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- (71) Applicant: **GDANSKI UNIWERSYTET MEDYCZNY** [PL/PL]; ul. M. Skłodowskiej-Curie 3a, 80-210 Gdańsk (PL).
- (72) Inventors: **HINC, Krzysztof**; ul. Ks. Bernarda Sychty 16/1, 80-276 Gdańsk (PL). **OBUCHOWSKI, Michał**; ul. Bereniki 81, 80-299 Gdańsk (PL). **RICCA Ezio**; ia Giro-lamo Santacroce 6, 80129 Naples (IT).
- (74) Agent: **KANCELARIA PRAWNO-PATENTOWA**; Małgorzata Matyka, ul. Kurierów AK 4a/7, 80-041 Gdańsk (PL).

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(54) Title: ORAL VACCINE CONTAINING THE *BACILLUS SUBTILIS* SPORES AND ITS APPLICATION TO IMMUNISE AGAINST *HELICOBACTER PYLORI*

(57) Abstract: The oral vaccine containing the *Bacillus subtilis* spores which serve as the antigen carrier, UreA, UreB proteins of the *Helicobacter pylori* or *Helicobacter acinonychis* urease serving as the antigen, and coat proteins CotB, CotC, CotG, CotZ. Using the *Bacillus subtilis* spores to produce the oral vaccine serving immunisation of humans and animals against *Helicobacter pylori* in patients suffering from the duodenal and gastric ulcer disease.



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Oral vaccine containing the *Bacillus subtilis* spores and its application to immunise against *Helicobacter pylori*

Field of the invention

The invention concerns the oral vaccine containing *Bacillus subtilis* spores which serve as the antigen carrier, and UreA or UreB proteins of the *Helicobacter pylori* or *Helicobacter acinonychis* urease serving as the antigen, the latter combined with coat proteins CotB, CotC, CotG, CotZ, and application of the vaccine to prevent/ treat *Helicobacter pylori* infections, designated for mammals both exposed to the infection (protective effect) and pathogen-infected (therapeutic effect).

Background of the Invention

Helicobacter pylori (*H.pylori*) infections are common world wide, although the size of the affected population differs from country to country. The development of *H. pylori* infection in the stomach leads to gastric mucosa inflammation, mainly in the prepyloric part, and may then spread onto the stomach. At a further stage, approximately 80-90% of the infected persons develop chronic gastric mucosa inflammation. In long-lasting infections chronic inflammation may lead to atrophy of the mucosa (the so-called atrophic inflammation). Aggravated inflammation may cause mucosa defects called erosive gastritis or even lead to haemorrhages (haemorrhagic inflammation). Moreover, *H. pylori* infections are seen as the main cause of the chronic duodenal and gastric ulcer disease.

In *H. pylori*-infected persons, there is a correlation observed between the presence of the bacteria and the occurrence of early cancer of the stomach http://pl.wikipedia.org/wiki/Helicobacter_pylori - cite note-48 The World Health Organisation has found *H. pylori* a factor of proven carcinogenic effects.

Sometimes *H. pylori* infections may lead to uncontrolled proliferation of the lymphatic system tissue in the stomach - the so-called MALT lymphoma (Mucosa Associated Lymphoid Tissue). The complication of the type is a particularly acute problem in the so-called developing countries, especially China and India, where the infection affects up to 80-90% of the population.

Although *H. pylori* infections are common worldwide, the difference in the size of the infected population is substantial between highly developed and developing countries. On the country-wide level, the distribution of the infection cases within the infected population differs by age. In general, the occurrence of *H. pylori* infection is strongly correlated with socioeconomic conditions. While more than 80% of middle-aged individuals in developing countries suffer from the infection, only 20 to 50% of the same age group population are infected in the highly developed countries.

The publication by Duc and associates of 2003 describes methods of using the spores for the presentation of a tetanus toxin fragment (*Clostridium tetani*) administered intragastrically to laboratory animals (mice). A cycle of immunisation with recombinant spores resulted in the development of resistance to tetanus toxin, thanks to which 50% of the animals which had received 20 times the LD₅₀ dose survived. The obtained results unquestionably prove that the spores may be effectively used as macroparticles for antigen presentation in oral immunisation.

Patent description WO 2008/040155 (patent EP 2 082 750 B) disclosed a recombinant protein used for immunoprophylaxis of *Helicobacter pylori* infections in humans. Specifically claimed is the recombinant protein formed by combination of A2 and B subunits of heat-labile *Escherichia coli* enterotoxin with B subunits of *Helicobacter pylori* urease. The pharmaceutical preparation takes the form of slow release microspheres.

Patent description WO 94/09823 (PL 174448 B) disclosed a *Helicobacter pylori* infection vaccine which contained *H.pylori* or *H. felis* urease, or a polypeptide containing its subunit. It also contained a mucosal adjuvant (cholera toxin).

Similarly, disclosed in publication WO 95/22987 (PL 179149 B) is a vaccine against *Helicobacter pylori* infection, which contains *H.pylori* or *H. felis* urease, or a polypeptide containing its subunit. Moreover, it contains a mucosal adjuvant in the form of heat-labile *Escherichia coli* toxin or a polypeptide containing its subunits or fragments.

One of the key factors which prevent proposing an effective vaccine is the fact that the place where the bacteria colonise in the human body is the stomach. Its acid environment kills the antibodies, which makes the traditional immunisation methods evoking humoral response fail.

Creating an effective vaccine against *Helicobacter pylori* infections is one of the challenges of the contemporary medicine. *H. pylori* is responsible for ulcer disease, it is also believed to lead to stomach cancer.

The treatment method commonly used today is an antibiotic therapy. It entails, however, many disadvantageous effects. Antibiotic therapy is burdensome and carries many highly undesirable side effects. One of them is the development of bacteria strains resistant to the specific antibiotic. The fact is extremely important since it makes it necessary to use ever new antibiotics, while many specialists claim the arsenal of adequate antibiotics at disposal in medicine is exhausting. Moreover, the therapy of the type used today is time consuming, which is highly undesirable in the case of antibiotics and causes additional complications. A serious disadvantage of the antibiotic therapy consists in its short-lived effects and the resulting relapses of *H. pylori* infections.

As at today, there is no vaccine against *H. pylori* infections available, and the therapy at hand comes down to administering antibiotics.

The National Institute of Health, USA, registered 104 clinical trials on *Helicobacter*. Absolute majority of the trials concern treatment with two, three, or any larger number of antibiotics. None of the registered clinical trials seems to have been conducted to the purpose of registering a vaccine against *Helicobacter pylori*. Some of the trials and active

tests were discontinued for various reasons, e.g. 69 trials/ tests were abandoned in phases I to IV, with 8 discontinued in phase I, 17 in phase II, 18 in phase III, and 26 in phase IV.

The gist of the invention

Quite unexpectedly, it has been found that *Bacillus subtilis* spores may be used as peculiar antigen carriers. To that end, selected *H. acinonychis* UreA and UreB proteins constituting antigens (bacterial urease subunits) will be presented on the spore surface as fusion proteins with natural coat components– the external building structure of the *B. subtilis* spores. *Helicobacter acinonychis* proteins have been proposed for application considering that in genetic terms *H. pylori* differs from *H. acinonychis* by a mere 8%, which makes it a convenient model for tests on animal models. The obtained results should be identical when the method is used on the human *H. pylori*.

The essence of the invention consists in using the recombined spores of the globally found non-pathogenic *Bacillus subtilis* soil bacterium as carriers of the oral vaccine against infections caused by *Helicobacter pylori*.

To that end, selected UreA and UreB proteins (in the role of antigens) – subunits of the bacterial urease of the *Helicobacter* type – will be presented on the spore surface as fusion proteins with Cot proteins – natural components of the coat, i.e. the external structure building the spore. The immunisation trials were based on the use of both the whole UreA and UreB proteins, and those fragments thereof which were most likely to contain the antigen epitopes. The fusion proteins were prepared with the use of integration vectors. The vectors contain CotB, CotC, CotG and CotZ coding genes copied under the PCR method together with the promoter regions. Moreover, restriction sites were introduced during the PCR reaction, which facilitated both the process of cloning to the vectors, and the formation of the fusion proteins. The obtained fusion genes were subject to transformation to *B. subtilis* cells, where upon the occurrence of double crossing-over they were incorporated into *locus amyE*, *thrC* or *lacA*. The obtained fusion genes are controlled by the physiological *B. subtilis* promoter activated exclusively at late stages of the sporulation process. The formed fusion proteins were used in the formation of the coat, thanks to which they are present on the spore surface.

Using the spores as vaccine carriers constitutes a new approach to immunisation of humans and animals and may found significant application particularly in immunisation aimed at providing protection against infections of the gastrointestinal tract. In this case oral administration of the vaccine enables achieving both mucosal, and systemic immunity and extension of the half-life of the administered antigen. An unquestionable advantage of the *B. subtilis* spores used as the vaccine carrier is that they demonstrate adjuvant effects, which manifests itself in enhancing the cellular-type immune response (Th1). The fact can be of significance for vaccine effectiveness.

Moreover, using the *B. subtilis* spores as the antigen carriers facilitates the production of endospores - *B. subtilis* is a bacterium of low trophic requirements, which translates to a relatively low cost of producing the spores in industrial quantities. Moreover, spore production is a natural process and does not require human intervention in bacterial physiology.

Omnipresence of spores in the natural environment, human food included, testifies to their low immunogenicity, which will prevent the development of allergic reactions during administration of the antigen-presenting spores.

High resistance of the spores to various environmental conditions enables their easy storage without the need to freeze them or keep the cold chain.

The possibility of mixing spores presenting different antigens enables producing many quantitative and qualitative vaccine combinations. Moreover, it enables instant preparation of the desired antigen epitope combination by mixing recombinant spores in the proper proportions in the standard laboratory conditions.

Administration of spore-containing vaccines may bring about additional positive health effects, since the *B. subtilis* spores are used as probiotics, and their modification so that they can present antigens will not reduce their beneficial effects on the gastrointestinal tract of the immunised animals or humans.

Preparation of the *B. subtilis* spores containing fragments of UreA and/or UreB proteins in the form of fusion proteins with e.g. CotB, CotC, CotG, or CotZ. The thus prepared material is administered orally to the patient and in this way delivered to its destination, i.e. the stomach. The spore and its inherent resistance to digestion protects the antigens which will then be able to stimulate the immune response against *H. pylori*, thus building immunity of the organism to that particular pathogen.

The creation of a specific drug to treat *H. pylori* infections is hindered because the pathogen has an adapting mechanism, enabling e.g. expression of antigens structurally similar to the carrier – bacterial LPS and antigens of the Lewis group found on gastric mucosal cells. The antigen mimicry may lead to effective extinguishment of the specific immune response.

An additional obstacle to the development of an effective vaccine comes from the environment in which *H. pylori* colonises the body, i.e. the stomach. This is where the food is digested, and as such its environment may be considered 'caustic'. Moreover, the same digestion process makes the traditional immunisation methods futile in terms of their therapeutic and protective effects.

The method proposed in the invention enables the vaccine to 'survive' in the hostile environment of the stomach, and eliminate the need to resort to antibiotic therapy.

The fundamental benefit of the vaccine will consist in building immunity to *H. pylori* infection and re-infection among the convalescents. This will enable to reduce the number of new infections substantially, and thus reduce the costs of treating the infected patients. An additional tangible benefit of using the vaccine will consist in the possibility to abandon antibiotic therapy to the extent it has been resorted to so far. In effect, the process of developing drug resistance by *H. pylori* will be slowed down. This is even more important if one bears in mind that many physicians and scientists note that the possibilities of antibiotic therapy are getting gradually exhausted because of the development of multi-drug resistant strains. This means that in the relatively near future we may come to the point when it will not be possible to treat the infection effectively since the bacterium will be resistant to all available antibiotics. In approximately 50% cases *H. pylori* infections lead

to the development of the stomach cancer. The global population of patients suffering from this type of cancer is estimated at over 970 000 cases a year, which represents ca. 10% of all cancer incidents. An effective vaccine against *H. pylori* will enable attaining a substantial reduction in the number of new stomach cancer incidents. Moreover, the invention has the potential of being used for therapeutic purposes in the treatment of already infected patients. The possibility of therapeutic use of the vaccine carries many advantages, such as the substantial limitation of the antibiotic therapy and the side effects thereof, as well as the related financial benefits.

The form in which the vaccine/ drug is taken will offer yet another benefit. The goal of the invention is to obtain an orally administered preparation and avoid such medical procedures as injections. Such solution is advantageous to both the patient, and the 'health care system', particularly in the developing countries where medical disposables are rarely used (needles in particular), which fosters other infections. The possibility to abandon using the disposables (needles, syringes, antiseptics) will allow reducing the costs of immunising large groups of patients. Moreover, oral administration of the vaccine will mitigate the irrational fear of injections, which causes that a part of the population postpones the vaccination to a later unspecified date. Thanks to the use of bacterial spores as antigen carriers the ready vaccine preparation will not require storing in any specific conditions, such as low temperature, which will facilitate its transport and enable long storage (a few to an odd dozen years). The patient will be able to buy the vaccine in the form of e.g. powder or suspension and take it at any time of his/her choice (e.g. before, or after the meal). Yet another benefit of using the vaccine in development will come down to the fact the *B. subtilis* bacteria for years used as a probiotic helping to regulate the functioning of the gastrointestinal tract will be introduced into the tract of the treated patients.

The invention concerns the oral vaccine containing the *Bacillus subtilis* spores which serve as the antigen carrier, and UreA or UreB proteins of the *Helicobacter pylori* or *Helicobacter acinonychis* urease serving as the antigen combined with CotB, CotC, CotG, CotZ proteins of the spore coat.

The oral vaccine, in which the spore coat protein CotB and the UreA urease protein are sequenced as in Fig.5.

The oral vaccine, in which the spore coat protein CotC and the UreA urease protein are sequenced as in Fig.6.

The oral vaccine, in which the spore coat protein CotG and the UreA urease protein are sequenced as in Fig.7.

The oral vaccine, in which the spore coat protein CotZ and the UreA urease protein are sequenced as in Fig.8

The oral vaccine, in which the spore coat protein CotC and the UreB urease protein fragment are sequenced as in Fig.17

The oral vaccine in which UreA and/or UreB urease proteins are located on the spore surface as fusion proteins with proteins CotB or CotC or CotG or CotZ.

The oral vaccine, in which UreA and/or UreB proteins of the *Helicobacter pylori* or *Helicobacter acinonychis* urease constitute its subunits.

The oral vaccine, which contains adjuvant as an additional element.

The oral vaccine, where *Bacillus subtilis* spores constitute an adjuvant, preferably contains another adjuvant.

The oral vaccine, the additional adjuvant of which is cytokine IL-2, the latter being a fusion protein.

The oral vaccine, where the vaccine containing the IL-2 adjuvant is sequenced as in Fig.24.

Using the *Bacillus subtilis* spores according to the invention to produce the oral vaccine applied to immunise patients suffering from the duodenal and gastric ulcer disease against *Helicobacter pylori*.

Using the oral vaccine containing the *Bacillus subtilis* spores which serve as the antigen carrier, UreA, UreB proteins of the *Helicobacter pylori* or *Helicobacter acinonychis* urease

serving as the antigen, the latter combined with the spore coat protein CotB or CotC or CotG or CotZ in the amount suitable for administration in the therapeutically effective dose.

Using the oral vaccine, where the oral vaccine is produced as powder, suspension, or in any other form suitable for the environment in which *Helicobacter pylori* bacteria occur.

The advantages of implementing the invention are as follows:

- the possibility to immunise large populations and thus to eliminate or substantially reduce the occurrence of *H. pylori* infections and the development of ulcer disease or stomach cancer;
- easy administration of the vaccine produced as an orally administered suspension;
- the possibility to store the vaccine/ drug in normal conditions without the need to keep any specific environment.
- limited application of antibiotic therapy,
- reduction of the therapy costs.

The terms used above, as well as in the patent description and claims are defined as follows:

Oral vaccine – enterally-administered preparation evoking a specific immune response in the recipient organism.

The *Bacillus subtilis* strain – strain No. 168 (wild laboratory strain)

Helicobacter pylori – strain No. NTC 11637

Helicobacter acinonychis – strain No. ATCC 51101

Description of the figures:

Fig.1 – shows the fusion proteins UreA-CotB recognised by antibodies directed against both CotB, and UreA proteins.

Fig.2 – shows the fusion proteins UreA-CotC recognised by antibodies directed against both CotC, and UreA proteins.

Fig.3 – shows the fusion proteins UreA-CotG recognised by antibodies directed against both CotG, and UreA proteins.

Fig.4 – presents quantification of the fusion proteins UreA-CotC presented on the surface of the recombined spores formed in the strains of different genetic backgrounds. 1 – purified UreA protein, 2 – extract of the proteins of the wild-type spore coat, 3 – extract of the *cotB::ureA1* strain spores, 4 – extract of the *cot::ureA1* strain spores, 5 – extract of the *cotC::spc cotC::ureA1* strain spores, 6 – extract of the *cotC::spc cotU::erm cotC::ureA1* strain spores, 7 – extract of the *cotG::urea* strain spores, 8 – extract of the *cotZ::urea* strain spores.

Fig.5 – shows the nucleotide and amino acid sequences of the recombined CotG gene and UreA protein or its fragments.

Fig.6 – shows the nucleotide and amino acid sequences of the recombined CotC gene and UreA protein or its fragments.

Fig.7 – shows the nucleotide and amino acid sequences of the recombined CotB gene and UreA protein or its fragments.

Fig.8 – shows the nucleotide and amino acid sequences of the recombined CotZ gene and UreA protein or its fragments.

Fig.9 – presents the maps of the following integration vectors: pKH20 (A), pKH03 (B), pKH14 (C), pKH102 (D). Expression of the recombined genes is ensured by the following respective physiological gene promoters: *cotG*, *cotC*, *cotB* and *cotZ*.

Fig.10 – shows the nucleotide sequence of the *rrnOP2* promoter, and the nucleotide and amino acid sequences of the UreA gene and protein.

Fig.11 – shows integration vector pKH115 containing the *ureA* gene controlled by the *rrnO* P2 ribosomal promoter.

Fig.12 – presents Western Blotting with the use of protein extracts obtained from the spore coat. The samples applied: 1- purified UreA, 2- 168 (wild-type strain), 3- BKH102 (*amyE::cotZ-ureA*), 4- BKH115 (*thrC::rrnOP2 – ureA*), 5- BKH116 (*amyE::cotZ-ureA, thrC::rrnOP2 – ureA*).

Fig.13 – presents Western Blotting with the use of protein extracts obtained from vegetative cells. The samples applied: 1- purified UreA, 2- 168 (wild-type strain), 3- BKH116 (*amyE::cotZ-ureA, thrC::rrnOP2 – ureA*).

Fig.14 – shows the immunofluorescences using spores of the BKH102 strain purified through rinsing with detergents. The experiment used mouse anti-UreA primary antibodies and anti-mouse IgG-Cy3 secondary antibodies which enabled their visualisation in the fluorescence microscope. The left photograph shows the image seen in the visible light (phase contrast), while the right presents the fluorescent image of the same field of vision.

Fig.15 – shows the results of the ELISPOT assay. Each spot is the ‘footprint’ of a single cell that has secreted IFN- γ in response to the present antigen. Splenocytes were isolated from non-immunised mice (blue), those immunised with wild-type spores – 168 (red), and yet others immunised with BH102 presenting the antigen (green).

Fig.16 – presents map of the pKH28 integration vector. Expression of the recombined gene is ensured by the physiological promoter of the CotC gene.

Fig.17 – shows the nucleotide and amino acid sequences of the recombined gene and the CotC-UreB3 protein.

Fig.18 – shows Western Blotting with the use of protein extracts obtained from the spore coat. The samples applied: 1 - 168 (wild-type strain), 2- BKH28 (*amyE::cotC-ureB3*), 3- RH101 (*cotC::spc*) 4- BKH48 (*cotC::spc, amyE::cotC-ureB3*), 4- BKH48 (*cotC::spc, amyE::cotC-ureB3*), 5- RH209 (*cotC::spc cotU::erm*), 6- BKH49 (*cotC::spc, cotU::erm, amyE::cotC-ureB3*).

Fig.19 – shows the immunofluorescences using spores of the BKH28 strain purified through rinsing with detergents. The experiment used mouse anti-UreB primary antibodies and anti-mouse IgG-Cy3 secondary antibodies which enabled their visualization in the

fluorescence microscope. The left photograph shows the image seen in the visible light (phase contrast), while the right depicts the fluorescent image of the same field of vision.

Fig.20 – shows the pKH101 integration vector containing the *ureB* gene controlled by the *rrnO* P2 ribosomal promoter.

Fig.21 – presents the nucleotide sequence of the *rrnOP2* promoter and the nucleotide and amino acid sequences of the *UreB* gene and protein.

Fig.22 – shows Western Blotting with the use of protein extracts obtained from the endospore coat (1 and 2) and vegetative cells (3 and 4). The samples applied: (1 and 3) - 168 (wild-type strain), (2 and 4)- BKH108 (*amyE:: cotC-ureB3, thrC:: rrnOP2 – ureB*).

Fig.23 – presents the map of the pKH124 integration vector. Expression of the recombined gene is ensured by the physiological promoter of the *CotB* gene.

Fig.24 – shows the nucleotide and amino acid sequences of the recombined *CotB-linker-IL-2* gene and protein.

Fig.25 – presents the *CotB-linker-IL-2* fusion protein recognised by the antibodies directed against both the *CotB* protein and the *IL-2* protein.

Fig.26 – shows the immunofluorescences using spores of the BKH121 strain purified through rinsing with detergents. The experiment used mouse anti-IL-2 primary antibodies and anti-mouse IgG-Cy3 secondary antibodies which enabled their visualization in the fluorescence microscope. The left photograph shows the image seen in the visible light (phase contrast), while the right depicts the fluorescent image of the same field of vision.

Fig.27 – shows the results of the ELISPOT assay. Each spot is the 'footprint' of a single cell that has secreted IFN- γ in response to the present antigen. Splenocytes were isolated from non-immunised mice (blue), mice immunised with wild-type spores – 168 (red), those immunised with BH108 presenting the *ureB* antigen (green), and others immunised with BH108 presenting antigen *ureB* and BHK121 presenting cytokine *IL-2* (grey).

The invention is illustrated with the following embodiments which do not restrict the invention in any way.

Example 1:

Preparation of the fusion proteins based on the *H. acinonychis* UreA subunit with the spore coat proteins, and verification of the level of expression and presence of the recombined proteins on the spore surface.

It was established that fusions of proteins CotB, CotC, CotG, CotZ with the *H. acinonychis* UreA subunit and its fragments were effectively presented on the surface of the *B. subtilis* endospores. Moreover, depending on the applied genetic background the number of the recombined proteins isolated from the spore coat was close to or higher than that reported in the literature. The conducted immunofluorescence experiments enabled identification of two types of spores. In one type, the antigen was exposed directly on the surface, whereas in the second type the fusion proteins were inside the coat, hence were not exposed outside the endospores (Fig. 1, Fig.2, Fig.3, Fig.4).

Both types of spores (presenting fragments of the UreA or UreB proteins) were used in the immunological part of the research. The spores where the antigen is not exposed directly on the surface offer more effective antigen protection from degradation on its way along the upper elements of the gastrointestinal tract down to its destination (stomach, intestines), which should consequently enable the attainment of the optimal induction of the immune response.

Example 2:

Tests of immunisation of laboratory animals with vaccines produced according to the invention.

The pDL integration vector was used as the starting point to prepare the fusion protein (CotZ-UreA). The *cotZ* gene from *B. subtilis*, together with the promoter region, was copied under the PCR method using primers: cZ-F 5'- GCT TAG GAT CCA TGA TGA TGT GTA CGA TTG -3' and cY-R 5'- CGT AGC GAA TTC AGT TAT CAC TCT TGT CCT C -3'. Restriction sites (*Bam*HI and *Eco*RI, marked in the primer sequence) were introduced during the PCR reaction facilitating both the process of cloning to the vectors and the formation of the fusion protein. The UreA subunit of the *H. aciononyhis* urease became the second component of the recombined protein. The *ureA* gene was copied under the PCR method using primers: uA-F 5'- GAG GGA TCC ATG AAA CTC ACC CCA AAA G-3' and uA-R 5'- CGC GAG CTC TAG GGC CAT ACA TAG AAA C- 3'. Restriction sites (*Bam*HI and *Sac*I, marked red in the primer sequence) were introduced during the PCR reaction facilitating the fusions of the *cotZ* and *ureA* genes in the correct reading frame.

The obtained fusion gene was sequenced so as to verify the attainment of the planned product. The obtained plasmid carrying the fusion gene was called pKH102.

The fusion proteins (CotG-UreA, CotC-UreA1, and CotB-UreA1) were prepared in an analogical manner (Fig.5, Fig.6, Fig.7, Fig.8).

The immediately following step consisted in transformation of the *B. subtilis* cells with the pKH102 plasmid containing the fusion gene. In effect, the recombined gene was incorporated into locus *amyE* of the bacterial chromosome. The obtained bacterial strain was called BKH102. On the other hand, transformation of the *B. subtilis* cells with the pKH20, pKH03, and pKH14 plasmids (Fig.9) containing the recombined genes *cotB-ureA*, *cotC-ureA1*, and *cotB-ureA1*, enabled obtaining strains BKH20, BKH03, and BKH14. The analysis of the expression level and presence of the recombined proteins on the spore surface using the strains is illustrated in example 1.

In order to reinforce the response attained upon administration of the preparation to laboratory animals, a vector was prepared based on the pDG1662 plasmid, which enabled incorporation of the UreA subunit of the *H. acinonychis* urease into the *B. subtilis* chromosome, in locus *thrC* of the coding gene under the control of a powerful active promoter in the vegetative cells (ribosomal RNA *rrnO* P2 gene promoter). Administered as vaccine, the spores germinate in the gastrointestinal tract forming vegetative cells, which can express additional quantities of the antigen. The promoter region was copied under the PCR method using primers: rOP2-F 5'- GAT GGC TAA GCT TCA TGG GTC TCA CCT CCT TGT TC -3' and rOP2-R 5'- GGC GTA GAG GAA TTC GAT CTG CAT GAC CAT TAT GAC -3'. Restriction sites (*HindIII* and *EcoRI*, marked in the primer sequence) were introduced during the PCR reaction. Then, the PCR method was employed to copy the *ureA* gene using primers: ruA-F 5'- GGC ATT AAG CTT AAC TAT GTA G -3' and ruA-R 5'- CTA AAC ATG TTC ATT TCT TAC TC -3'. During the PCR reaction restriction sites were introduced (*HindIII* and *PciI*, marked in the primer sequence) and the control of the ribosomal promoter imposed. The obtained vector was called pKH115.

The subsequent step consisted in transformation of the *B. subtilis* cells strain BKH102 (*amyE::cotZ-ureA*) with plasmid pKH115. In effect, the recombined gene was incorporated into locus *thrC* of the bacterial chromosome. This solution enabled creating a strain that expressed the UreA antigen on the spore surface (fusion protein CotZ-UreA) and in the vegetative cell. The obtained strain was called BKH116 (*amyE::cotZ-ureA, thrC::p_{rrnO2}-ureA*). The cultures of the BKH102 and BKH116 strains were analysed for expression of the recombined protein CotZ-UreA and protein UreA using anti-UreA mouse antibodies obtained in our laboratory. The presence of the fusion protein was tested in the protein extracts obtained from the spore coat of the BKH102 strain, and the UreA protein was tested in protein extracts prepared from the vegetative cells of the BKH116 strain. In both cases the western-blotting method was applied, consisting in detection of the specific protein bound to the membrane using enzyme-bound antibodies, where the colour staining reaction enables its location.

The suitability of the spores carrying the fusion protein in oral immunisation stems from the location of the fusion protein within the spore structures. It is assumed that the fusion

proteins present on the spore surface demonstrate a higher potential of stimulating the immune system. The presence of the recombinant protein CotZ-UreA on the surface of the spores produced by strain BKH102 was confirmed by the immunofluorescence method (Fig.14, right panel). The visible red fluorescence forming around the spore proves the surface location of the protein in question.

Upon verification of the expression and location of the fusion protein in the spore structures, the procedure turned to purification of the spores in the quantity sufficient to immunise a group of 10 mice. To that end, cultivation to a larger scale was initiated (15 litres of the DSM culture medium), which enabled isolation of an adequate quantity of spores. The spores were purified by rinsing in water and detergents, and then counted in the Thoma chamber. Each batch of the purified spores was tested for the presence of the appropriate fusion proteins on their surface (the data not shown). Spore purification completed the stage of preparing the material used to immunise the laboratory animals (mice) at a later stage. The procedures of immunising the laboratory animals with the created vaccine and assessing the immune response (the degree and type of immune response induction) followed subsequently with assessment of the vaccine effectiveness were conducted on mice of the BALB/c stock (females aged 6 weeks +). The vaccine in nine doses of 1.0×10^{10} (equivalent to 5mg of spores per dose), containing antigen in the form of *H. acinonychis* urease subunit UreA on the surface, was administered into the stomach with a syringe connected to a polyethylene cannula. The animals were vaccinated on the following days: 0, 3, 5, 14, 16, 18, 30, 32, and 34. On day 56 they were euthanised. The experimental group was made up of 10 animals. The reaction of the immune system was analysed for the humoral and cellular responses. Blood, excreta, and tissue of the animals (the spleen and stomach) served as the material for testing.

The ELISPOT assay (Fig.15) combining the enzyme-linked immunosorbent assay technique with short-term cell cultivation revealed an increased number of cytokines (IFN- γ) secreted by splenocytes (immune system cells isolated from the spleen) of the mice immunised with the spores presenting fusion protein CoZ-UreA. The attained result proves activation of the cellular immune response (th1). The fact prognosticates well for the vaccine, since analysis of the information available from the literature indicates that this particular type of the immune response is most effective in elimination of the pathogen.

Example 3:

Preparation of the fusion proteins based on the *H. acinonychis* UreB subunit with the spore coat proteins, and verification of the level of expression and presence of the recombined proteins on the spore surface.

The pDG364 integration vector was used as the starting point to prepare the fusion protein (CotC-UreB3). The *cotC* gene from *B. subtilis*, together with the promoter region, was copied under the PCR method using primers: cC-F 5'- ACC CAA GCT TTG TAG GAT AAA TCG TTT G -3' and cC-R 5'- GAT ATC GTA GTG TTT TTT ATG CTT -3'. Restriction sites (*HindIII* and *EcoRV*, marked in the primer sequence) were introduced during the PCR reaction facilitating both the process of cloning to the vectors and the formation of the fusion protein. The UreB subunit of the *H. acinonychis* urease became the second component of the recombined protein. A fragment of the *ureB* gene was copied under the PCR method using primers: uB-F 5'- GCT GGT GAT ATC GGC TTT AAA ATC CAC-3' and uB-R 5'- GCA CCT TAC TAG TAA CTT TTG TTG CTT GAG C -3'. Restriction sites (*EcoRV* and *SpeI*, marked red in the primer sequence) were introduced during the PCR reaction facilitating the fusions of the *cotC* and *ureB* genes in the correct reading frame

The obtained fusion gene was sequenced so as to verify the attainment of the planned product. The obtained plasmid carrying the fusion gene was called pKH28 (Fig.16).

The immediately following step consisted in transformation of the *B. subtilis* cells with the pKH28 plasmid containing the fusion gene. In effect, the recombined gene was incorporated into locus *amyE* of the bacterial chromosome. The obtained bacterial strain was called BKH28.

The culture of the BKH28 strain was analysed for expression of the recombined protein CotC-UreB3 of the mouse anti-UreB antibodies obtained in our laboratory. The presence of the fusion protein was tested in the protein extracts obtained from the spore coat of the BKH28 strain applying the western-blotting method, consisting in detection of the specific

protein bound to the membrane using enzyme-bound antibodies, where the colour staining reaction enables its location (Fig.18).

The presence of the recombined protein CotC-UreB3 on the surface of the spores produced by strain BKH28 was confirmed by the immunofluorescence method (Fig.19, right panel).

In order to reinforce the response attained upon administration of the spores presenting antigen UreB on the surface to laboratory animals, a vector was prepared based on the pDG1662 plasmid, which enabled incorporation of the UreB subunit of the *H. acinonychis* urease into the *B. subtilis* chromosome, in locus *thrC* of the coding gene under the control of a powerful active promoter in the vegetative cells (ribosomal RNA *rrnO* P2 gene promoter). Administered as vaccine, the spores germinate in the gastrointestinal tract forming vegetative cells, which can express additional quantities of the antigen. The promoter region was copied under the PCR method using primers: rOP2-F 5'- GAT GGC TAA GCT TCA TGG GTC TCA CCT CCT TGT TC -3' and rOP2-R 5'- GGC GTA GAG GAA TTC GAT CTG CAT GAC CAT TAT GAC -3'. Restriction sites (*Hind*III and *Eco*RI, marked in the primer sequence) were introduced during the PCR reaction. Then, the PCR method was employed to copy the *ureB* gene using primers: uB-F 5'- CCA TAA GCT TAA AAA GAT TAG CAG AAA AG -3' and uB-R 5'- CTC CAC ATG TAT CCT AGA AAA TGC TAA AG -3'. During the PCR reaction restriction sites were introduced (*Hind*III and *Pci*I, marked red in the primer sequence) and the control of the ribosomal promoter imposed (Fig.21). The obtained vector was called pKH101 (Fig.20).

The subsequent step consisted in transformation of the *B. subtilis* cells strain BKH28 (*amyE::cotC-ureB3*) with plasmid pKH101. In effect, the recombined gene was incorporated into locus *thrC* of the bacterial chromosome. This solution enabled creating a strain that expressed the UreB antigen on the spore surface (fusion protein CotC-UreB3) and in the vegetative cell. The obtained strain was called BKH108 (*amyE::cotC-ureB3*, *thrC::p_{rrnO2}-ureB*).

The culture of the BKH108 strain was analysed for expression of the recombined protein CotC-UreB3 and protein UreB using mouse anti-UreB antibodies obtained in our laboratory. The presence of the fusion protein was tested in the protein extracts obtained from the

spore coat, and the UreB protein was tested in protein extracts prepared from the vegetative cells. In both cases the western-blotting method was applied, consisting in detection of the specific protein bound to the membrane using enzyme-bound antibodies, where the colour staining reaction enables its location (Fig.22).

The quality of vaccines is frequently attained at the expense of reducing their ability to stimulate specific immune responses. The immunogenicity of the antigen can, however, be enhanced when administered together with adjuvants, which improve the vaccine's effectiveness in many ways. The substances can, e.g. stimulate the cells of the immune system non-specifically and pertain to more efficient absorption and presentation of the antigen. By selecting an appropriate adjuvant one can obtain the immune response of the specific type - Th1 or Th2.

Here, cytokines served as the adjuvant, though instead of stimulating their production with appropriate agents, they were administered directly, together with the antigen. In this way, it was possible to control the antigen processing and APC activation at the level of one and the same cell. In our research we used spores carrying IL-2, in the function of the adjuvant, on their surface in the oral vaccines.

The pDG1663 integration vector was used as the starting point to prepare the fusion protein (CotB-linker-IL-2). The *cotB* gene from *B. subtilis*, together with the promoter region, was copied under the PCR method using primers: cB-F 5'- GCG GAT CCG GAT GAT TGA T -3' and cB-R 5'- GAT GAA TTC ACG GAT TAG -3'. Restriction sites (*Bam*HI and *Eco*RI, marked red in the primer sequence) were introduced during the PCR reaction facilitating both the process of cloning to the vectors and the formation of the fusion protein. The other component of the recombined protein, cytokine IL-2, was synthesised (EUROGENTEC) together with the sequences coding the peptide linkers sequenced GGGEAAKGGG at the beginning and end of the formed protein. Then, gene *IL-2* (containing the linker coding sequence 5' at the end) was copied under the PCR method using primers: IL2link-F 5'- GCT TCA CAT GTT TAC GTC AGT GTA GAG ATG ATA GAT TGG C -3' and IL2link-R 5'- CAT ATG GAT CCG GTG GAG GAG AAG CAG CAG CG -3'. Restriction sites (*Pci*I *Bam*HI, marked red in the primer sequence) were introduced during the PCR reaction facilitating the fusions of the *cotB-linker-IL2* genes in the correct reading frame (Fig.24).

The obtained fusion gene was sequenced so as to verify the attainment of the planned product. The obtained plasmid carrying the fusion gene was called pKH122 (Fig.23).

The subsequent step consisted in transformation of the *B. subtilis* cells with the pKH122 plasmid. In effect, the recombined gene *cotB-linker-IL-2* was incorporated into locus *thrC* of the bacterial chromosome. The obtained bacterial strain was called BKH121.

The purified spores produced by the above strain were tested for the presence of recombined coat proteins using mouse anti-IL-2 antibodies under the western-blotting method (Fig. 25).

The quantity of the recombined proteins located in the outer spore coat was assessed by the dot-blotting technique where proteins are applied directly on the membrane and then detected by antibodies. The assays revealed that the quantity of the recombined proteins CotB-linker-IL-2 isolated from the spore coat stood at 1.1×10^4 molecules/spore.

The usefulness of the spores carrying fusion proteins in oral immunisation is due to the location of the fusion protein in the spore structures. It is assumed that the fusion proteins present on the spore surface demonstrate a higher potential of stimulating the immune system. The presence of the recombined proteins on the surface of the spores was confirmed by the immunofluorescence method. The fusion of CotB-linker-IL-2 is deposited on the surface of the recombined spores (Fig.26).

Upon verification of the expression and location of the fusion proteins in the spore structures, the procedure turned to purification of the spores in the quantity sufficient to immunise a group of 10 mice. To that end, cultivation to a larger scale was initiated (15 litres of the DSM culture medium per strain), which enabled isolation of an adequate quantity of spores. The spores were purified by rinsing in water and detergents, and then counted in the Thoma chamber. Each batch of the purified spores was tested for the presence of the appropriate fusion proteins on their surface (the data not shown). Spore purification completed the stage of preparing the material used to immunise the laboratory animals (mice) at a later stage. The procedures of immunising the laboratory animals with the created vaccine and assessing the immune response (the degree and type of immune response induction) followed subsequently with assessment of the vaccine effectiveness were conducted on mice of the BALB/c stock (females aged 6 weeks +). The vaccine of

recombined spores of the BKH108 (*amyE::cotC-ureB3 thrC::rnnOP2-UreB*) and BKH121 (*thrC::cotB-linker-IL-2*) strains, at 1.0×10^{10} , i.e. 2.0×10^{10} (equivalent to 10mg of spores per dose) was administered into the stomach with a syringe connected to a polyethylene cannula. The animals were vaccinated on the following days: 0, 3, 5, 14, 16, 18, 30, 32, and 34. On day 56 they were euthanised. The experimental group was made up of 10 animals. The reaction of the immune system was analysed for the humoral and cellular responses. Blood, excreta, and tissue of the animals (the spleen and stomach) served as the material for testing.

The ELISPOT assay combining the enzyme-linked immunosorbent assay technique with short-term cell cultivation revealed an increased number of cytokines (IFN- γ) secreted by splenocytes (immune system cells isolated from the spleen) of the mice immunised with the spores presenting fusion protein *cotC-ureB3* in the presence of spores presenting cytokine IL-2 on the surface (Fig. 27). The attained result proves activation of the cellular immune response (Th1). The fact prognosticates well for further work on the vaccine development, since analysis of the information available from the literature indicates that this particular type of the immune response is most effective in elimination of the pathogen.

Patent Claims

1. Oral vaccine containing the *Bacillus subtilis* spores **characterised in that** it contains the *Bacillus subtilis* spores which serve as the antigen carrier, and UreA, UreB proteins of the *Helicobacter pylori* or *Helicobacter acinonychis* urease serving as the antigen combined with the spore coat protein CotB or CotC or CotG or CotZ.
2. Oral vaccine according to Claim 1 **characterised in that** the spore coat protein CotB and the UreA urease protein are sequenced as in Fig.5
3. Oral vaccine according to Claim 1 **characterised in that** the spore coat protein CotC and the UreA urease protein are sequenced as in Fig.6
4. Oral vaccine according to Claim 1 **characterised in that** the spore coat protein CotG and the UreA urease protein are sequenced as in Fig.7
5. Oral vaccine according to Claim 1 **characterised in that** the spore coat protein CotZ and the UreA urease protein are sequenced as in Fig.8
6. Oral vaccine according to Claim 1 **characterised in that** the spore coat protein CotC and a fragment of the UreB urease protein are sequenced as in Fig.17
7. Oral vaccine according to Claim 1 **characterised in that** the UreA and/or UreB urease proteins are located on the spore surface as fusion proteins with proteins CotB or CotC or CotG or CotZ.
8. Oral vaccine according to Claim 1, **characterised in that** the UreA and/or UreB proteins of the *Helicobacter pylori* or *Helicobacter acinonychis* urease constitute its subunits.
9. Oral vaccine according to Claims 1-8, **characterised in that** it contains adjuvant as an additional element.
10. Oral vaccine according to Claims 1-9, **characterised in that** the *Bacillus subtilis* spores constitute its adjuvant, and preferably it contains another adjuvant.
11. Oral vaccine according to Claims 1-10, **characterised in that** its additional adjuvant is IL-2 cytokine, the latter being a fusion protein.

12. Oral vaccine according to Claims 1-11, **characterised in that** the vaccine containing the IL-2 adjuvant is sequenced as in Fig.24.
13. Using the *Bacillus subtilis* spores according to the invention to produce the oral vaccine applied to immunise patients suffering from the duodenal and gastric ulcer disease against *Helicobacter pylori*.
14. Using the vaccine according to Claim 13, **characterised in that** the oral vaccine contains the *Bacillus subtilis* spores which serve as the antigen carrier, UreA, UreB proteins of the *Helicobacter pylori* or *Helicobacter acinonychis* urease serving as the antigen combined with the spore coat protein CotB or CotC or CotG or CotZ in the amount suitable for administration in the therapeutically effective dose.
15. Using the vaccine according to Claims 13-14, **characterized in that** the oral vaccine is produced as powder, suspension, or in any other form suitable for the environment in which *Helicobacter pylori* bacteria occur.

Fig.1

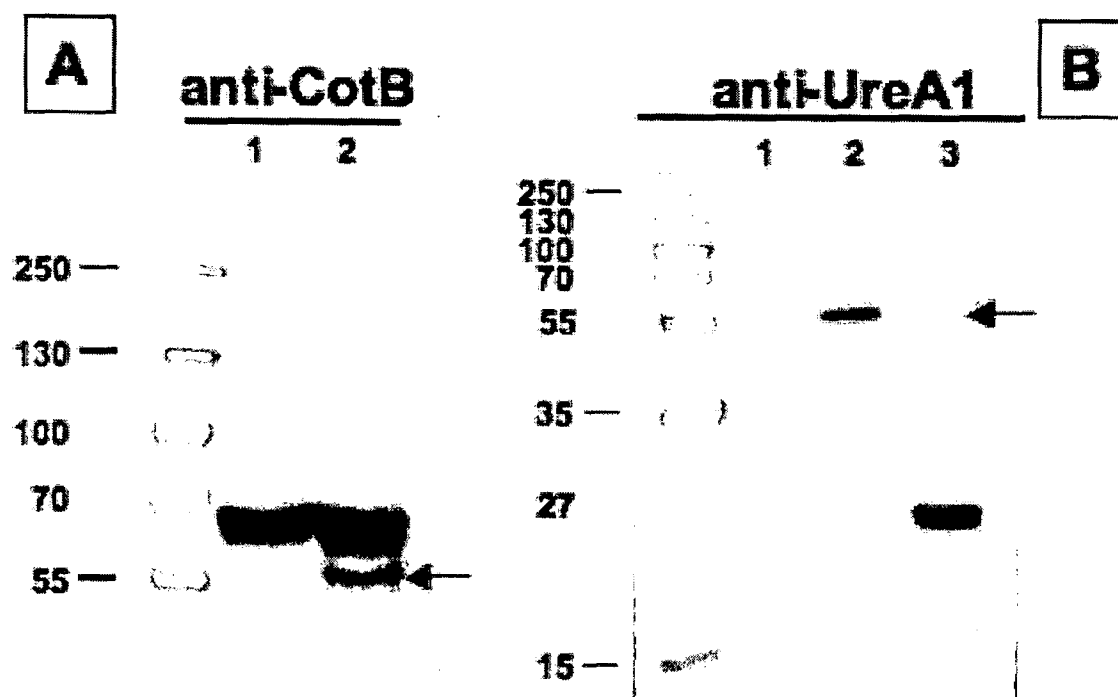


Fig.2

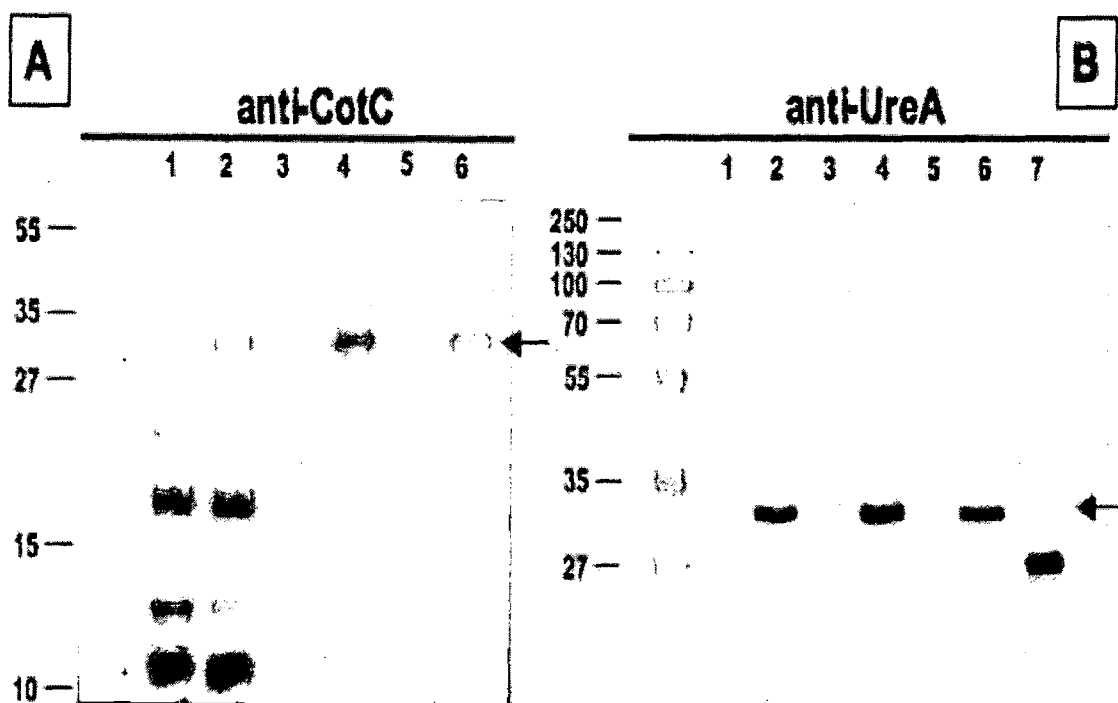


Fig.3

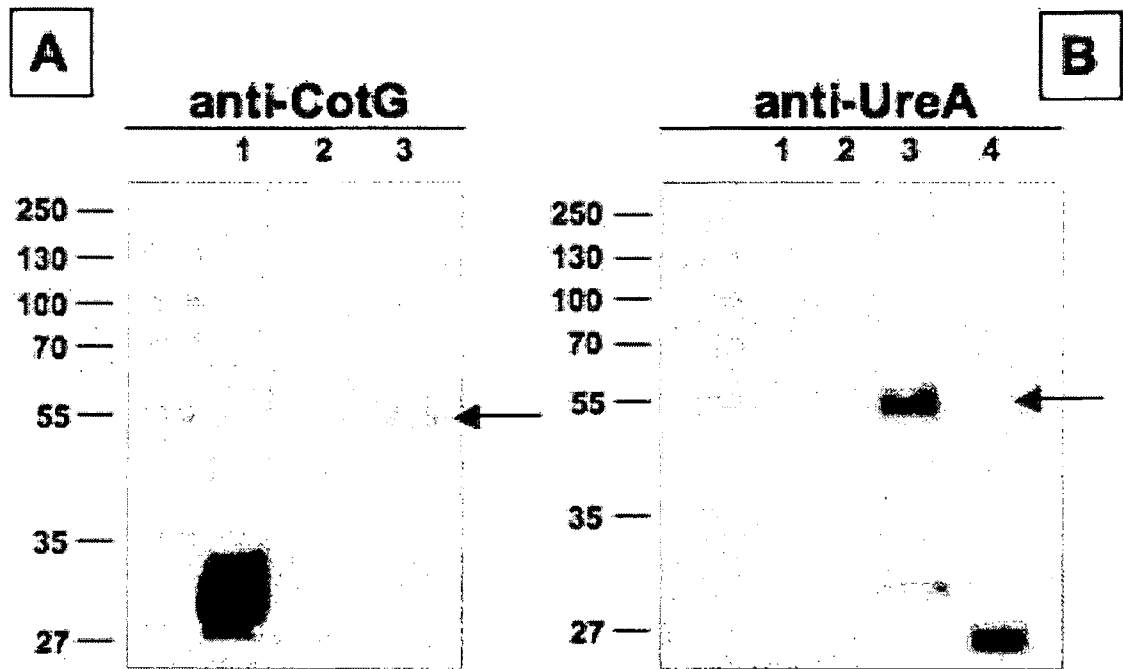


Fig.4

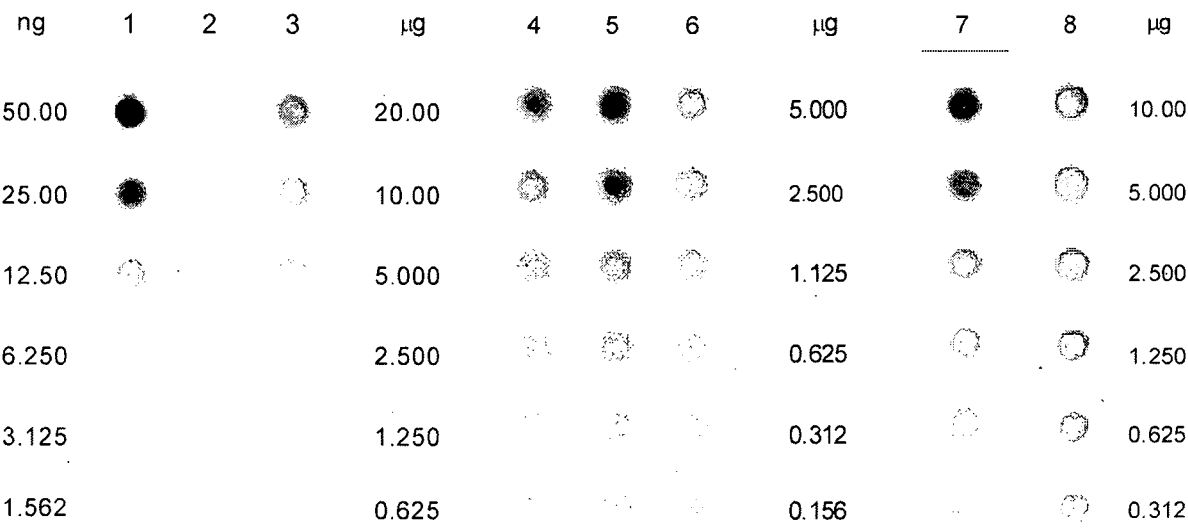


Fig.5

CotB-UreA1

```
1  atg agc aag agg aga atg aaa tat cat tca aat aat gaa ata tcg tat tat
   >>.....cotB-ureA1.....>
   >>.....cotB.....>

52  aac ttt ttg cac tca atg aaa gat aaa att gtt act gta tat cgt gga ggt
   >.....cotB-ureA1.....>
   >.....cotB.....>

103 ccg gaa tct aaa aaa gga aaa tta aca gct gta aaa tca gat tat ata gct
   >.....cotB-ureA1.....>
   >.....cotB.....>

154 tta caa gct gaa aaa aaa ata att tat tat cag ttg gag cat gtg aaa agt
   >.....cotB-ureA1.....>
   >.....cotB.....>

205 att act gag gat acc aat aat agc acc aca aca att gag act gag gaa atg
   >.....cotB-ureA1.....>
   >.....cotB.....>

256 ctc gat gct gat gat ttt cat agc tta atc gga cat tta ata aac caa tca
   >.....cotB-ureA1.....>
   >.....cotB.....>

307 gtt caa ttt aac caa ggg ggt ccg gaa tct aaa aaa gga aga ttg gtc tgg
   >.....cotB-ureA1.....>
   >.....cotB.....>

358 ctg gga gat gat tac gct gcg tta aac aca aat gag gat ggg gta gtg tat
   >.....cotB-ureA1.....>
   >.....cotB.....>

409 ttt aat atc cat cac atc aaa agt ata agt aaa cac gag cct gat ttg aaa
   >.....cotB-ureA1.....>
   >.....cotB.....>

460 ata gaa gag cag acg cca gtt gga gtt ttg gaa gct gat gat tta agc gag
   >.....cotB-ureA1.....>
   >.....cotB.....>

511 gtt ttt aag agt ctg act cat aaa tgg gtt tca att aat cgt gga ggt ccg
   >.....cotB-ureA1.....>
   >.....cotB.....>

562 gaa gcc att gag ggt atc ctt gta gat aat gcc gac ggc cat tat act ata
   >.....cotB-ureA1.....>
   >.....cotB.....>

613 gtg aaa aat caa gag gtg ctt cgc atc tat cct ttt cac ata aaa agc atc
   >.....cotB-ureA1.....>
   >.....cotB.....>

664 agc tta ggt cca aaa ggg tcg tac aaa aaa gag gat caa aaa aat gaa caa
```

```
>.....cotB-ureA1.....>
>.....cotB.....>

715  aac cag gaa gac aat aat gat aag gac agc aat tcg ttc att tct tca aaa
>.....cotB-ureA1.....>
>.....cotB.....>

766  tca tat agc tca tca aaa tca tct aaa cga tca cta aaa tct tca gat gat
>.....cotB-ureA1.....>
>.....cotB.....>

817  caa tca tcc gat atc ggt aaa aag act gcg gct gaa ttg atg caa gaa ggg
>.....cotB-ureA1.....>
>..cotB..>>
>>.....ureA1.....>

868  cgc act ctt tta aaa ccg gat gat gtg atg gat ggt gtg gca agc atg atc
>.....cotB-ureA1.....>
>.....ureA1.....>

919  cat gaa gtg ggt att gaa gcg atg ttt cct gat ggg acc aaa ctc gta acc
>.....cotB-ureA1.....>
>.....ureA1.....>

970  gtg cat acc cct att gag gct aat ggt aaa ttg gtt cct ggt gag ttg ttc
>.....cotB-ureA1.....>
>.....ureA1.....>

1021 tta aaa aat gaa gac atc act atc aac gaa ggc aaa aaa gcc gtt agc gtg
>.....cotB-ureA1.....>
>.....ureA1.....>

1072 aaa gtt aaa aac gtg ggc gac aga ccg gtt caa atc ggt tca cac ttc cat
>.....cotB-ureA1.....>
>.....ureA1.....>

1123 ttc ttt gaa gtg aat aga tgc cta gac ttt gac aga gaa aaa act ttc ggc
>.....cotB-ureA1.....>
>.....ureA1.....>

1174 aaa cgc tta gac att gcg agc ggg aca gcg gta agg ttt gag cct ggc gaa
>.....cotB-ureA1.....>
>.....ureA1.....>

1225 gaa aaa tcc gta gaa ttg att gac att ggt ggc aac aga aga atc ttt gga
>.....cotB-ureA1.....>
>.....ureA1.....>

1276 ttt aac gca ttg gtt gat agg caa gca gac aac gaa agc aaa aaa att gct
>.....cotB-ureA1.....>
>.....ureA1.....>

1327 tta cac aga gct aaa gag cgt ggt ttt cat ggt gct aaa agc gat gac aac
>.....cotB-ureA1.....>
>.....ureA1.....>

1378 tat gta aaa aca att aag gag taa
>.....cotB-ureA1.....>>
>.....ureA1.....>>
```

Fig. 6

CotC-UreA1

```

1  atg ggt tat tac aaa aaa tac aaa gaa gag tat tat acg gtc aaa aaa acg
   M  G  Y  Y  K  K  Y  K  E  E  Y  Y  T  V  K  K  T
   >>.....cotC.....>
   >>.....cotC-ureA1.....>

52  tat tat aag aag tat tac gaa tat gat aaa aaa gat tat gac tgt gat tac
    Y  Y  K  K  Y  Y  E  Y  D  K  K  D  Y  D  C  D  Y
    >.....cotC.....>
    >.....cotC-ureA1.....>

103  gac aaa aaa tat gat gac tat gat aaa aaa tat tat gat cac gat aaa aaa
     D  K  K  Y  D  D  Y  D  K  K  Y  Y  D  H  D  K  K
     >.....cotC.....>
     >.....cotC-ureA1.....>

154  gac tat gat tat gtt gta gag tat aaa aag cat aaa aaa cac tac gat atc
     D  Y  D  Y  V  V  E  Y  K  K  H  K  K  H  Y  D  I
     >.....cotC.....>>
     >.....cotC-ureA1.....>

205  ggt aaa aag act gcg gct gaa ttg atg caa gaa ggg cgc act ctt tta aaa
     G  K  K  T  A  A  E  L  M  Q  E  G  R  T  L  L  K
     >.....cotC-ureA1.....>
     >>.....ureA1.....>

256  ccg gat gat gtg atg gat ggc gtg gca agc atg atc cat gaa gtg ggt att
     P  D  D  V  M  D  G  V  A  S  M  I  H  E  V  G  I
     >.....cotC-ureA1.....>
     >.....ureA1.....>

307  gaa gcg atg ttt cct gat ggg aca aaa ctc gta acc gtg cat acc cct att
     E  A  M  F  P  D  G  T  K  L  V  T  V  H  T  P  I
     >.....cotC-ureA1.....>
     >.....ureA1.....>

358  gag gcc aat ggt aaa tta gtt cct ggt gag ttg ttc tta aaa aat gaa gac
     E  A  N  G  K  L  V  P  G  E  L  F  L  K  N  E  D
     >.....cotC-ureA1.....>
     >.....ureA1.....>

409  atc act atc aac gaa ggc aaa aaa gcc gtt agc gtg aaa gtt aaa aat gtt
     I  T  I  N  E  G  K  K  A  V  S  V  K  V  K  N  V
     >.....cotC-ureA1.....>
     >.....ureA1.....>

460  ggc gac aga ccg gtt caa atc ggc tca cac ttc cat ttc ttt gaa gtg aat
     G  D  R  P  V  Q  I  G  S  H  F  H  F  F  E  V  N
     >.....cotC-ureA1.....>
     >.....ureA1.....>

511  aga tgc tta gac ttt gac aga gaa aaa act ttc ggt aaa cgc tta gac att
     R  C  L  D  F  D  R  E  K  T  F  G  K  R  L  D  I
     >.....cotC-ureA1.....>

```

```
>.....ureA1.....>
562 gcg agc ggg aca gcg gta agg ttt gag cct ggc gaa gaa aaa tcc gta gaa
    A  S  G  T  A  V  R  F  E  P  G  E  E  K  S  V  E
>.....cotC-ureA1.....>
>.....ureA1.....>
613 ttg att gac att ggc ggt aac aga aga atc ttt gga ttt aac gcg ttg gtt
    L  I  D  I  G  G  N  R  R  I  F  G  F  N  A  L  V
>.....cotC-ureA1.....>
>.....ureA1.....>
664 gat agg caa gca gac aac gaa agc aaa aaa att gct tta cac aga gct aaa
    D  R  Q  A  D  N  E  S  K  K  I  A  L  H  R  A  K
>.....cotC-ureA1.....>
>.....ureA1.....>
715 gag cgt ggt ttt cat ggc gct aaa agc gat gac aac tat gta aaa aca att
    E  R  G  F  H  G  A  K  S  D  D  N  Y  V  K  T  I
>.....cotC-ureA1.....>
>.....ureA1.....>
766 aag gag taa
    K  E  -
>.....>> cotC-ureA1
>..ureA1.>>
```

Fig. 7

CotG-UreA

```

1  ttg ggc cac tat tcc cat tct gac atc gaa gaa gcg gtg aaa tcc gca aaa
   L  G  H  Y  S  H  S  D  I  E  E  A  V  K  S  A  K
>>.....cotG-ureA.....>
>>.....cotG.....>

52  aaa gaa ggt tta aag gat tat tta tac caa gag cct cat gga aaa aaa cgc
   K  E  G  L  K  D  Y  L  Y  Q  E  P  H  G  K  K  R
>.....cotG-ureA.....>
>.....cotG.....>

103 agt cat aaa aag tcg cac cgc act cac aaa aaa tct cgc agc cat aaa aaa
   S  H  K  K  S  H  R  T  H  K  K  S  R  S  H  K  K
>.....cotG-ureA.....>
>.....cotG.....>

154 tca tac tgc tct cac aaa aaa tct cgc agt cac aaa aaa tca ttc tgt tct
   S  Y  C  S  H  K  K  S  R  S  H  K  K  S  F  C  S
>.....cotG-ureA.....>
>.....cotG.....>

205 cac aaa aaa tct cgc agc cac aaa aaa tca tac tgc tct cac aag aaa tct
   H  K  K  S  R  S  H  K  K  S  Y  C  S  H  K  K  S
>.....cotG-ureA.....>
>.....cotG.....>

256 cgc agc cac aaa aaa tcg tac cgt tct cac aaa aaa tct cgc agc tat aaa
   R  S  H  K  K  S  Y  R  S  H  K  K  S  R  S  Y  K
>.....cotG-ureA.....>
>.....cotG.....>

307 aaa tct tac cgt tct tac aaa aaa tct cgt agc tat aaa aaa tct tgc cgt
   K  S  Y  R  S  Y  K  K  S  R  S  Y  K  K  S  C  R
>.....cotG-ureA.....>
>.....cotG.....>

358 tct tac aaa aaa tct cgc agc tac aaa aag tct tac tgt tct cac aag aaa
   S  Y  K  K  S  R  S  Y  K  K  S  Y  C  S  H  K  K
>.....cotG-ureA.....>
>.....cotG.....>

409 aaa tct cgc agc tat aag aag tca tgc cgc aca cac aaa aaa tct tat cgt
   K  S  R  S  Y  K  K  S  C  R  T  H  K  K  S  Y  R
>.....cotG-ureA.....>
>.....cotG.....>

460 tcc cat aag aaa tac tac aaa aaa ccg cac cac cac tgc gac gac tac aaa
   S  H  K  K  Y  Y  K  K  P  H  H  H  C  D  D  Y  K
>.....cotG-ureA.....>
>.....cotG.....>

511 aga cac gat gat tat gac agc aaa aaa gaa tac tgg aaa gac ggc aat tgc
   R  H  D  D  Y  D  S  K  K  E  Y  W  K  D  G  N  C
>.....cotG-ureA.....>
>.....cotG.....>

```

562 tgg gta gtc aaa aag aaa tac aaa gga tcc atg aaa ctc acc cca aaa gag
W V V K K K Y K G S M K L T P K E
>.....cotG-ureA.....>
>.....cotG.....>>
>>.....ureA.....>

613 tta gac aag ttg atg ctc cac tat gct gga gaa ttg gct aaa aaa cgc aaa
L D K L M L H Y A G E L A K K R K
>.....cotG-ureA.....>
>.....ureA.....>

664 gaa aaa ggc att aag ctt aac tat gta gaa gcg gta gct ttg att agt gcc
E K G I K L N Y V E A V A L I S A
>.....cotG-ureA.....>
>.....ureA.....>

715 cat att atg gaa gaa gcg aga gct ggt aaa aag act gcg gct gaa ttg atg
H I M E E A R A G K K T A A E L M
>.....cotG-ureA.....>
>.....ureA.....>

766 caa gaa ggg cgc act ctt tta aaa ccg gat gat gtg atg gat ggc gtg gca
Q E G R T L L K P D D V M D G V A
>.....cotG-ureA.....>
>.....ureA.....>

817 agc atg atc cat gaa gtg ggt att gaa gcg atg ttt cct gat ggg aca aaa
S M I H E V G I E A M F P D G T K
>.....cotG-ureA.....>
>.....ureA.....>

868 ctc gta acc gtg cat acc cct att gag gcc aat ggt aaa tta gtt cct ggt
L V T V H T P I E A N G K L V P G
>.....cotG-ureA.....>
>.....ureA.....>

919 gag ttg ttc tta aaa aat gaa gac atc act atc aac gaa ggc aaa aaa gcc
E L F L K N E D I T I N E G K K A
>.....cotG-ureA.....>
>.....ureA.....>

970 gtt agc gtg aaa gtt aaa aat gtt ggc gac aga ccg gtt caa atc ggc tca
V S V K V K N V G D R P V Q I G S
>.....cotG-ureA.....>
>.....ureA.....>

1021 cac ttc cat ttc ttt gaa gtg aat aga tgc tta gac ttt gac aga gaa aaa
H F H F F E V N R C L D F D R E K
>.....cotG-ureA.....>
>.....ureA.....>

1072 act ttc ggt aaa cgc tta gac att gcg agc ggg aca gcg gta agg ttt gag
T F G K R L D I A S G T A V R F E
>.....cotG-ureA.....>
>.....ureA.....>

1123 cct ggc gaa gaa aaa tcc gta gaa ttg att gac att ggc ggt aac aga aga
P G E E K S V E L I D I G G N R R
>.....cotG-ureA.....>
>.....ureA.....>

```
1174  atc ttt gga ttt aac gcg ttg gtt gat agg caa gca gac aac gaa agc aaa
      I  F  G  F  N  A  L  V  D  R  Q  A  D  N  E  S  K
      >.....cotG-ureA.....>
      >.....ureA.....>

1225  aaa att gct tta cac aga gct aaa gag cgt ggt ttt cat ggc gct aaa agc
      K  I  A  L  H  R  A  K  E  R  G  F  H  G  A  K  S
      >.....cotG-ureA.....>
      >.....ureA.....>

1276  gat gac aac tat gta aaa aca att aag gag taa
      D  D  N  Y  V  K  T  I  K  E  -
      >.....cotG-ureA.....>>
      >.....ureA.....>>
```

Fig.8

CotZ-UreA

```

1  atg agc cag aaa aca tca agc tgc gtg cgt gaa gct gta gaa aat att gaa
   M  S  Q  K  T  S  S  C  V  R  E  A  V  E  N  I  E
   >>.....cotZ.....>
   >>.....cotZ-ureA.....>

52  gat ctg caa aac gct gtt gaa gaa gac tgc ccg acc ggc tgc cac tct aag
   D  L  Q  N  A  V  E  E  D  C  P  T  G  C  H  S  K
   >.....cotZ.....>
   >.....cotZ-ureA.....>

103  ctt tta tct gta agc cat tcg tta ggc gac aca gtg cct ttt gca ata ttt
   L  L  S  V  S  H  S  L  G  D  T  V  P  F  A  I  F
   >.....cotZ.....>
   >.....cotZ-ureA.....>

154  aca tca aaa tca acg cca tta gtc gcc ttc gga aat gtc ggc gaa ctc gat
   T  S  K  S  T  P  L  V  A  F  G  N  V  G  E  L  D
   >.....cotZ.....>
   >.....cotZ-ureA.....>

205  aac ggc cct tgc ttt aat aca gta ttt ttc agg gtc gaa aga gtg cat gga
   N  G  P  C  F  N  T  V  F  F  R  V  E  R  V  H  G
   >.....cotZ.....>
   >.....cotZ-ureA.....>

256  agc tgt gca aca ctg tca tta tta atc gca ttt gac gaa cac aaa cac att
   S  C  A  T  L  S  L  L  I  A  F  D  E  H  K  H  I
   >.....cotZ.....>
   >.....cotZ-ureA.....>

307  ttg gac ttc acc gat aaa gat acg gtg tgt gaa gtg ttc cga ctc gaa aaa
   L  D  F  T  D  K  D  T  V  C  E  V  F  R  L  E  K
   >.....cotZ.....>
   >.....cotZ-ureA.....>

358  acg aac tac tgt att gaa gtt gac tta gac tgc ttc tgc gca atc aac tgc
   T  N  Y  C  I  E  V  D  L  D  C  F  C  A  I  N  C
   >.....cotZ.....>
   >.....cotZ-ureA.....>

409  tta aat cct cga tta atc aat cgt aca cat cat cat gga tcc atg aaa ctc
   L  N  P  R  L  I  N  R  T  H  H  H  G  S  M  K  L
   >.....cotZ.....>>
   >.....cotZ-ureA.....>
                                     >>.ureA...>

460  acc cca aaa gag tta gac aag ttg atg ctc cac tat gct gga gaa ttg gct
   T  P  K  E  L  D  K  L  M  L  H  Y  A  G  E  L  A
   >.....cotZ-ureA.....>
   >.....ureA.....>

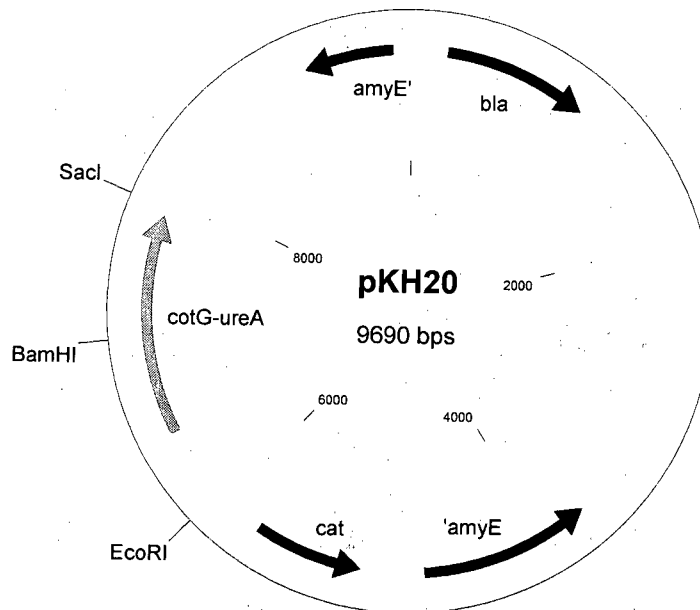
511  aaa aaa cgc aaa gaa aaa ggc att aag ctt aac tat gta gaa gcg gta gct
   K  K  R  K  E  K  G  I  K  L  N  Y  V  E  A  V  A
   >.....cotZ-ureA.....>

```

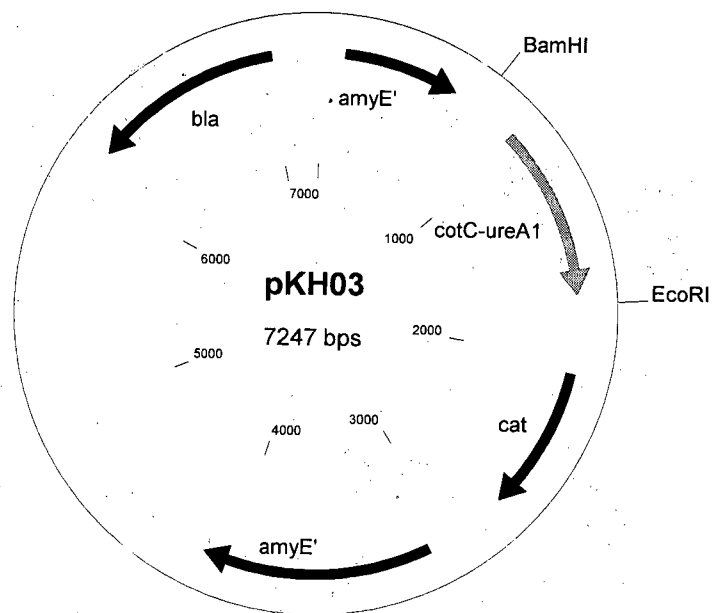
```
>.....ureA.....>
562 ttg att agt gcc cat att atg gaa gaa gcg aga gct ggt aaa aag act gcg
    L I S A H I M E E A R A G K K T A
>.....cotZ-ureA.....>
>.....ureA.....>
613 gct gaa ttg atg caa gaa ggg cgc act ctt tta aaa ccg gat gat gtg atg
    A E L M Q E G R T L L K P D D V M
>.....cotZ-ureA.....>
>.....ureA.....>
664 gat ggc gtg gca agc atg atc cat gaa gtg ggt att gaa gcg atg ttt cct
    D G V A S M I H E V G I E A M F P
>.....cotZ-ureA.....>
>.....ureA.....>
715 gat ggg aca aaa ctc gta acc gtg cat acc cct att gag gcc aat ggt aaa
    D G T K L V T V H T P I E A N G K
>.....cotZ-ureA.....>
>.....ureA.....>
766 tta gtt cct ggt gag ttg ttc tta aaa aat gaa gac atc act atc aac gaa
    L V P G E L F L K N E D I T I N E
>.....cotZ-ureA.....>
>.....ureA.....>
817 ggc aaa aaa gcc gtt agc gtg aaa gtt aaa aat gtt ggc gac aga ccg gtt
    G K K A V S V K V K N V G D R P V
>.....cotZ-ureA.....>
>.....ureA.....>
868 caa atc ggc tca cac ttc cat ttc ttt gaa gtg aat aga tgc tta gac ttt
    Q I G S H F H F F E V N R C L D F
>.....cotZ-ureA.....>
>.....ureA.....>
919 gac aga gaa aaa act ttc ggt aaa cgc tta gac att gcg agc ggg aca gcg
    D R E K T F G K R L D I A S G T A
>.....cotZ-ureA.....>
>.....ureA.....>
970 gta agg ttt gag cct ggc gaa gaa aaa tcc gta gaa ttg att gac att ggc
    V R F E P G E E K S V E L I D I G
>.....cotZ-ureA.....>
>.....ureA.....>
1021 ggt aac aga aga atc ttt gga ttt aac gcg ttg gtt gat agg caa gca gac
    G N R R I F G F N A L V D R Q A D
>.....cotZ-ureA.....>
>.....ureA.....>
1072 aac gaa agc aaa aaa att gct tta cac aga gct aaa gag cgt ggt ttt cat
    N E S K K I A L H R A K E R G F H
>.....cotZ-ureA.....>
>.....ureA.....>
1123 ggc gct aaa agc gat gac aac tat gta aaa aca att aag gag taa
    G A K S D D N Y V K T I K E -
>.....cotZ-ureA.....>>
>.....ureA.....>>
```

Fig.9

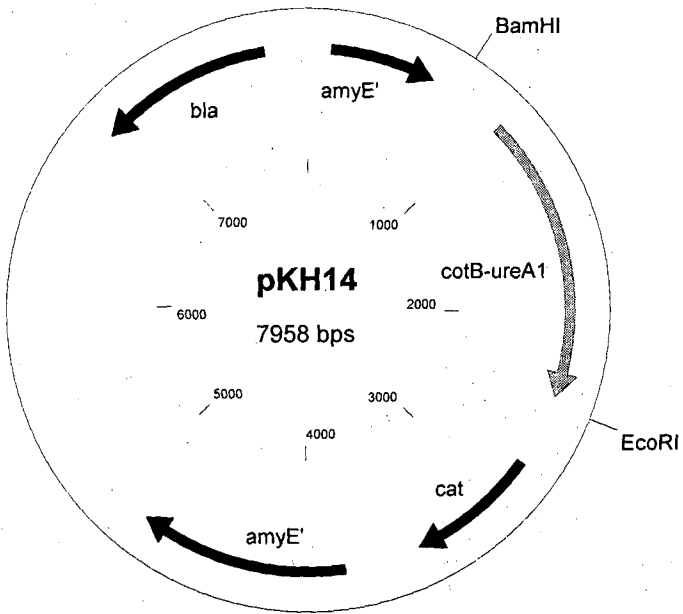
A



B



C



D

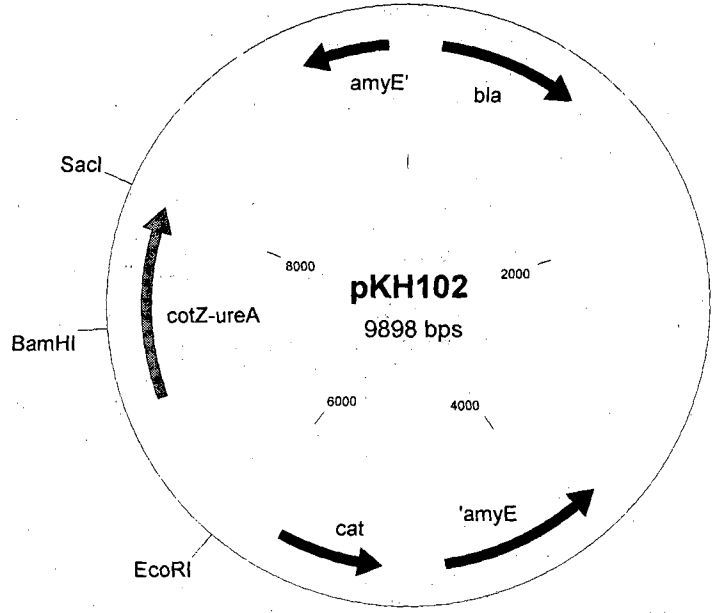


Fig.10

```

1  cga tct gca tga cca tta tga cta gta aaa act ttt tca aaa aag tat tga
                                     rrn0 P2 >>...>

52  cct agt taa cta aaa atg tta cta tta agt agt cgc ttt gag aga agc aca
>.....rrn0 P2.....>>

103  caa agt tct ttg aaa act aaa caa gac aaa acg tac ctg tta att cat ttt

154  tat aaa tcg cac agc gat gtg cgt agt cag tca aac tag ggc ctg cac gac

205  gca ggt cac aca ggt gtc gcc gca gga tgc ggt gaa ctt aac ctg ttc tag

256  aac aag gag gtg aga ccc atg aag ctt aac tat gta gaa gcg gta gct ttg
      M  K  L  N  Y  V  E  A  V  A  L
      >>.....ureA.....>
      >>RBS sspA.>>

307  att agt gcc cat att atg gaa gaa gcg aga gct ggt aaa aag act gcg gct
      I  S  A  H  I  M  E  E  A  R  A  G  K  K  T  A  A
      >.....ureA.....>

358  gaa ttg atg caa gaa ggg cgc act ctt tta aaa ccg gat gat gtg atg gat
      E  L  M  Q  E  G  R  T  L  L  K  P  D  D  V  M  D
      >.....ureA.....>

409  ggc gtg gca agc atg atc cat gaa gtg ggt att gaa gcg atg ttt cct gat
      G  V  A  S  M  I  H  E  V  G  I  E  A  M  F  P  D
      >.....ureA.....>

460  ggg aca aaa ctc gta acc gtg cat acc cct att gag gcc aat ggt aaa tta
      G  T  K  L  V  T  V  H  T  P  I  E  A  N  G  K  L
      >.....ureA.....>

511  gtt cct ggt gag ttg ttc tta aaa aat gaa gac atc act atc aac gaa ggc
      V  P  G  E  L  F  L  K  N  E  D  I  T  I  N  E  G
      >.....ureA.....>

562  aaa aaa gcc gtt agc gtg aaa gtt aaa aat gtt ggc gac aga ccg gtt caa
      K  K  A  V  S  V  K  V  K  N  V  G  D  R  P  V  Q
      >.....ureA.....>

613  atc ggc tca cac ttc cat ttc ttt gaa gtg aat aga tgc tta gac ttt gac
      I  G  S  H  F  H  F  F  E  V  N  R  C  L  D  F  D
      >.....ureA.....>

664  aga gaa aaa act ttc ggt aaa cgc tta gac att gcg agc ggg aca gcg gta
      R  E  K  T  F  G  K  R  L  D  I  A  S  G  T  A  V
      >.....ureA.....>

715  agg ttt gag cct ggc gaa gaa aaa tcc gta gaa ttg att gac att ggc ggt
      R  F  E  P  G  E  E  K  S  V  E  L  I  D  I  G  G
      >.....ureA.....>

766  aac aga aga atc ttt gga ttt aac gcg ttg gtt gat agg caa gca gac aac
      N  R  R  I  F  G  F  N  A  L  V  D  R  Q  A  D  N

```

```

>.....ureA.....>
817 gaa agc aaa aaa att gct tta cac aga gct aaa gag cgt ggt ttt cat ggc
    E  S  K  K  I  A  L  H  R  A  K  E  R  G  F  H  G
>.....ureA.....>
868 gct aaa agc gat gac aac tat gta aaa aca att aag gag taa gaa atg aac
    A  K  S  D  D  N  Y  V  K  T  I  K  E  -
>.....ureA.....>>
919 atg

```

Fig.11

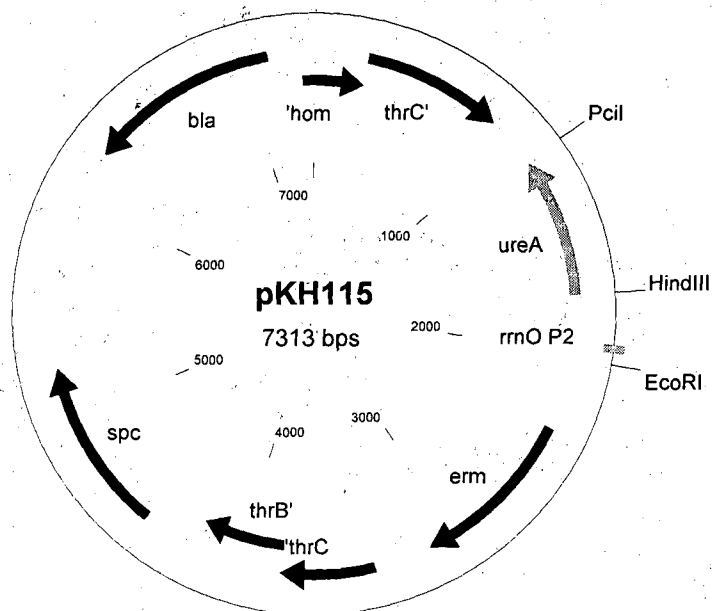


Fig.12

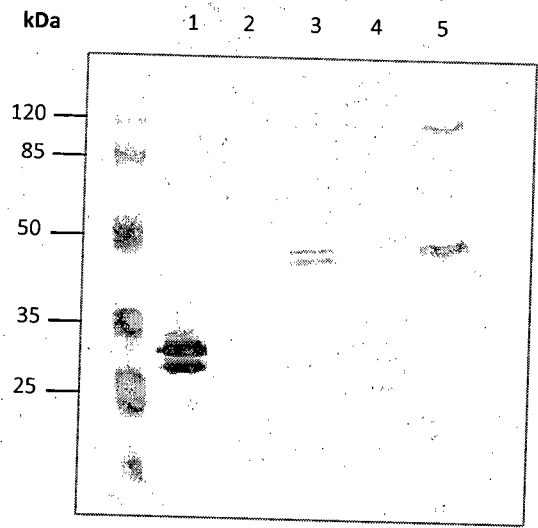


Fig.13

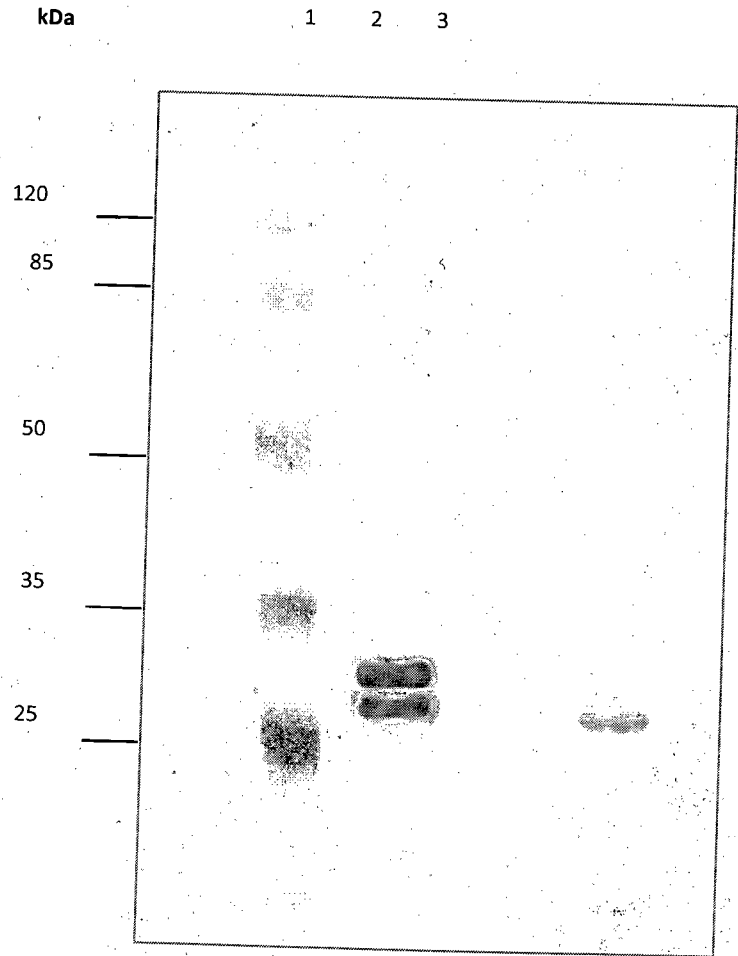


Fig.14

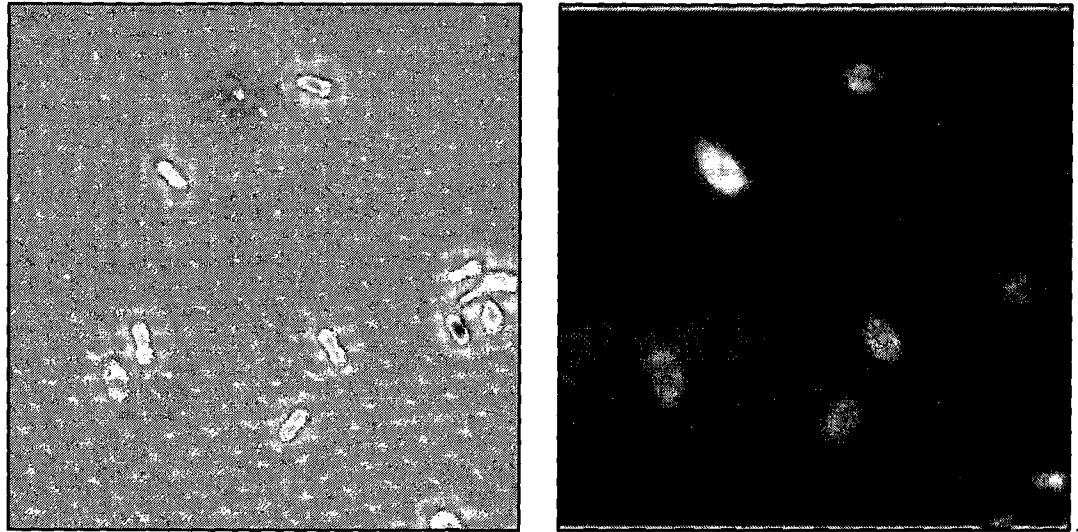


Fig.15

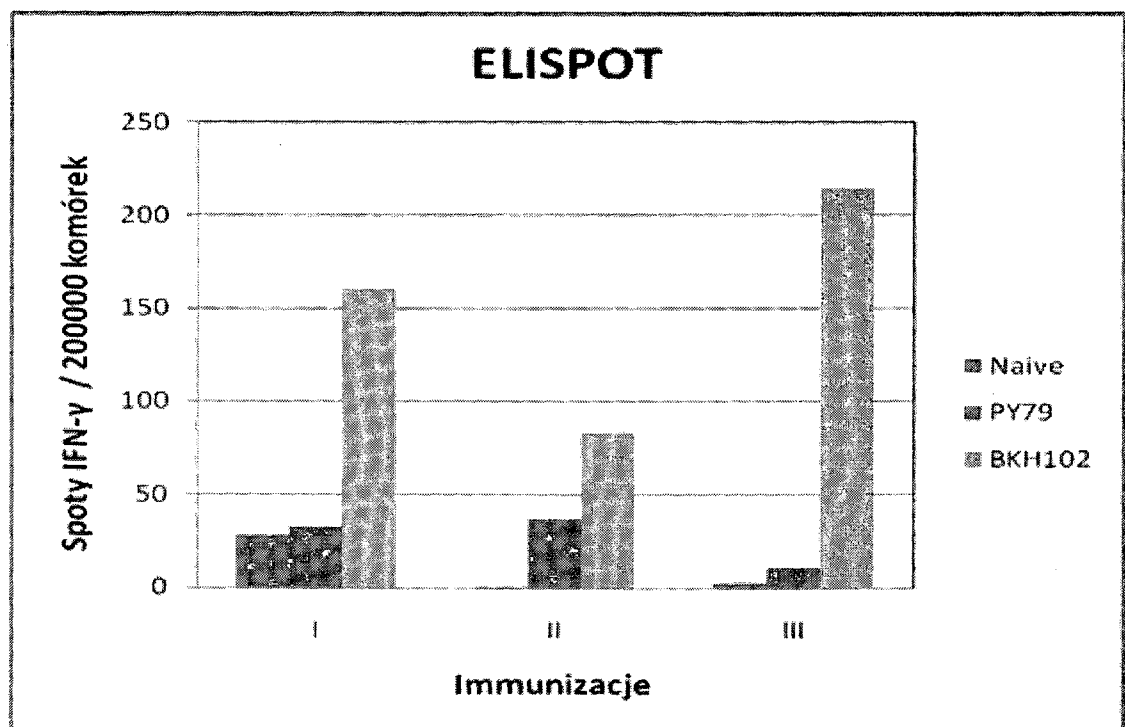


Fig.16

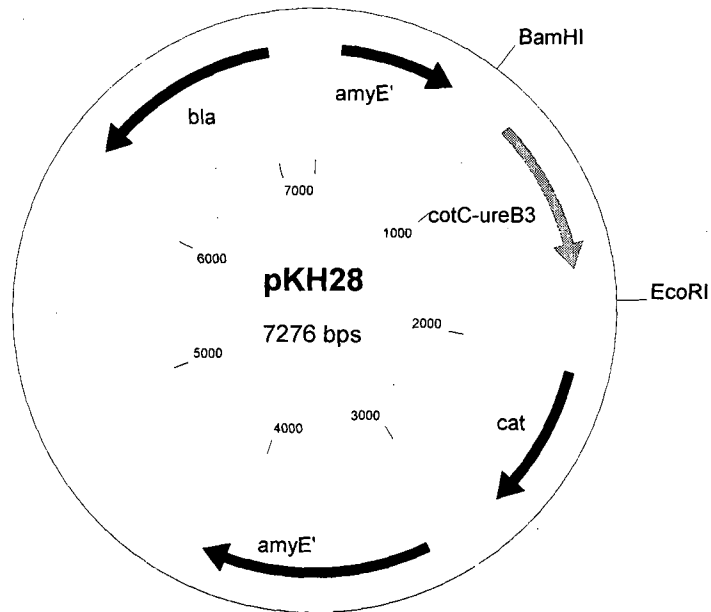


Fig.17

```

1  atg ggt tat tac aaa aaa tac aaa gaa gag tat tat acg gtc aaa aaa acg
   M  G  Y  Y  K  K  Y  K  E  E  Y  Y  T  V  K  K  T
>>.....cotC-ureB3.....>
>>.....cotC.....>

52  tat tat aag aag tat tac gaa tat gat aaa aaa gat tat gac tgt gat tac
   Y  Y  K  K  Y  Y  E  Y  D  K  K  D  Y  D  C  D  Y
>.....cotC-ureB3.....>
>.....cotC.....>

103  gac aaa aaa tat gat gac tat gat aaa aaa tat tat gat cac gat aaa aaa
     D  K  K  Y  D  D  Y  D  K  K  Y  Y  D  H  D  K  K
>.....cotC-ureB3.....>
>.....cotC.....>

154  gac tat gat tat gtt gta gag tat aaa aag cat aaa aaa cac tac gat atc
     D  Y  D  Y  V  V  E  Y  K  K  H  K  K  H  Y  D  I
>.....cotC-ureB3.....>
>.....cotC.....>

205  aaa tac acc att aac cca gcg atc gct cat ggg att agc gag tat gta ggt
     K  Y  T  I  N  P  A  I  A  H  G  I  S  E  Y  V  G
>.....cotC-ureB3.....>
>>.....ureB3.....>

```

256 tct gta gaa gtg ggc aaa gtg gct gac ttg gta ttg tgg agt ccc gca ttc
S V E V G K V A D L V L W S P A F
>.....cotC-ureB3.....>
>.....ureB3.....>

307 ttt ggc gta aaa ccc aac atg atc atc aaa ggc ggg ttc att gcg ttg agt
F G V K P N M I I K G G F I A L S
>.....cotC-ureB3.....>
>.....ureB3.....>

358 caa atg ggt gac gcg aac gct tct atc cct acc cca caa cca gtt tat tac
Q M G D A N A S I P T P Q P V Y Y
>.....cotC-ureB3.....>
>.....ureB3.....>

409 aga gaa atg ttc gct cat cat ggt aaa gcc aaa tac gat gca aac atc act
R E M F A H H G K A K Y D A N I T
>.....cotC-ureB3.....>
>.....ureB3.....>

460 ttt gtg tct caa gcg gct tat gac aaa ggc att aaa gaa gaa tta ggg ctt
F V S Q A A Y D K G I K E E L G L
>.....cotC-ureB3.....>
>.....ureB3.....>

511 gaa aga caa gtg ttg ccg gta aaa aat tgc aga aac atc act aaa aaa gac
E R Q V L P V K N C R N I T K K D
>.....cotC-ureB3.....>
>.....ureB3.....>

562 atg caa ttc aac gac act acc gct cac att gaa gtc aat cct gaa act tac
M Q F N D T T A H I E V N P E T Y
>.....cotC-ureB3.....>
>.....ureB3.....>

613 cat gtg ttc gtg gat ggc aaa gaa gta act tct aaa cca gcc aat aaa gtg
H V F V D G K E V T S K P A N K V
>.....cotC-ureB3.....>
>.....ureB3.....>

664 agc ttg gcg caa ctc ttt agc att ttc tag
S L A Q L F S I F -
>.....cotC-ureB3.....>>
>.....ureB3.....>>

Fig.18

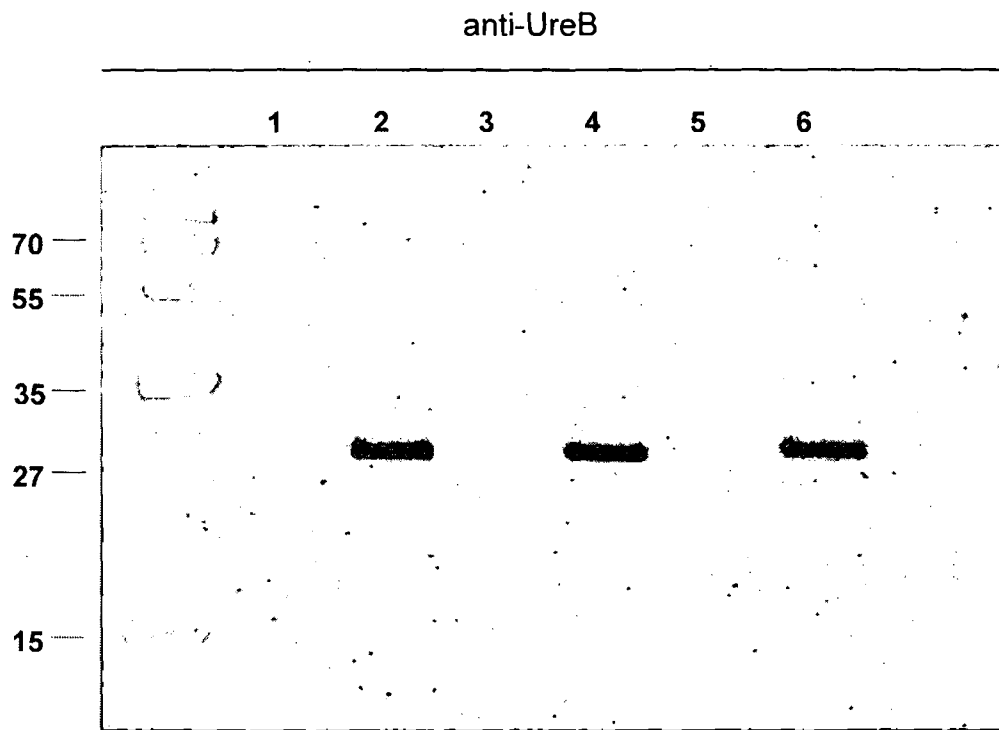


Fig.19

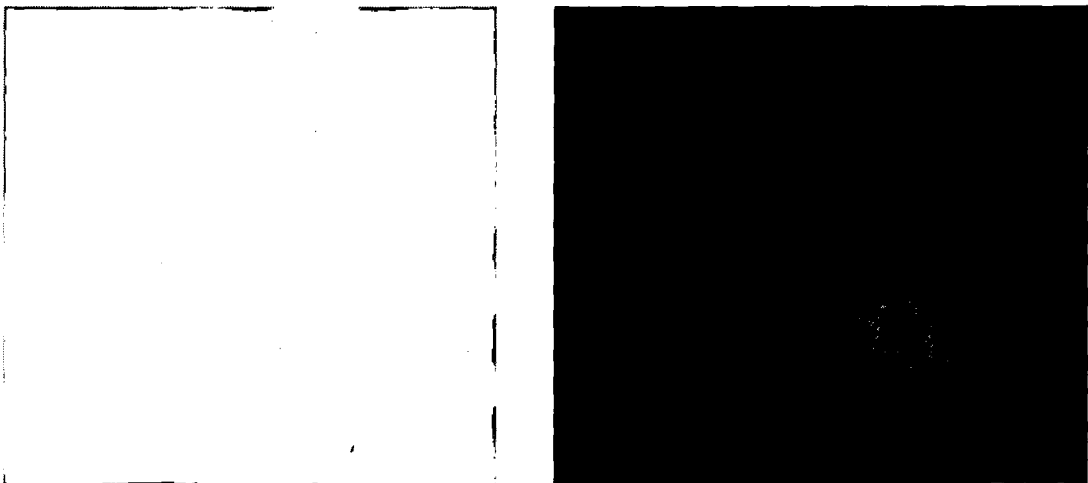


Fig.20

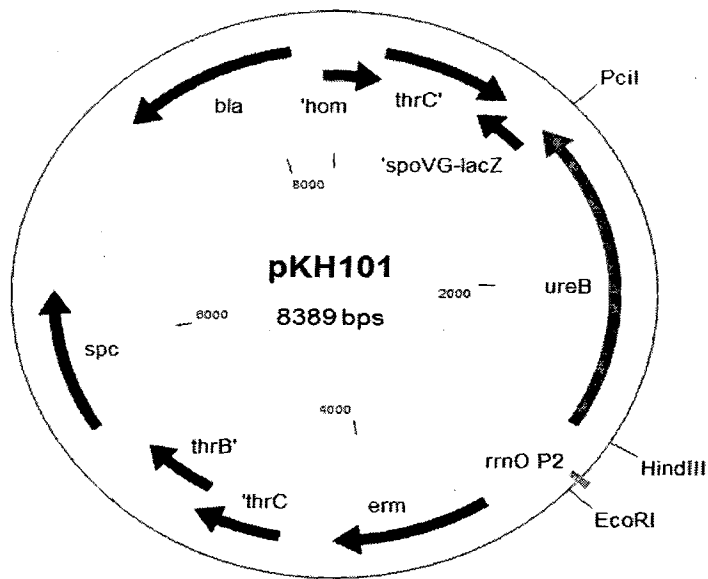


Fig.21

```

1  tta tga cta gta aaa act ttt tca aaa aag tat tga cct agt taa cta aaa
   >>.....rrnO P2.....>

52 atg tta cta tta agt agt cgc ttt gag aga agc aca caa agt tct ttg aaa
   >...rrnO P2.>>

103 act aaa caa gac aaa acg tac ctg tta att cat ttt tat aaa tcg cac agc

154 gat gtg cgt agt cag tca aac tag ggc ctg cac gac gca ggt cac aca ggt

205 gtc gcc gca gga tgc ggt gaa ctt aac ctg ttc tag aac aag gag gtg aga
   >>sspA RBS.>>

256 ccc atg aag ctt aaa aag att agc aga aaa gaa tat gtt tct atg tat ggc
    M  K  L  K  K  I  S  R  K  E  Y  V  S  M  Y  G
    >>.....ureB.....>

307 cct act aca ggc gat aaa gtg aga ttg ggc gat aca gac ttg atc gct gaa
    P  T  T  G  D  K  V  R  L  G  D  T  D  L  I  A  E
    >.....ureB.....>

358 gta gaa cat gac tac acc att tat ggc gaa gag ctt aaa ttc ggt ggc ggt
    V  E  H  D  Y  T  I  Y  G  E  E  L  K  F  G  G  G
    >.....ureB.....>

409 aaa acc ctg aga gaa ggc atg agc caa tcc aac aac cct agc aaa gaa gaa
    K  T  L  R  E  G  M  S  Q  S  N  N  P  S  K  E  E
    >.....ureB.....>

460 ttg gat cta atc atc act aac gct tta atc gtg gat tac acc ggt att tat
    L  D  L  I  I  T  N  A  L  I  V  D  Y  T  G  I  Y
  
```

>.....ureB.....>

511 aaa gcg gat att ggt att aaa gat ggc aaa atc gct ggc att ggt aaa ggc
K A D I G I K D G K I A G I G K G
>.....ureB.....>

562 ggt aac aaa gac atg caa gat ggc gtt aaa aac aat ctt agc gta ggt cct
G N K D M Q D G V K N N L S V G P
>.....ureB.....>

613 gct act gaa gcc tta gcc ggt gaa ggt ttg atc gta act gct ggt ggt att
A T E A L A G E G L I V T A G G I
>.....ureB.....>

664 gac aca cac atc cac ttc att tca ccc caa caa atc cct aca gct ttt gca
D T H I H F I S P Q Q I P T A F A
>.....ureB.....>

715 agc ggt gta aca acc atg att ggt ggc gga act ggt cct gct gat ggc act
S G V T T M I G G G T G P A D G T
>.....ureB.....>

766 aat gcg act act atc act cca ggc aga aga aat tta aaa tgg atg ctc aga
N A T T I T P G R R N L K W M L R
>.....ureB.....>

817 gcg gct gaa gaa tat tct atg aac tta ggt ttc ttg gct aaa ggt aac gct
A A E E Y S M N L G F L A K G N A
>.....ureB.....>

868 tot aac gac gcg agc tta gcc gat caa att gaa gct ggt gcg att ggc ttt
S N D A S L A D Q I E A G A I G F
>.....ureB.....>

919 aaa atc cac gaa gac tgg ggc acc act cct tct gca atc aat cat gcg tta
K I H E D W G T T P S A I N H A L
>.....ureB.....>

970 gat gtt gca gac aaa tac gat gtg caa gtc gct atc cac aca gac act ttg
D V A D K Y D V Q V A I H T D T L
>.....ureB.....>

1021 aat gaa gcc ggt tgc gtg gaa gac act atg gca gct att gcc gga cgc act
N E A G C V E D T M A A I A G R T
>.....ureB.....>

1072 atg cac act ttc cac act gaa ggt gct ggc ggc gga cac gct cct gat att
M H T F H T E G A G G G H A P D I
>.....ureB.....>

1123 att aaa gta gct ggt gaa cac aac att ctt ccc gct tcc act aac ccc act
I K V A G E H N I L P A S T N P T
>.....ureB.....>

1174 atc cct ttc act gtg aat aca gaa gca gaa cac atg gac atg ctt atg gtg
I P F T V N T E A E H M D M L M V
>.....ureB.....>

1225 tgc cac cac ttg gat aaa agc att aaa gaa gat gtt cag ttc gct gat tca
C H H L D K S I K E D V Q F A D S
>.....ureB.....>

1276 agg atc cgc cct caa acc att gcg gct gaa gac act ttg cat gac atg ggg
R I R P Q T I A A E D T L H D M G
>.....ureB.....>

1327 att ttc tca atc acc agc tct gac tct caa gct atg ggt cgt gtg ggt gaa
I F S I T S S D S Q A M G R V G E
>.....ureB.....>

1378 gtt atc act aga act tgg caa aca gct gac aaa aac aaa aaa gaa ttt ggc
V I T R T W Q T A D K N K K E F G
>.....ureB.....>

1429 cgc ttg aaa gaa gaa aaa ggc gat aac gac aac ttc agg atc aaa cgc tac
R L K E E K G D N D N F R I K R Y
>.....ureB.....>

1480 ttg tct aaa tac acc att aac cca gcg atc gct cat ggg att agc gag tat
L S K Y T I N P A I A H G I S E Y
>.....ureB.....>

1531 gta ggt tct gta gaa gtg ggc aaa gtg gct gac ttg gta ttg tgg agt ccc
V G S V E V G K V A D L V L W S P
>.....ureB.....>

1582 gca ttc ttt ggc gta aaa ccc aac atg atc atc aaa ggc ggg ttc att gcg
A F F G V K P N M I I K G G F I A
>.....ureB.....>

1633 ttg agt caa atg ggt gac gcg aac gct tct atc cct acc cca caa cca gtt
L S Q M G D A N A S I P T P Q P V
>.....ureB.....>

1684 tat tac aga gaa atg ttc gct cat cat ggt aaa gcc aaa tac gat gca aac
Y Y R E M F A H H G K A K Y D A N
>.....ureB.....>

1735 atc act ttt gtg tct caa gcg gct tat gac aaa ggc att aaa gaa gaa tta
I T F V S Q A A Y D K G I K E E L
>.....ureB.....>

1786 ggg ctt gaa aga caa gtg ttg ccg gta aaa aat tgc aga aac atc act aaa
G L E R Q V L P V K N C R N I T K
>.....ureB.....>

1837 aaa gac atg caa ttc aac gac act acc gct cac att gaa gtc aat cct gaa
K D M Q F N D T T A H I E V N P E
>.....ureB.....>

1888 act tac cat gtg ttc gtg gat ggc aaa gaa gta act tct aaa cca gcc aat
T Y H V F V D G K E V T S K P A N
>.....ureB.....>

1939 aaa gtg agc ttg gcg caa ctc ttt agc att ttc tag
K V S L A Q L F S I F -
>.....ureB.....>>

Fig.22

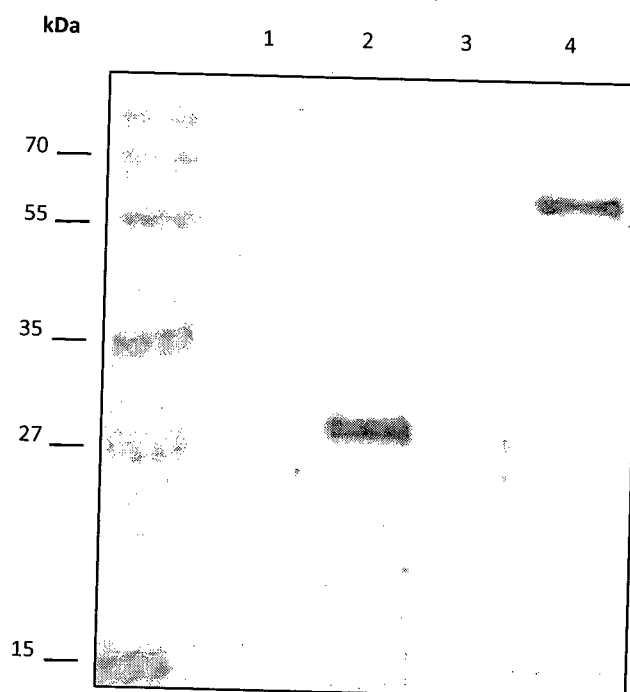


Fig.23

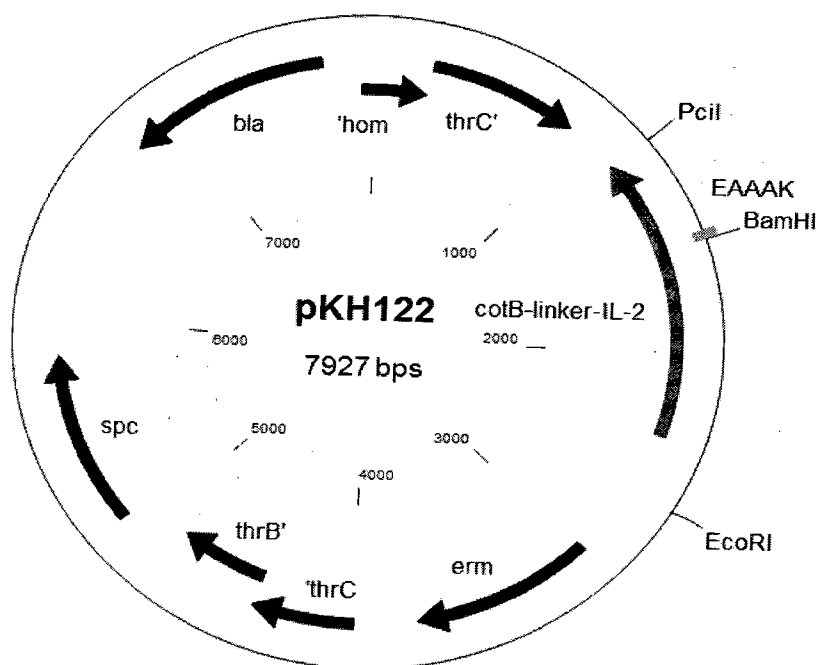


Fig.24

CotB-linker-IL2

```

1  atg agc aag agg aga atg aaa tat cat tca aat aat gaa ata tcg tat tat
   M  S  K  R  R  M  K  Y  H  S  N  N  E  I  S  Y  Y
   >>.....cotB'.....>

52  aac ttt ttg cac tca atg aaa gat aaa att gtt act gta tat cgt gga ggt
   N  F  L  H  S  M  K  D  K  I  V  T  V  Y  R  G  G
   >.....cotB'.....>

103  ccg gaa tct aaa aaa gga aaa tta aca gct gta aaa tca gat tat ata gct
   P  E  S  K  K  G  K  L  T  A  V  K  S  D  Y  I  A
   >.....cotB'.....>

154  tta caa gct gaa aaa aaa ata att tat tat cag ttg gag cat gtg aaa agt
   L  Q  A  E  K  K  I  I  Y  Y  Q  L  E  H  V  K  S
   >.....cotB'.....>

205  att act gag gat acc aat aat agc acc aca aca att gag act gag gaa atg
   I  T  E  D  T  N  N  S  T  T  T  I  E  T  E  E  M
   >.....cotB'.....>

256  ctc gat gct gat gat ttt cat agc tta atc gga cat tta ata aac caa tca
   L  D  A  D  D  F  H  S  L  I  G  H  L  I  N  Q  S
   >.....cotB'.....>

307  gtt caa ttt aac caa ggg ggt ccg gaa tct aaa aaa gga aga ttg gtc tgg
   V  Q  F  N  Q  G  G  P  E  S  K  K  G  R  L  V  W
   >.....cotB'.....>

358  ctg gga gat gat tac gct gcg tta aac aca aat gag gat ggg gta gtg tat
   L  G  D  D  Y  A  A  L  N  T  N  E  D  G  V  V  Y
   >.....cotB'.....>

409  ttt aat atc cat cac atc aaa agt ata agt aaa cac gag cct gat ttg aaa
   F  N  I  H  H  I  K  S  I  S  K  H  E  P  D  L  K
   >.....cotB'.....>

460  ata gaa gag cag acg cca gtt gga gtt ttg gaa gct gat gat tta agc gag
   I  E  E  Q  T  P  V  G  V  L  E  A  D  D  L  S  E
   >.....cotB'.....>

511  gtt ttt aag agt ctg act cat aaa tgg gtt tca att aat cgt gga ggt ccg
   V  F  K  S  L  T  H  K  W  V  S  I  N  R  G  G  P
   >.....cotB'.....>

562  gaa gcc att gag ggt atc ctt gta gat aat gcc gac ggc cat tat act ata
   E  A  I  E  G  I  L  V  D  N  A  D  G  H  Y  T  I
   >.....cotB'.....>

613  gtg aaa aat caa gag gtg ctt cgc atc tat cct ttt cac ata aaa agc atc
   V  K  N  Q  E  V  L  R  I  Y  P  F  H  I  K  S  I
   >.....cotB'.....>

```

664 agc tta ggt cca aaa ggg tcg tac aaa aaa gag gat caa aaa aat gaa caa
S L G P K G S Y K K E D Q K N E Q
>.....cotB'.....>

715 aac cag gaa gac aat aat gat aag gac agc aat tcg ttc att tct tca aaa
N Q E D N N D K D S N S F I S S K
>.....cotB'.....>

766 tca tat agc tca tca aaa tca tct aaa cga tca cta aaa tct tca gat gat
S Y S S S K S S K R S L K S S D D
>.....cotB'.....>

817 caa tca tcc gga tcc ggt gga gga gaa gca gca gcg aaa ggt gga gga gcg
Q S S G S G G G E A A A K G G G A
>.....cotB'.....>>
>>.....GGGEAAAKGGG.....>>
IL-2 >>>

868 ccg acg agc agc agc acg aaa aaa aca cag tta cag ttg gaa cat tta ctg
P T S S S T K K T Q L Q L E H L L
>.....IL-2.....>

919 ctt gat ttg caa atg atc ctg aac ggc atc aac aac tac aaa aac ccg aaa
L D L Q M I L N G I N N Y K N P K
>.....IL-2.....>

970 ctt aca cgt atg ttg acg ttt aaa ttt tac atg cct aaa aaa gct aca gaa
L T R M L T F K F Y M P K K A T E
>.....IL-2.....>

1021 ttg aaa cat ttg caa tgc ctg gaa gaa gaa ctt aaa ccg ttg gaa gaa gtc
L K H L Q C L E E E L K P L E E V
>.....IL-2.....>

1072 ttg aac ttg gca cag tca aaa aac ttt cat ttg aga cct cgc gat ctt att
L N L A Q S K N F H L R P R D L I
>.....IL-2.....>

1123 agc aat atc aac gtt att gtg ctg gaa ctt aaa gga tca gaa aca acg ttt
S N I N V I V L E L K G S E T T F
>.....IL-2.....>

1174 atg tgt gaa tat gca gac gaa aca gcg acg atc gta gaa ttt ctg aat aga
M C E Y A D E T A T I V E F L N R
>.....IL-2.....>

1225 tgg att acg ttt tgc caa tct atc atc tct aca ctg acg taa
W I T F C Q S I I S T L T -
>.....IL-2.....>>

Fig.25

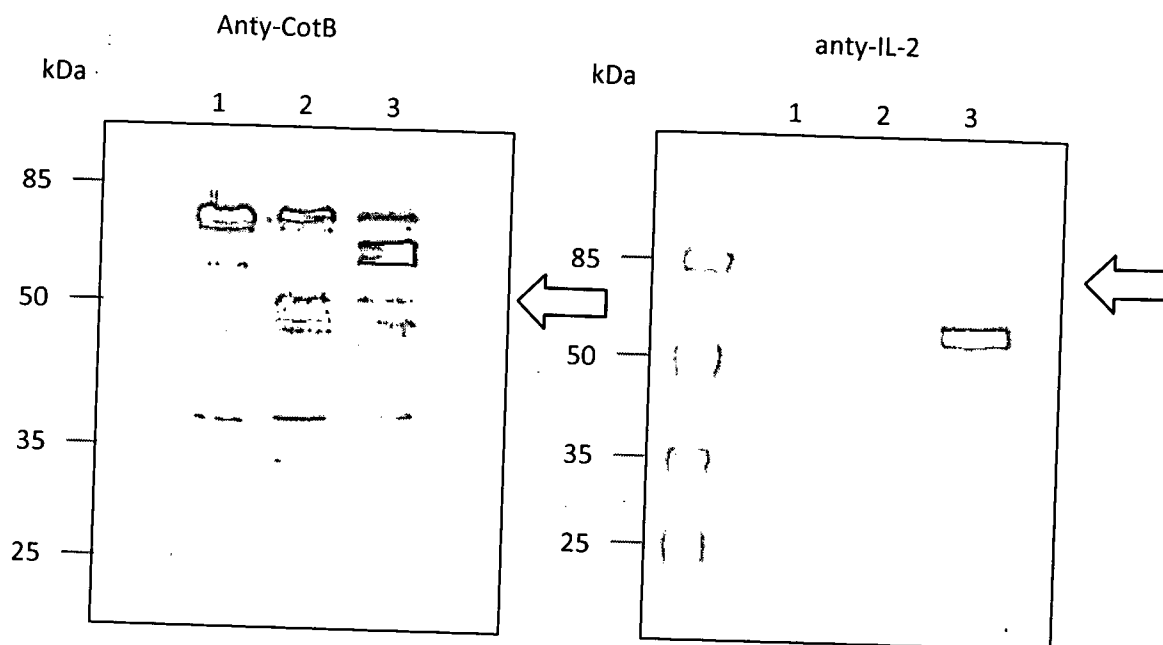


Fig.26

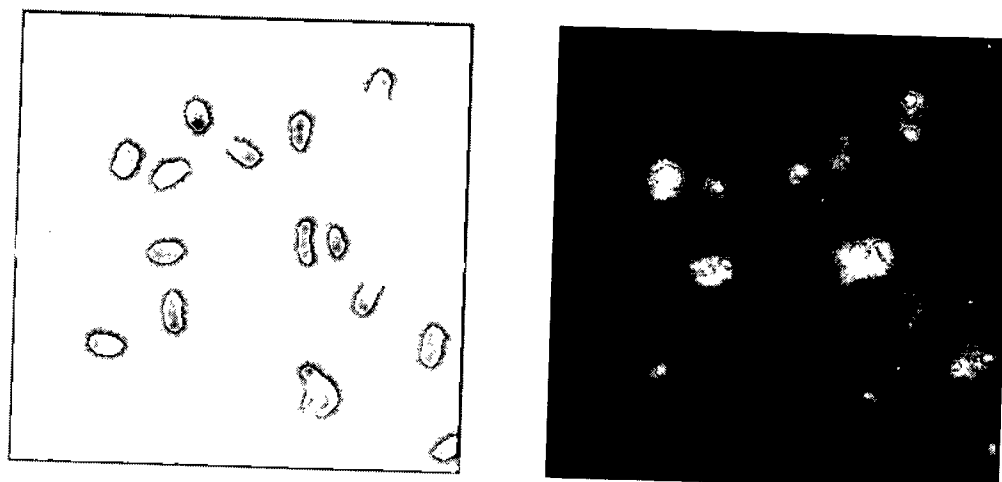


Fig.27

