare bacterial lysates, a suspension (\(A_{600}=0.6\)) of *H. pylori* cells was prepared in 200 \(\mu\)l of sterile distilled water. Samples were boiled in a water bath for 5 min, cooled on ice, and centrifuged at 15,000 rpm for 5 min. Suspensions containing liberated DNA were stored at -20°C and 10 \(\mu\)l were used per PCR reaction.

PCR reactions were carried out in 50\(\mu\)l of an amplification reaction mixture containing 350 pmol of each primer (degenerated oligonucleotides), 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl\(_2\), 0.2 mM of each deoxynucleotide, 10 \(\mu\)l of a DNA preparation and 2.5 Units of Taq DNA polymerase. PCR consisted of 25 cycles of the following program : 94°C for 2 min, 50°C for 2 min, and 72°C for 2 min.

Direct sequencing of the PCR products was achieved following treatment of the PCR products (7\(\mu\)l) with 10 units of Exonuclease I and 1 unit of Shrimp Alkaline Phosphatase according to the manufacturer's instructions (Sequenase PCR Product Sequencing kit, Amersham). 100 pmoles of each oligonucleotide H36 and H37 were used for sequencing. Samples were loaded on a 6% acrylamide gel and run in Taurine Buffer (0.1 M Tris-base, 0.03 M Taurine, 0.5 mM EDTA).

5. **Hybridization**

Colony blots for screening of the *H. pylori* cosmid bank were prepared on nitrocellulose membranes according to the procedure of Sambrook et al. (1989) as were the Southern blots. Radioactive labeling of PCR products was performed by random priming with \(^{32}\)P(dCTP) using the Megaprime DNA system (Amersham). Colony hybridizations were performed under high stringency conditions (5 x SSC, 0.1% SDS, 50% formamide, 42°C). Southern hybridizations were performed under high stringency conditions (5 x SSC, 2 x Denhardt's Solution, 0.02% ATP, 0.1%SDS) at 65°C. After hybridization, filters were washed 2 times for 5 minutes at room temperature with a solution of 1 x SSC, 0.1% SDS (w/v), and 2 times at 65°C with a solution of 0.1 x SSC, 0.1% SDS.
6. **Measurement of the amidase activity**

An amidase activity assay was adapted from the urease assay described by Cussac et al. (1992), which made use of the Berthelot Reaction. A very similar enzymatic assay has previously been described for amidase activity determination (Silman et al., 1989). Amidase activity was measured as the release of ammonia after cleavage from its amide substrate.

Bacteria were harvested in 2 ml of PEB (100 mM phosphate buffer pH 7.4, 10 mM EDTA) and washed 2 times in the same buffer. To prepare sonicated extracts, cells were disrupted by four 30 sec bursts with a Branson Sonifier at 30 W at a 50 % cycle. Cell debris was removed by centrifugation prior to the amidase assay. Protein concentration of the sonicated extracts was determined with a commercial version of the Bradford Assay (Sigma Chemicals). Samples (5 to 50 µl) were added to 200 µl of an amide substrate solution. Acrylamide, acetamide, propionamide, formamide, isobutyramide, and nicotinamide were used as substrate at 100 mM in PEB. The reaction was carried out at room temperature for up to 30 min. The reaction mixture was then treated by addition of 400 µl of phenol-nitroprusside reagent and the color was developed by the addition of 400 µl of alkaline hypochlorite reagent after 6 min incubation at 50°C. Reaction mixture blanks, in which the amidase activity was inactivated by boiling 5 min prior to addition of substrate, were treated in the same way. The absorbance was read 625 nm. The amount of ammonia released was determined from a standard curve. One unit of the amidase activity was defined as the amount of enzyme required for the formation of one µmol of ammonia from the substrate per min per mg of total proteins.

7. **Computer work**

DNA and protein sequences were treated with the DNA Strider (1.2) program. Searches in the data banks and sequence alignments were performed with the Genetics Computer Group Sequence Analysis Software Package, version 7-UNIX.
8. Microsequencing of the N-terminus of two internal peptides of a *H. pylori* protein
A systematic analysis of the amino acid sequence of *H. pylori* proteins was performed after separation of whole *H. pylori* 85P proteins by two-dimensional denaturing gel electrophoresis. One spot corresponding to a protein with an apparent molecular mass of 49 kDa was detected, purified, and endoproteolysed. The N-terminus of two major products were microsequenced and the corresponding peptidic sequences were analyzed. The two amino acid sequences (Fig. 1) show strong similarity with the *P. aeruginosa* and *Rhodococcus* sp. R312 aliphatic amidases. This suggested the existence of an aliphatic amidase in *H. pylori*. A pair of degenerate oligonucleotides, H36 and H37 (Fig. 1), were deduced from this sequence based on the *H. pylori* codon usage.

9. PCR-amplification of an internal amidase sequence with H36 and H37 degenerated oligonucleotides
PCR-amplification was performed with oligonucleotides H36 and H37 on chromosomal DNA of two different *H. pylori* strains, N6 and 85P. A single 240 bp-PCR-product was visualized on agarose gel with both strains and the nucleotide sequence of the PCR-fragment generated from strain 85P was determined (Fig. 2). The deduced amino acid sequence encoded by this PCR fragment (Fig. 2) was very similar to an internal sequence of the *P. aeruginosa* and *Rhodococcus* sp. R312 amidases. Boiled bacterial lysates of 45 clinical isolates of *H. pylori* were used for PCR-amplification with oligonucleotides H36 and H37. A single 240 bp-product was generated by gene amplification in all the lysates tested suggesting that amidase is a common trait to all *H. pylori* isolates.

10. Screening of a *H. pylori* genomic library and identification of a cosmid (IIG5) carrying the complete amidase gene of *H. pylori*
A cosmid library of *H. pylori* strain 85P was previously constructed in our laboratory (Labigne et al., 1991). The 240 bp-DNA fragment generated by PCR using H36 and H37 was randomly labeled with α<sup>32</sup>P(dCTP) and used as a probe to screen the 480 clones of the *H. pylori* genomic library for colony hybridization. Only the colony harboring cosmid IIG5 (Labigne et al., 1991) showed clear hybridization. The purified IIG5 cosmid used as a template in PCR-amplification using the H36 and H37 oligonucleotides generated a single
240 bp-fragment.

11. **Southern blot analysis of the amidase gene on the *H. pylori* N6 and 85P genomic DNA and on cosmid IIIG5**

   The same probe corresponding to an internal segment of the amidase encoding gene was Southern hybridized on *HindIII*-restricted DNA from the *H. pylori* strains N6 and 85P, and from cosmid IIIG5. Hybridization with a single and identical 2.6 kb *HindIII* restriction fragment was obtained with cosmid IIIG5 and with 85P genomic DNA. This suggested the existence of a single gene coding for an aliphatic amidase in *H. pylori* and confirmed that the PCR-amplified DNA fragment was indeed amplified from *H. pylori*. The same probe hybridized to two *HindIII* restriction fragments of the N6 genomic DNA; this indicates that in N6, the amidase gene sequence is slightly different and that this difference is associated with the presence of an *HindIII* restriction site.

12. **Subcloning of a DNA fragment carrying the entire amidase gene**

   The 2.6 kb-*HindIII* fragment hybridizing with the PCR-generated probe was subcloned into the *HindIII* site of vector pILL570 (Labigne et al., 1991). The resulting plasmid was designated pILL400 (Fig. 3a). Several subclones generated from pILL400 were constructed using vector pUC19 (Yanisch-Perron et al., 1985). The smallest plasmid still able to produce the 240 bp-PCR-fragment with H36-H37, and that was likely to contain the entire amidase gene, was selected and designated pILL405. This plasmid carries a 1.5 kb *XhoI-BgII* fragment of *H. pylori* DNA and its restriction map is shown in Fig. 3b.

   Plasmid pILL405 was deposited with the Collection Nationale de Cultures de Microorganismes (CNCM) under number I-1863 on March 18, 1997.

13. **Determination of the nucleotide sequence of the *H. pylori* amidase gene *amiE* and comparison of AmiE with its homologs**

   A DNA preparation of plasmid pILL405 was sent to the "Institut d'Analyses Génétiques, Genome express SA" for nucleotide sequence determination of the 1520 bp *H. pylori* DNA insert (automatic sequencer, Applied Biosystems) (Fig. 4a). The 1520 bp-insert included a 1017 bp-long open reading frame (ORF) coding for a protein of 339 amino acids with a calculated molecular mass of 37,746 da (Fig. 5). The amino acid sequence deduced
from this ORF was very similar to the *P. aeruginosa* and *Rhodococcus* sp. R312 amidases, with as much as 75% of identity with each of them (Fig. 6). The DNA sequence of the *H. pylori* amiE gene has 65 % homology with the amidase genes of *P. aeruginosa* and *Rhodococcus* sp. R312. This confirmed that the encoded protein is indeed an aliphatic amidase. The 1017 bp-ORF was designated amiE and the corresponding protein AmiE.

A truncated ORF situated upstream from the amiE gene, corresponding to the 3'-extremity of another *Helicobacter* gene, has also been detected (Fig. 7). No homologs of the deduced protein could be found in the data banks.

14. **Overproduction of the *H. pylori* amidase**

In order to overexpress the *H. pylori* amidase, the amiE gene was put under control of the *Plac* promoter of the high copy number vector pUC19 by cloning the *EcoRI*-*BstEII* fragment from pILL405 into the *EcoRI*-*SmaI* sites of pUC19. The resulting plasmid was designated pILL417 (Fig. 3c). The proteins expressed by the *E. coli* strain MC1061 harboring either pILL417 or pUC19 grown overnight in liquid Luria medium, were examined and compared by SDS-PAGE. The amiE gene product was visualized as a large band corresponding to a protein with an apparent molecular mass of 40 kDa. The *H. pylori* amidase can thus be stably overproduced in *E. coli* without affecting cell viability.

Plasmid pILL417 was deposited with the Collection Nationale de Cultures de Microorganismes (CNLM) under number I-1864 on March 27, 1997.

15. **Amidase activity and substrate specificity in *H. pylori* and in recombinant *E. coli* strains**

Amidase activity was measured as the release of ammonia after cleavage from its amide substrate. Substrate specificity of *H. pylori* amidase was tested with crude extracts of *H. pylori* strain N6. Amidase activity was approximately 6 units (U) for acrylamide, 5 U for propionamide, 3 U for acetamide, and was very low for formamide (0.1U) and isobutyramide (0.02U). No activity was detected on nicotinamide. Amidase activity was also measured on crude extracts of *E. coli* recombinant strains. Strain MC1061 carrying plasmid pILL405 has an amidase activity on acrylamide of 100 U; the *H. pylori* amidase is thus fully active in *E*
coli. The high activity level of MC1060(pILL405) compared to that of *H. pylori* resulted from the expression of *amiE* under the control of the *Plac* promoter and probably also from the high copy number of this plasmid. Urea, although structurally related to amides, is not hydrolyzed by the *H. pylori* amidase expressed in strain MC1061(pILL405). In addition, a *H. pylori* urease negative mutant (N6-ureB) is not affected in its amidase activity. These results suggest that there is no obvious interference in ammonia release due to the activity of the two enzymes (amidase and urease).

16. **Construction of a N6-836 *H. pylori* mutant carrying a disrupted *amiE* gene**

A *H. pylori* mutant carrying a disrupted *amiE* gene was constructed by allelic exchange. In order to obtain a plasmid with a unique *Xmal* restriction site situated within the *amiE* open reading frame (at 147 bp from its initiation codon), we constructed plasmid pILL835 (Fig. 3d). This plasmid carries the *Pstl*-*Xhol* restriction fragment of plasmid pILL405 (containing the 1.5 kb *H. pylori* DNA insert) cloned into the *Pstl*-*Sspl* restriction sites of pBR322. Plasmid pILL836 (Fig. 3e) resulted from the introduction of a 1.5 kb-fragment carrying a kanamycin resistance [*aph(3')-III*] gene under control of its own promoter (Trieu-Cuot et al., 1985), into the *Xmal* site of plasmid pILL835. A concentrated DNA preparation of pILL836 was used to transform *H. pylori* strain N6 by electroporation. *H. pylori* transformants resistant to 20 μg/ml of kanamycin were selected on plates. All of the eight transformants examined carried an *amiE* gene disrupted by the kanamycin gene attesting to allelic exchange between the mutated *amiE* allele of pILL836 and the chromosomal *amiE* copy. The correct insertion of the cassette in the *amiE* gene on the chromosome was controlled by PCR with primers corresponding to sequences flanking the *Xmal* restriction site within the *amiE* gene and divergent primers corresponding to sequences within the kanamycin resistance gene. One of these strains was further studied and designated N6-836. No amidase activity on acrylamide was detected in the amidase negative mutant N6-836.

The growth rate of this amidase negative mutant N6-836 on blood agar medium (a rich medium) was not significantly affected when compared to that of the parental strain N6. The amidase function is thus not essential for *H. pylori* growth *in vitro*.

To determine whether the *amiE* gene is essential for *H. pylori* survival and
colonization in its natural environment, the gastric mucosa, the same mutation is introduced into the amiE gene of the *H. pylori* SS1 strain (Sydney Strain; Buck et al., 1996) used in a *H. pylori*/mouse model. Colonization and local inflammation are compared after infection of mice with the *H. pylori* parental strain and its amidase mutant.

17. Information obtained from the analysis of the total genome sequence of *Helicobacter pylori*

In August 1997, Tomb et al. (TIGR) published the complete genome sequence of *Helicobacter pylori* strain 26695. Analysis of these data indicated the presence of an amiE gene (ORF number: HP294). This sequence presents 97.8% identical nucleotides with our amiE sequence from strain 85P, the two corresponding proteins sharing 99% identical amino acids. The amiE gene seems thus to be highly conserved in *H. pylori*. Unlike the amiE gene of *Pseudomonas aeruginosa*, the *H. pylori* amiE gene is not part of an amidase operon.

More interesting, there is an amiE-parologue (paralogues correspond to proteins having similar functions, present in a single genome), HP1238, which is not genetically linked to amiE. We decided to name this 1002bp-long gene, amiF. There are 48.7% identical nucleotides between amiE and amiF and the two corresponding proteins share 34% identical amino acids. Three oligonucleotides (oligo 1, 2 and 3, see Fig. 12) corresponding to amiE sequences and showing strong similarity with the corresponding amiF regions have been defined by the inventors. These oligonucleotides are supposed to be able to hybridize to the amiF gene in Southern blot experiments in stringent conditions.

The sequence of AmiF (37,266 Da) suggests that it is an aliphatic amidase due to conservation of the predicted active site region. However, it is not known at present whether amiF is expressed during *H. pylori* in vitro growth. If amiF was expressed in these conditions, our results obtained with an *H. pylori* amiE deficient mutant might indicate that the substrate specificity of AmiF is different from that of AmiE.

Analysis of the AmiF sequence allowed us to define oligonucleotides H69 and H76 (see Table 1) which permitted PCR-amplification (using a high fidelity PCR kit, Boehringer) of the complete amiF gene (1002 bp) from a DNA preparation of strain 26695.

This 1044 bp-long PCR fragment was cloned into vector pUC19 between the BamHI
and EcoRI restriction sites in order to express the amiF gene under control of the lac promoter. The resulting plasmid is named pILL439. Amidase activity assays, on different amide substrates (acrylamide, propionamide, acetamide, formamide, isobutyramide) can be performed on crude extracts of an E. coli strain harbouring pILL439.

A H. pylori mutant (N6-437) carrying a kanamycin resistance cassette inserted in the amiF open reading frame could be constructed, indicating that the amiF gene is not essential for H. pylori growth in vitro.

18. Connection between urease and amidase activity

Amidase activity, as measured in vitro, is enhanced 2 to 3 times in a H. pylori strain deficient in urease activity (N6-ureB). Western blot experiments using anti-AmiE serum (see paragraph 23) showed that higher amidase activity in strain N6-ureB is perfectly correlated with an increase in the amount of AmiE protein produced. This indicates that the expression of amiE is regulated, we propose that this regulation could be dependent on intracellular ammonium or nitrogen concentration.

Preliminary kinetic experiments were performed on crude extracts of an E. coli strain overexpressing AmiE from plasmid pILL417. As shown on Fig. 11, competitive inhibition with urea (at 0.5 mM) was shown.

19. The amiE gene is not necessary for H. pylori colonization in the mouse animal model

The model of colonization of the mouse stomach by H. pylori strain SS1 (Sydney Strain, Lee et al., 1997) has been applied and validated (Ferrero et al., 1998). This model was used to test the role of AmiE in vivo. Plasmid pILL836 which permitted the construction of an amidase deficient mutant, N6-836, has been used to construct (following the same experimental procedures) the SS1-836 strain carrying a mutated amiE gene. The absence of amidase activity in SS1-836 was verified by an assay with acrylamide as substrate.

Mice were infected with the amiE mutant, SS1-836, and the parental strain, SS1, used as a positive control. Aliquots (100 μl) containing 10^6 bacteria from each H. pylori strain were administered orogastrically to 10 mice each (six to eight-weeks old Swiss specific-pathogen-free mice) as described by Ferrero et al., 1998. Mice were killed four weeks after inoculation. We tested for the presence of H. pylori with a direct urease test on biopsies performed on half
the stomach. The remaining gastric tissues were used for quantitative culture of *H. pylori* (Ferrero et al., 1998). The stomachs of the ten SS1-infected mice and the ten SS1-836 infected mice all tested positive for urease. The bacterial load was between \(5 \times 10^4\) and \(5 \times 10^5\) colony forming units (CFU) per g of stomach for strain SS1 and between \(1 \times 10^4\) and \(1 \times 10^5\) CFU per g of stomach for strain SS1-836.

20. Representation of the AmiE amidase among different *Helicobacter* species

We have shown that the *amiE* gene was present in all the *H. pylori* strains tested (45 independent strains analysed). We then asked whether other *Helicobacter* strains colonizing animals also possessed an aliphatic amidase similar to AmiE. The presence of amidase was tested in *Helicobacter felis, Helicobacter mustelae, Helicobacter muridarum, Helicobacter hepaticus, Helicobacter bilis* and *Helicobacter canis*. A couple of degenerate oligonucleotides, H36 and H37 corresponding to well-conserved regions of the amidase were used to test by PCR-amplification for the presence of an *amiE*-homolog. In addition, an amidase activity assay was performed on crude extracts of these strains with acrylamide as a substrate. As an internal control of the quality of the crude extracts, urease activity was assayed confirming strong urease activity for all these strains except for *H. canis* where the absence of urease had already been described.

Only *Helicobacter muridarum*, colonizing the intestinal tractus of rats and mice without known associated pathology, was found to possess an AmiE-homolog. AmiE is thus not conserved among the *Helicobacter* species. Our finding was surprising since *Helicobacter muridarum* has not been classified as phylogenetically close to *H. pylori*. Whether the other *Helicobacter* gastric species contain amidases encoded by genes which had strongly diverged from *amiE* or with different substrate specificity is not known.

21. Complementation in trans of the *H. pylori* ammE mutant by plasmid pILL839

In order to test whether an intact amidase gene could complement *in trans* the *H. pylori* amidase mutant N6-836, the *amiE* gene was introduced into *E. coli/H. pylori* shuttle vector (containing a BglII site) to produce plasmid pILL839 (Figure 8). The shuttle vector contains the *cat* gene conferring the chloramphenical resistance to *E. coli* and *H. pylori* strains. To obtain pILL839, a BglII restriction fragment (2.25 Kb) of plasmid pILL400, carrying the
entire *amiE* gene, was introduced into the unique *Bgl*II restriction site of the shuttle vector. Strong amidase activity was measured in *E. coli* strains transformed by pILL839. Plasmid pILL839 was introduced into *H. pylori* strains N6 (parental strain) and N6-836 (an amidase defective mutant) by electroporation following the procedure described above. Transformants, selected on plates in the presence of chloramphenicol (4μg/ml), were assayed for their amidase activity *in vitro*. We observed that strain N6-836 harbouring plasmid pILL839 produced strong amidase activity (10 times that of the parental N6 strain). The *amiE* gene of plasmid pILL839 is thus able to complement *in trans* the amidase deficient *H. pylori* mutant N6-836.

22. Overproduction and purification of a 6 His-AmiE fusion

A plasmid carrying a fusion between 6 His codons and the 5'-end of *amiE* has been constructed. Therefore, plasmid pILL419 was first obtained by introduction of a 900 pb-long *SmaI*-*Hind*III restriction fragment from plasmid pILL405 into vector pQE30 (Qiagen). A PCR fragment was then amplified from DNA of pILL405 with primers H59 and H40 (Table 1). This PCR product was digested with the *Bam*HI and *SmaI* restriction enzymes and the fragment produced (147 bp) was introduced into pILL419. In the resulting plasmid, pILL420 (Fig. 9), a complete *amiE* was reconstituted and this ORF was in frame with the 6 His codons of vector pQE30. The 6 His-AmiE fusion protein was then purified by affinity with Cobalt columns in denaturing conditions (following the instructions of the supplier, Clontech) and subsequently dialysed against PBS buffer. About 6.5 mg of 6 His-AmiE fusion protein were thus purified and the degree of purity of this preparation was controled by SDS-PAGE stained with Coomassie Blue (Fig. 10A).

23. Production of anti-AmiE polyclonal antibodies

Following a classical procedure, a preparation of purified 6 His-AmiE fusion protein (100μg) was injected to a New Zealand rabbit in order to raise polyclonal antibodies directed against AmiE. Western blots were performed (as described in Suerbaum et al., 1994) using the anti-serum of this rabbit diluted 1:6000. As shown on Figure 10B, this serum recognizes AmiE very specifically on whole cell extracts of *H. pylori* strain N6. As a negative control, no protein was recognized on whole cell extracts of *H. pylori* strain N6-836 deficient in amidase.
This confirmed the high specificity of the anti-AmiE serum and suggested that there is no cross-reaction with AmiF, given that this latter protein is expressed in the conditions tested. A first experiment of AmiE localization in the \textit{H. pylori} cells was performed. AmiE was only found in the soluble protein fraction while it was absent from the culture supernatant and from the membrane fractions. These results strongly suggested that like the other amidases (and in the absence of a peptide signal sequence at the AmiE N-terminus), the \textit{H. pylori} AmiE amidase is cytoplasmic.

24. Immune response against amidase in \textit{H. pylori} infected patients

We asked whether patients infected by \textit{H. pylori} developed immune response against the AmiE aliphatic amidase. This was tested by Western blot experiments with about 1\mu g of purified 6 His-AmiE protein loaded per well on SDS-PAGE. After transfer on nitrocellulose membranes, these membranes were incubated overnight with serum (diluted 1:100) of each patient infected by \textit{H. pylori} (infection was attested by serology). In a first series of experiments, 4 sera out of 20 recognized AmiE. In a second series of experiments, on 26 sera tested, 8 recognized the amidase very strongly while for 13 others the recognition was weaker. These results indicated that between 26\% and 54\% of the \textit{H. pylori} infected patients had antibodies directed against linear epitopes of the AmiE amidase. This clearly demonstrated the immunogenic potentiality of the \textit{H. pylori} amidase.
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Table 1: Name and nucleotide sequence of oligonucleotides used in this study.
REFERENCES


WE CLAIM:

1. A purified polynucleotide corresponding to a Helicobacter aliphatic amidase amiE.

2. A purified polynucleotide selected from the group consisting of:
   (a) all or part of the DNA sequence depicted in Fig. 5 encoding Helicobacter pylori aliphatic amidase AmiE;
   (b) all or part of the DNA sequence depicted in Fig. 4;
   (c) a DNA sequence that hybridizes with all or part of DNA sequence (a) or (b) under stringent conditions and encodes a polypeptide having the biological or immunological properties of Helicobacter pylori aliphatic amidase or a fragment thereof; and
   (d) an analog of DNA sequence (a), (b), or (c) resulting from the degeneracy of the genetic code.

3. A purified polynucleotide selected from the group consisting of:
   (a) ATTTTCCCTGAATACAGCACGCA
       TGGTATCCTGGGAT
       CCTGTGGCGATTTG
   (b) a DNA sequence that hybridizes with DNA sequence (a) under stringent conditions and encodes a polypeptide having the biological or immunological properties of Helicobacter pylori aliphatic amidase or a fragment thereof; and
   (c) an analog of DNA sequence (a) or (b) resulting from the degeneracy of the genetic code.

4. A polypeptide having an amidase activity, which is encoded by a polynucleotide of Helicobacter.

5. A polypeptide according to claim 2 which is encoded by a DNA sequence selected from the group consisting of:
   (a) all or part of the DNA sequence encoding AmiE in Helicobacter pylori depicted in Fig. 5;
   (b) all or part of the DNA sequence depicted in Fig. 4;
(c) a DNA sequence that hybridizes with all or part of DNA sequence (a) or (b) under stringent conditions and encodes a polypeptide having the biological or immunological properties of *Helicobacter pylori* aliphatic amidase or a fragment thereof.

6. A pharmaceutical composition comprising a polypeptide according to anyone of claims 4 or 5 or a fragment thereof, in combination with a pharmaceutically acceptable excipient.

7. A pharmaceutical composition according to claim 6 further comprising *Helicobacter* UreB protein or a fragment thereof.

8. A monoclonal antibody specific for a polypeptide according to anyone of claims 4 to 6 or a fragment thereof.

9. A kit for the detection of an *Helicobacter* infection comprising a polypeptide according to anyone of claims 4 or 5, or a fragment thereof, capable of reacting with antibodies present in a serum of a patient or an animal infected by *Helicobacter*.

10. A process of degrading an amide selected from the group consisting of acrylamide, acetamide, propionamide and isobutyramide, said process comprising the step of contacting the amide with a polypeptide, according to claim 4 or claim 5.

11. An immunogenic composition capable of inducing antibodies against *Helicobacter* infection comprising a polypeptide according to anyone of claims 4 or 5, or a fragment thereof, recognized by antibodies reacting with *Helicobacter* amidase.

12. An immunogenic composition comprising

   a) a polypeptide according to anyone of claims 4 or 5, or a fragment thereof, recognized by antibodies reacting with *Helicobacter* amidase;

   b) a urease Ure B polypeptide, or fragment thereof, recognized by antibodies reacting with *Helicobacter* urease; and optionally including

   c) a HspA polypeptide or fragment thereof recognized by antibodies reacting with *Helicobacter* heat shock protein.

13. The immunogenic composition according to claim 11 capable of inducing protective antibodies.

14. The immunogenic composition according to claim 12 capable of inducing
protective antibodies.

15. An expression vector comprising a polynucleotide according to anyone of claims 1 to 3.

16. An expression vector according to claim 15 which is a plasmid selected from the group consisting of pILL400, pILL835 or pILL836.

17. An expression vector according to claim 15 which is plasmid pILL405 (CNCM I-1863).

18. An expression vector according to claim 15 which is plasmid pILL417 (CNCM I-1864).

19. An expression vector according to claim 15 which is plasmid pILL420 (CNCM I-1998).

20. A kit for the detection of an Helicobacter infection comprising at least one polynucleotide according to anyone of claims 1 to 3 or a vector according to anyone of claims 15 to 19.

21. A procaryotic or eucaryotic host cell stably transformed by an expression vector according to anyone of claims 15 to 19.

22. A process for the preparation of a polypeptide according to anyone of claims 4 or 5, or a fragment thereof, comprising the steps of:

a) culturing under suitable conditions a procaryotic or eucaryotic host cell transformed or transfected with a polynucleotide according to anyone of claims 1 to 3 in a manner allowing the host cell to express said polypeptide; and

b) optionally isolating the desired polypeptide expression product.

23. A therapeutic composition comprising an antibacterial substrate capable of inhibiting an amidase activity of Helicobacter sp. amidase.

24. A therapeutic composition comprising an active molecule capable of reacting with Helicobacter sp. amidase to produce a product toxic to Helicobacter sp.

25. A process for screening for an active substrate capable of inhibiting the activity of Helicobacter sp. amidase which comprises contacting a test substrate with a polypeptide according to claim 4 or claim 5.
26. A method of treating or preventing *Helicobacter* sp. infection in a man or an animal comprising the step of administering an effective amount of a pharmaceutical composition according to claim 6 or 7.

27. A method of treating or preventing *Helicobacter* sp. infection in a human or an animal comprising the step of administering an effective amount of an immunogenic composition according to anyone of claims 11 to 14.

28. A method of treating or preventing *Helicobacter* sp. infection in a human or an animal according to claim 27, wherein said composition is administered by any route such as oral route, intradermal route, intramuscular route, intravenous route or mucosal route.
a) first internal peptide VWGVFLTGEK

oligonucleotide H36 :
5' GTITGGGgiGTIT(T/C)(A/T)(C/G)(iC/T)TiACiGG 3'

b) second internal peptide VSLIIICDDGNYPEI

oligonucleotide H37 :
5' CCAiAT(C/T)TCiGG(A/G)TA(A/G)ATiCC(A/G)TC(A/G)TC(A/G)C 3'

c) non-degenerate oligonucleotides

oligonucleotide H46 :
5'-CCTTATAACACTTTGATTCTTGTC-3'

oligonucleotide H49 :
5'-CAAGCCCTTAGGCCCATCAACC-3'

Figure 1
Figure 2
Plasmid name: pILL400
Plasmid size: 8 000 bp

Comments: Insertion of the 2 600 bp HindIII fragment of cosmid IIIG5 (carrying the complete amiE gene) into the HindIII site of vector pILL570.

Figure 3(a)
Plasmid name: pILL405
Plasmid size: 4200 bp

Comments: Insertion of the 1520 bp Xhol/BgIII fragment from pILL400 (carrying the complete amIE gene) into the SalI/BamHI sites of vector pUC19.

Figure 3(b)
Plasmid name: pILL417
Plasmid size: 3 900 bp

Comments: Insertion of the 1 230 bp BstEII/EcoRI fragment from plasmid pILL405 into the SmaI/EcoRI sites of vector pUC19.

Figure 3(c)
Plasmid name: pILL835
Plasmid size: 5,400 bp

Comments: Replacement of the PstI/SpeI fragment of vector pBR322 by the 1,570 bp XhoI/PstI fragment of pILL400.

Figure 3(d)
Plasmid name: pILL836
Plasmid size: 6 900 bp

Comments: Insertion of a 1 500 kb Xmal fragment containing the gene conferring kanamycin resistance (from pILL200, unpublished) into the Xmal site, situated at 147 bp from the amiE start codon in pILL835.

Figure 3(e)
Figure 4(a)
Figure 4(b)
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Figure 4(b) (cont'd)
Figure 4(b) (cont'd)
Figure 4(b) (cont'd)
Figure 5
Figure 7
FIGURE 11
ATGGGAAGTATCGGTAGTATGGGCAAACCTATTGAGGGTTTTTA
5'-CGGGATCCATGGGGAAGTATCGGTAGTATGGG-3' primer H69

GTGGCAGCCATTCAGTTTCCGTGCAATAGCGCTAAAGGTATTTGATCATCAC
AATATTGAAAGCATTATTTAGAACCCCTGACATCGGACTAAAGCAGGGGTATCCGGAGTG
GAGCTTATCATTTTCCCCTGAGTATAGCAGCGCAAGGTTTGAATACCCGCTAAGTGGCTT
ATTATCCCTGAAATACAGCACGCAG oligo 1

AGCGAAGAGTTTTATATAGATGTCCCGGTTAAAGGAAGACAGAGCTATACGCTAAGGC
GTGTAAGAGGCGAAGTTATATGGTGGTTTTTTCAATCATGGAAAGCAATCTGTATT
CTAACAACAAAACCTTCAACACCCGGCCATTATCATGTAGTCCGGCAAGTTACATT
TTAATAACGCAAGCTATTTTCCTATGGAAATCCCATTTGAGCAGCAGTGTCTCGGGGAT
oligo 2 TGGTATCTCGGGGAT

TTAAGGAATGCTCTGTGTCCGAGGACTCCCGGCGGATCAAAAATTAGCCGCTGTGCATT
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CAACCGCTCAAACGCTGGAACAAATTGGATGTATACCCGGCCTAGTGGAAATTAGCCG
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ACCTAGGCCCATAGAGGGTATGTGGCTAAACCGGCGGAGAACATGCAGCGCCTTAA
ACCTATATCAAAGACTTACGCGCGGCTAAATACAAATGGCCTTTGGGAGATCATCAGT
GAAAAATCAGAGCAGCTCTATTATTAGGCTACCCTACCACCAGGGGTGGCGTTTTGAG
oligo 3 5'-CCTGTGGCGATTG-3'

***
AAATAATCCCTAACCTTGGCATTTTGTGCTGAAACCCGTTTTTAAAGCT
primer H76 3'-CGATCTTGGGCAAATTTCCCCCTAAGG-5'

FIGURE 12