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DATE OF RECEIPT-

WO 93/11242 PCT/BE92/00052

METHOD FOR SELECTING RECOMBINANT MICROORGANISMS CONTAINING AT THEIR SURFACE AT LEAST ONE MOLECULE HAVING ENZYMATIC ACTIVITY

Subject of the invention

The invention relates to a method for selecting recombinant microorganisms containing at their surface at least one molecule having enzymatic activity.

The invention also extends to the means for implementing this method and to methods for obtaining them.

Prior state of the art and technological background forming the basis of the invention

The discovery of enzymes having new properties (catalytic activity, new enhanced stability, altered specificity, shifted pH profile and the like) is of great interest for the improvement of biochemical processes.

Although very substantial progress has been achieved in the understanding of the structure-activity relationship for proteins (Atkins et al., Current opinion in structural biology (1991), 1, p. 611 to 623), the prediction of the type of modification to be introduced into a protein in order to alter its properties in a desired direction remains difficult.

Consequently, a strategy is usually adopted in which an existing enzyme, whose gene is mutated, is used as starting material. This mutagenesis is carried out in a random manner, thus creating a vast population of mutated enzymes. Among these, it is hoped that some of them will have the desired new properties (Cunningham et al., Protein Engineering (1987), 1, p. 319 to 325 and Lehtovaara et al. Protein Engineering (1988), 2, p. 263 to 268).

In order to increase the chances of success, it is necessary to test a very large number of mutants and to have available a technique which makes it possible to select, within the largest possible population, the enzymes having the appropriate properties.

Proteins having new enzymatic properties can also be produced by resorting to the monoclonal antibody

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technique. In this case, the selection of the antibodies-enzymes (also called abzymes) having the appropriate properties also necessitates an effective screening technique.

A technique for detection of the mutated enzymes which is frequently used is based on the development of a sensitive test for the enzymatic activity. A search is then directly carried out on a population of mutants plated on a Petri dish for those which produce the desired enzyme. However, this technique is laborious and permits only a limited number of clones to be tested (Pollack et al., J. Am. Chem. Soc., 111, p. 5961-5962 (1989) and Huse et al., Science, 246, p. 1275-1281 (1989)).

An advantageous alternative arises when the desired catalytic activity may be useful for the survival or for the development of the microorganism expressing the corresponding gene. It is sufficient, in this case, to exert a selection pressure on the medium for culturing the population of mutants in order to select the strains which produce the desired enzyme (Hermès et al., PNAS, USA, 87, p. 696-700 (1990) and Evnin et al., PNAS, USA, 87, p. 6659-6663 (1990)).

This technique has the disadvantage of not being applicable to all the molecules having enzymatic activity.

It is also known (Smith, Science (1985), 228, p. 1315-1317, Parmley and Smith, Gene (1985), 76, 305-318, de la Cruz et al., J. Biol. Chem. (1988), 263, p. 4318-4322, Bass et al., Proteins (1990), 8, p. 309 to 314, Cwirla et al., Proc. Natl. Acad. Sci. USA (1990), 87, p. 6378-6382, Devlin et al., Science (1990), 249, 404-406, McCafferty et al., Nature (1990), 348, p. 552-554, Scott and Smith, Science (1990), 249, p. 386-390, Clackson et al., Nature (1991), 352, p. 624-628, Lowman et al., Biochemistry, (1991), 30, p. 10832-10838, J. McCafferty et al., Prot. Engng (1991) 955-961, Kang et al., (1991) Proc. Natl. Acad. Sci. USA, vol. 88, p. 4363-4366; Barbas et al. (1991), Proc. Natl. Acad. Sci. USA, vol. 88,

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p. 7978-7982; Roberts et al., Proc. Natl. Acad. Sci. USA (1992), 89, p. 2429-2433) to be able to expose certain foreign peptide sequences, in particular proteins, at the surface of "filamentous" phages and of phagemids.

However, the in vitro selection of phages which correctly expose active polypeptide sequences at their surface is not resolved either.

Various affinity chromatography techniques have been described. Among the affinity supports developed, several use immobilized streptavidin or avidin, the latter being advantageously in monomeric form (EP-A-0,423,938). Phages with peptide sequences having an affinity for streptavidin have thus been selected with the aid of agarose carrying this protein (Devlin et al., Science (1990), 249, 404-406).

However, these techniques have the disadvantage that the selection, during affinity chromatography, of these phages is carried out on the basis of their ability to form a complex with peptide receptors or specific antibodies which indiscriminately bind active or non-active molecules.

With the aim of detecting specific microorganisms, various other labeling techniques have been developed. For example, certain microorganisms have been detected with the aid of bacteriophages which are specific for these hosts and labeled (WO-A-8,804,326). Transducing particles of known host specificity and containing genes which alter the phenotype can be used for labeling bacteria (WO-A-9,004,041).

The sensitivity to certain toxic substances which is associated with the detection by bioluminescence encoded by a plasmid has also been used to detect specific microorganisms.

However, none of these techniques permits the selection of microorganisms carrying catalytic peptides.

Moreover, covalent inhibitors, used as substrate by certain enzymes, are also known for their capacity to inhibit all enzymatic reactions by binding covalently to the active site of the enzyme.

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Some covalent inhibitors are also called "suicide" inhibitors (ATOR et al., The Enzyme, vol. 19, p. 214-282, edit. Sigman Boyer 3rd edition (1990)) because they bind irreversibly to the active site of the enzyme using the catalytic mechanism of the latter and thus render any reuse of the enzyme impossible.

It is also known that it is possible to immobilize a substrate by first binding it to biotin and by placing the product obtained in the presence of avidin bound to a water-insoluble support (JP-A-3,080,098). This immobilized substrate permits the easy determination of certain enzymatic activities, but cannot serve as chromatographic support to select microorganisms carrying catalytic peptides.

Aims of the invention

The invention aims to develop a method for selecting recombinant microorganisms containing at their surface at least one molecule having enzymatic activity and which does not carry the disadvantages of the prior state of the art.

The invention aims in particular to develop a method which permits the detection and selection of recombinant microorganisms exposing at their surface molecules having enzymatic activity, which are performed on the basis of the catalytic activity of these molecules rather than on the basis of their complexing activity.

Characteristic features of the invention

The invention relates to a method for selecting recombinant microorganisms containing at their surface at least one molecule having enzymatic activity, in which the recombinant microorganisms, preferably viruses such as filamentous phages or λ phages or phagemids, are immobilized on a solid support by a covalent inhibitor of the active site of the molecule having enzymatic activity, connected to the solid support by an arm; the recombinant microorganisms or all or part of their genome are then recovered and all or part of their genome is isolated.

The term "recombinant microorganism" is

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understood to mean any macromolecular structure which satisfies the English definition of "Genetic Display Package" (GDP), that is to say any macromolecular structure incorporating genetic material and containing a peptide sequence encoded by this genetic material.

This definition includes especially yeasts, bacteria, viruses, phages such as filamentous phages or phagemids.

The term "solid support" is understood to mean any solid structure on which the microorganisms can be temporarily immobilized.

Such a solid support can be made by a person skilled in the art according to the techniques known in the prior state of the art.

This support may consist of a chromatographic support, paramagnetic particles, a polystyrene support or any other support on which complexing proteins such as avidin or streptavidin or an antibody capable of binding the microorganism are immobilized.

Preferably, the molecules having enzymatic activity are chosen from the group consisting of enzymes, abzymes, catalytic peptides or a mixture thereof.

According to a preferred embodiment of the method of the invention, prior to their immobilization on the solid support, the recombinant microorganisms are labeled with the covalent inhibitor connected by an arm to a ligand which can be immobilized on the solid support.

According to another preferred embodiment of the method of the invention, the microorganisms or all or part of their genome are recovered by destruction of the microorganism or by detachment of the microorganism from its solid support.

This detachment can be performed according to several methods: either by detaching the ligand from the solid support with an eluting solution, preferably with a solution at an acidic pH or by cleaving the arm connecting the covalent inhibitor to the solid support; or by cleaving the bond between the covalent inhibitor and the molecule having enzymatic activity; or by

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cleaving a peptide linkage between the molecule with enzymatic activity and the microorganism.

The cleavage of the peptide linkage between the molecule having enzymatic activity is preferably performed with a specific protease such as factor 10A or collagenase; cyanogen bromide which cleaves a peptide linkage containing a methionine or hydroxylamine according [sic] to a peptide linkage containing the peptide asparagine-glycine have also been used.

Such cleavage makes it possible to advantageously increase the selectivity of the method according to the invention.

Indeed, the suicide inhibitor in the method according to the invention is capable of also binding to the microorganism at sites other than the active site of the molecule having enzymatic activity.

By cleaving the specific peptide linkage existing between the molecule having enzymatic activity and the microorganism, only the microorganism containing at its surface an active enzymatic molecule will be recovered during the elution.

Furthermore, this method of selection can be applied for the detection and selection of microorganisms which expose at their surface, on a native or recombinant protein of the microorganism, one or more specific peptides capable of inducing a cellular or humoral immune response.

Indeed, it was observed that a mere antibody-antigen recognition of such peptides did not permit selection of the peptides most capable of eliciting a cellular or humoral immune response (Wetzel (1991), Prot. Eng., vol 4, p. 371-374).

Consequently, a cleavage carried out according to the invention at the level of the peptide linkage existing between the said peptide and the native molecule of the microorganism also makes it possible to select in a specific manner the peptide(s) most capable of eliciting a cellular or humoral reaction.

In addition, all or part of the genome of the

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microorganisms can also be recovered by destroying the microorganism or by recovering all or part of its genome. This genome is then isolated, amplified (for example by PCR) and cloned.

The invention also relates to a marker consisting of a covalent, preferably irreversible, inhibitor of the active site of a molecule having enzymatic activity, connected by an arm to a ligand which can be immobilized on a solid support.

Preferably, the ligand is a biotin or an iminobiotin.

Advantageously, the arm contains a cleavable chemical functional group, preferably a disulfide functional group.

According to a preferred embodiment of the marker according to the invention, the covalent inhibitor is an analog of the specific transition state of the enzymatic reaction performed by the molecule having enzymatic activity.

Another aspect of the invention relates to a method for preparing the marker according to the invention, in which a covalent inhibitor of the active site of a molecule having enzymatic activity is coupled by an arm to a ligand which can be immobilized on a solid support.

The invention also relates to a solid support in which a covalent inhibitor of the active site of a molecule with enzymatic activity is connected by an arm to a solid support.

Advantageously, the covalent inhibitor connected to the solid support consists of the marker according to the invention.

According to a preferred embodiment of the invention, the arm is connected to the solid support by an ester linkage or contains a vicinal diol.

A final aspect of the invention relates to the method for preparing the solid support according to the invention in which a covalent inhibitor of the active site of a molecule having enzymatic activity is connected by an arm to a solid support.

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Brief description of the figures

- Figure 1 represents the nucleotide sequence of the β-lactamase-encoding gene of the plasmid pBR322.
- Figure 2 schematically represents the steps of the method for inserting the gene encoding the mature β-lactamase into the phage fdDOG1.
- Figure 3 represents the nucleotide sequences of the primers complementary to the gene encoding β-lactamase.
- Figures 4 to 9 schematically represent the method of synthesis of the markers according to the invention.
- Figure 10 schematically represents the binding of a microorganism onto a solid support, by the marker according to the invention, as well as the possible cleavage sites which make it possible to recover the said microorganism.

Description of a preferred embodiment of the invention

Figure 10 schematically represents a recombinant microorganism (1) containing at its surface a molecule having enzymatic activity (2), immobilized on a solid support (3) containing a complexing protein (4) such as avidin or streptavidin, by a marker consisting of a covalent inhibitor (5) of the active site of the molecule having enzymatic activity (2), an arm (6) and a ligand (7) which can be immobilized by the complexing protein (4) on the solid support (3).

The microorganisms or all or part of their genome can be recovered:

- by detaching the ligand (7) from the solid support with an eluting solution, such as a solution at a pH of the order of 4 (A);

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- by cleaving the arm (6) of the marker (B);
- by cleaving the bond between the inhibitor (5) and the molecule having enzymatic activity (2) (C);
- by cleaving the peptide linkage between the microorganism (1) and the molecule having enzymatic activity (2) (D);
- by destroying the microorganism (1) and by recovering all or part of its genome (E).

The method will be described in greater detail with reference to the accompanying figures, in the following examples.

Example I: Construction and selection of phages carrying active β-lactamase (fd-bla)

The viruses having at their surface molecules with enzymatic activity are constructed from "filamentous" phages.

The filamentous phages such as M13 or fd are bacterial viruses which, in the free form, contain a 6.4 kb single-stranded circular DNA encapsulated in a protein envelope in the form of a very long cylinder consisting of five proteins (Beck & Zink, Gene (1981), 16, p. 35 to 58; Grant et al., J. Biol. Chem. (1981), 256, p. 539 to 546; Lopez & Webster, Virology (1983), 127, p. 177 to 193.

Among these, the product of the gene III (pIII) is a complex protein, present in an amount of 3 to 5 copies, which closes one of the ends of the virion and is composed of three parts: a signal peptide which is used for exporting the protein to the periplasm of the bacterium and is cleaved during excretion; a globular part necessary for infection which recognizes the F-pilus of the E. coli target cell; a hydrophobic, transmembrane part during the virus morphogenesis phase which subsequently ensures anchorage of pIII in the particle (J. Armstrong et al., FEBS Letters (1981), 135, p. 167-172; C.W. Gray et al., J. Mol. Biol. (1981) 146 p. 621 to 627; G. Glasez-Wuttke et al., BBA (1989) 985, p. 239 to 247).

Following their work on the role and function of

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the protein pIII in the physiology of the phage fd, Smith and Crissman (Virology (1984), 132, p. 445 to 455) showed that it was possible to introduce, by recombinant DNA methods, a foreign peptide sequence inside the sequence of protein pIII (Science (1985) 228, p. 1315 to 1317) or between the signal peptide and the sequence of the mature protein (Gene (1988), 73, p. 305 to 318). This insertion does not prevent the viability of the phage and the introduced sequence is accessible.

The phage-enzyme fd-tet-β-lactamase (fd-bla is constructed from the phage fdDOG1 (Clackson et al, Nature, 352, p. 624 to 628 (1991) in which the sequence encoding the mutated β-lactamase has been introduced.

This phage is derived from the phage fd-tet (Beck et al., Nucl. Acids. Res., 5, p. 4495 to 4503 (1978)), in which a polylinker is inserted between the sequence encoding the signal peptide of protein III (pIII) and the sequence encoding the mature pIII; in this manner, the β-lactamase will form, with pIII, a fusion protein. The phage fd-tet is itself derived from the phage fd (Zacher et al., Gene, 9 p. 127 to 140 (1980)) by insertion of the tetracycline resistance gene of the transposon Tn 10.

The phage fdDOG1 comprises two restriction sites (NotI and ApaLI) constructed by mutations in the region encoding the protein pIII, between the signal peptide and the globular part.

The phage fdDOG1 also comprises the tetracycline gene inserted in the region of the origin of replication of the phage and permitting a propagation of the phage as plasmid.

Isolation of the nucleotide sequence encoding β-lactamase

The β-lactamase gene of the plasmid pBR322 of E. coli (whose sequence is given by Sutcliffe, PNAS USA, 75, p. 3737 to 3741 (1987) and in figure 1) was isolated from the phagemid pBluescript S/K+®.

Given that the cloning into fdDOG1 will use the ApaLI site and that the sequence of the β-lactamase contains such a site, the latter should first be removed by site-directed mutagenesis. This mutation conserves the

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protein sequence.

The phagemid pBluescript S/K+® (from the company Stratagène®, sold by Ozyme®), is mutated according to the method of Eckstein (Nucl. Acids Res., 14, p. 9679 to 9698 (1986) with the aid of a kit marketed by the company Amersham®.

After in vitro mutagenesis, the double strand of the mutated phagemid pBluescript S/K+® (pBluescript-M) is used to transform the strain TG1 of E. coli (Gibson, Studies on the Epstein-Barr virus genome, PhD Thesis, Cambridge University England (1984).

The transformed cells are plated on LB-Amp medium (Sambrook et al., Molecular Cloning, A laboratory manual, second edition, Cold Spring Harbor Laboratory Press (1989) Appendice A).

Colonies are recovered and cultured in LB-Amp medium. The single-stranded form of pBluescript-M is produced by addition of the helper phage R408 to the medium (Russel et al., 45, p. 333 to 338 (1986)).

The strand pBluescript-M is then sequenced according to the Sanger method (PNAS USA, 74, p. 5463 to 5467 (1977)) with the aid of the primer 5'-TTTAAAAGTGGCCATCATTGGA centered at the position encoding Ser 68 in order to verify that, in the chosen clones, the mutation is indeed introduced.

Cloning of β-lactamase into the phage fdDOG1

The sequence of the gene encoding the mature β-lactamase is inserted into the phage fdDOG1 between the sequence encoding the signal peptide of pIII and that encoding the mature pIII. The cloning sites ApaL1 and Not1 are used according to the scheme given in figure 2. To this effect, the β-lactamase gene is recovered from the phagemid pBluescript-M with introduction of the appropriate restriction sites by PCR.

The primers used, shown in Figure 3, contain the following characteristics:

The primer in 5' is a 30mer complementary over 18 bases of the mature β-lactamase gene starting from the second codon (encoding Pro 2). The 5' extension (12

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bases) introduces the ApaL1 site and causes a mutation which results in the replacement of His 1 by Gln 1. This mutation is introduced in order to avoid the phages carrying a β-lactamase having the terminal amino sequence His-Pro-Gln capable of exhibiting an affinity for avidin or streptavidin (Devlin et al. Science, 249, p. 404 to 406 (1990)) subsequently used for their selection.

The primer in 3' is a 47mer complementary over 21 bases from the end of the β-lactamase gene. The extension replaces the stop codon by a codon encoding a glycine, introduces four codons between the gene encoding the β-lactamase and the gene encoding the protein pIII, as well as the Not1 restriction site for the cloning. These four additional amino acids constitute a potential cleavage site between the two proteins for factor Xa. The protein pIII provides, indeed, the capacity for infection of the phages (Goldsmith et al., Biochemistry, 16, p. 2686-2694 (1977). While the presence of a protein as voluminous as β-lactamase affects the capacity for infection, an incubation with factor Xa makes it possible to restore the latter.

In addition, this site constitutes an additional cleavage point for recovering the microorganisms.

The phage fdDOG1 is digested for 18 hours with ApaL1 and Not1 and purified on agarose gel. Moreover, the sequence of the phagemid pBluescript-M is amplified with the primers described above using 34 PCR cycles (1 min. at 95°C, 1 min. at 60°C and 2.5 min. at 72°C) in the presence of Taqpolymerase® (Saiki et al., Science, 239, p. 487 (1988). After gel purification, the product is digested with ApaL1 and Not1 for 18h and repurified; then 1.5 µg of digested fdDOG1 and 250 ng of the β-lactamase gene are mixed in 60 µl with 4 units of T4 DNA ligase (Gibco-BRL®) and maintained overnight at 16°C. The purified ligation mixture is used to transform the strain TG1 of E. Coli.

The cells are plated on LB-tet and LB-Amp medium (Sambrook et al.) where it is observed that the presence of the phage provides the resistance to the antibiotic.

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Colonies are then cultured in 2xYT-Tet medium or in 2xYT-Amp medium in order to prepare the phage fd-bla.

Instead of constructing phages-enzymes by manipulation of the protein pIII, it is also possible to construct a fusion protein with the protein pVIII (1) which is a small protein of 5.2 kd constituting the cylinder body of the phage fd. and which is normally present in 2700 copies (Newman et al., J. Mol. Biol (1977), 116, p. 593 to 606). If the size of the DNA molecule is modified, the number of copies of the protein pVIII can be adjusted in order to include the said DNA molecule.

This variant is achieved with a phagemid in order to be able to control the number of copies of the fusion protein Kang et al., (1991) Proc. Natl. Acad. Sci. USA, vol. 88, p. 4363-4366.

Characterization of the phage carrying the active β-lactamase (fd-bla)

The phage thus constructed is characterized in the following manner. The fact that the infection by the phage confers on the cells a resistance to ampicillin is first demonstrated. Then it is shown directly that the phage itself exhibits an activity for hydrolyzing a β-lactam, nitrocefine. The fact that the enzymatic activity is not present in the ultrafiltrate after filtration on a membrane which allows the β-lactamase to pass through already indicates that the enzyme is bound to the phage. Finally, the phage proteins are analyzed by gel electrophoresis and Western Blotting and it is shown that the protein pIII is present in the form of a fusion protein with the β-lactamase. This confirms that the construction is indeed correct and that, during the morphogenesis of the virus, there is no proteolytic cleavage which would result in the detachment of the β-lactamase from the phage.

By infection of cells of E. Coli TG1 with this phage and plating on LB-Amp medium, ampicillin-resistant colonies are obtained. The β-lactamase activity is directly detected in the Petri dish by a nitrocefine test

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(Plückthun et al., J. Biol. Chem., 262, p. 3951 to 3957 (1987)). Likewise, the phage isolated from the culture medium after centrifugation of the cells, precipitated with 4 % PEG, 0.5M NaCl (final) and resuspended in 50 mM tris buffer, 150 mM NaCl at pH 7.5, at a concentration of the order of 10^{12} transducing units (tu) per ml, hydrolyzes the nitrocefine.

The specific activity of the enzyme carried by the phages was identical to that of the β-lactamase RTEM in solution (Boehringer). The measurement of the activity was also performed after ultrafiltration of the phages on Centri/por membrane with an MW cut-off of 100,000 which allows commercial β-lactamase RTEM to pass through. Less than 5 % of the β-lactamase activity was detected in the ultrafiltrate, which indicates that the enzyme remains attached to the phage.

Viruses fd-tet and fd-bla are prepared and concentrated (10^{12} tu/ml). 20 µl of each solution are loaded onto a 12.5 % SDS-polyacrylamide gel and analyzed (miniprotean® II from Biorad®). By Western Blotting, the proteins are transferred onto nitrocellulose (minitransblot® from Biorad®) and the revealing is performed with an anti-protein III rabbit antibody, followed by a goat antibody against a rabbit antibody coupled to HRP (horseradish peroxidase). The chromophoric substrate of HRP reveals bands of apparent MW of 70 kd and 105 kd for fd-tet and fd-bla respectively. The revealing was also performed with an anti-β-lactamase antibody; a band was found only for fd-bla at the position expected for the fusion protein.

Example II: Construction and characterization of phages carrying an inactive β-lactamase (fd-bla$^-$)

In order to test the efficiency of the selection method according to the invention, phages carrying an inactive β-lactamase (fd-bla$^-$) are constructed and characterized.

The phagemid pBluescript-M is subjected to a mutagenesis in which the codon encoding the essential

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serine 68 is muted to alanine in order to inactivate the enzyme.

The mutated gene is recovered according to the same technique as that used for the active β-lactamase (PCR amplification with introduction of restriction sites (cf. figure 2)). The phage carrying inactive β-lactamase (fd-bla-) is characterized by restriction fragment analysis, complete sequencing of the Bla gene, detection of enzymatic activity and analysis of the proteins.

The S68A mutation is introduced according to the same procedure as the mutation of the ApaL1 site. The transformed cells are plated on X-Gal medium (Horwitz et al., J. Med. Chem., 7, p. 574 (1964)) without ampicillin. The blue colonies are cultured in 2xYT medium and the single-stranded form is produced by addition of the helper phage R408. The mutation is sequenced by the Sanger method with the aid of the primer 5'-TAGTGTATGCGGCGACC complementary to the region encoding G90-Y95.

After amplification of the gene by PCR, cloning, transformation and plating on tet medium as performed for the active β-lactamase, colonies are recovered and cultured in 2xYT-tet medium. The double-stranded DNA of the phage is isolated and digested with the restriction nucleases BamH1 and EcoR1. The digestion products are separated on agarose; the transformants show 2 fragments of 4.7 kbp and 5.5 kbp, in agreement with what is expected. The complete sequencing of the mutated Bla gene is performed with the primers 5'-TAGTGTATGCGGCGACC, 5'-A[lacuna]GCGAGTTAC[lacuna]G, 5'-CCAGCCAGCCGGAAG and 5'-TGCTAAACAACTTTC located respectively in the regions encoding G90-Y65, H156-L160, L223-W227 of the β-lactamase and E5-A9 of the mature protein pIII). It thus is verified that the PCR amplification did not introduce mutations other than that aimed at inactivating the active site.

The presence of a β-lactamase activity is then detected by performing, on the supernatant of a culture of phage fd-bla-, a nitrocefine test as described above.

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In agreement with what is expected, no activity is observed.

Finally, analyses of the phage coat proteins by denaturing gel electrophoresis, Western Blotting and immunological detection is performed as for the phage fd-bla. The fusion protein PIII-β-LACTAMASE is correctly identified.

Example III: Synthesis of the active β-lactamase marker provided with a small-sized arm between suicide inhibitor and ligand

The phages whose DNA contains the active β-lactamase gene and which carry the corresponding enzyme attached to the coat protein pIII will be selected by a reaction with the marker according to the invention. This marker consists of a bifunctional molecule which contains, at one end, a covalent inhibitor of the β-lactamase and, at the other, a ligand, in this case biotin, which can be easily immobilized on a solid support.

The suicide inhibitor module consists of a penam sulfone (see Fisher et al., Biochemistry (1981), 20, 2726-2731, Mezes et al., FEBS Let. (1982), 143, 265-267, Clarke et al., (1983), 748, 389-397, Dmitrienko et al., Bioorg. Chem. (1985), 13, 34-46) functionalized so as to allow its attachment by an arm to a ligand or a support.

Imino-biotin can also be advantageously used because its affinity for streptavidin can be considerably reduced by decreasing the pH of the solution. This will give us an additional possibility to isolate the phages from the support after immobilization.

These two entities are connected by an arm of sufficient length to allow the interaction of the ligand carried by the marker with the immobilized protein.

The scheme for synthesis of the bifunctional molecule is given in figures 4 to 6. Most of the steps of this synthesis are described in the chemical literature or involve coventional procedures. The molecules are characterized by IR spectroscopy and 1H-NMR.

Sodium p-nitrobenzyloxycarbonyl-2-aminoethane-

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sulfonate (3) is prepared by addition of two equivalents of p-nitrobenzyl chloroformate to dissolved taurine (2) one equivalent [sic] of 1N NaOH at 0°C, continuously neutralizing the medium by addition of NaOH, under the Schotten-Baumann conditions. After extraction of the p-nitrobenzyl alcohol with ethyl acetate, the product is freeze-dried. The yield was 66 %.

The sulfonate (3) was converted to sulfonyl chloride (4) with phosphorous oxychloride according to the method of Fujita (Synthesis, 423-424 (1982)). By washing the oil obtained with dry ether, the product was obtained in the form of a white powder with a yield of 97 %.

In parallel, 6-aminopenicillanic acid (5) was converted to its methoxymethyl ester (6) according to the method of Manhas et al. (Synthesis, 549-552 (1983)) with the aid of methoxymethyl chloride after temporary protection with ethyl acetoacetate.

The acid chloride (4) is coupled with the 6-aminopenicillanic acid ester (5) in dichloromethane in the presence of triethylamine. The product (7) purified on a silica column (eluant: ethyl acetate-dichloromethane 80:20), was obtained with a yield of 50 %.

This product was oxidized into a sulfone (8) according to the method of Johnson et al. (J. Org. Chem., 28, 1927-28 (1963)); the product obtained was washed with ether to give a solid white [sic] which was purified on silica (eluant: ethyl acetate-dichloromethane 50:50). The yield was 75 %.

On deprotection of the amine functional group by hydrogenation on palladium/carbon according to a conventional method, the product (9) was obtained which was coupled directly with sulfosuccinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate (10) (marketed by PIERCE®), to give the marker precursor (11) which was purified by chromatography on silica (eluant: ethyl acetate-dichloromethane 50:50). The yield was 25 %.

The protection of the carboxylic functional group in position 3 of the penam ring in (11) was removed with

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the aid of magnesium bromide in dichloromethane according to the method of Kim et al. (Tetrahedron Letters, 32, 3099-3100(1991)). The crude product recovered (12) after evaporation of the solvent was used directly to inhibit the enzyme and to label the phages carrying active enzyme.

Example IV: Immobilization of the phages carrying active β-lactamase (fd-bla)

The use of a covalent marker irreversibly binding the active site of the enzyme and making impossible any reuse of the enzyme, does not constitute a disadvantage in the method for selecting the enzyme. Indeed, in this method the desire is only to select and recover the genetic sequence encoding the active molecule.

The optimum conditions for inhibition, by the bifunctional marker, of the phages carrying the active β-lactamase are first determined. To this end, the rate of disappearance of the β-lactamase activity of the phages is measured, by the nitrocefine test, as a function of time after addition of the inhibitor. Phages are then incubated in the presence of the marker.

Then the reaction product is passed through an agarose-coupled streptavidin column. The phages are then eluted by cleaving the disulfide functional group of the marker. They are used to reinfect an E. coli culture. From this culture, the gene encoding the active β-lactamase can be easily recovered.

A culture of bacteria infected with the fd-bla phages is prepared in a 2xYT medium containing 15 µg per ml of tetracycline and incubated, with stirring, at 37°C for 16 hours. After centrifugation of the culture for 10 minutes at 10000 rpm (revolutions per minute), the supernatant is recovered. The phages are precipitated by addition of 1/5 volume of 20 % polyethylene glycol solution, at 2.5M NaCl. After incubation for 1 hour at 4°C and centrifugation, the phages are resuspended in a 50 mM acetate buffer at pH 5.0.

The marker (12) in solution in DMSO, at a concentration of 1 mg/25 µl, was added at a final

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concentration of 0.25 mM to the solution containing the phages (approximately 10^{11} tu/ml) and 1 % BSA. The inhibition is continued for 30 minutes.

Moreover, a suspension of 250 µl of agarose-streptavidin (PIERCE®) is washed with 20 ml of PBS buffer containing 1 % BSA (Bovine Serum Albumin, Sigma). 0.3 ml of the solution, containing 1.25×10^9 phages, was loaded onto the column, washed with 6 ml of PBS/1 % BSA and 6 ml of PBS. The phages are then eluted with 1 ml of a 500 mM dithiothreitol solution in PBS buffer. One aliquot of recovered phages was incubated, after change of buffer, with 10 ml of E. coli TG1 cells in exponential growth at 37°C. The recovery of the phages was demonstrated by plating various dilutions on LB-tet medium and counting the colonies. 1.8×10^4 phages carrying active β-lactamase, equivalent to 14.4 ppm, were recovered in this manner.

Example V: Immobilization of phages carrying inactive β-lactamase (fd-bla⁻)

1.05×10^9 phages (df-bla⁻) contained in 0.3 ml of acetate buffer at pH 5 were placed in the presence of the marker (12) according to the invention and the reaction product was passed through an agarose-coupled streptavidin column and eluted according to a procedure identical to that described in example 4. 2.0×10^3 phages carrying inactive β-lactamase, equivalent to 1.9 ppm, were recovered in this case.

Comparison of the results of examples IV and V shows that the active phages are retained better than the inactive phages by a factor of 8.

Example VI: Enrichment, with active phages, of a mixture containing active (fd-bla) and inactive (fd-bla⁻) phages: elution with dithiothreitol

An experiment of the same type was carried out with a mixture of active phages and inactive phages to determine directly the active phage enrichment factor.

A mixture containing 6.4×10^9 active phages and 1.4×10^{10} inactive phages in 0.3 ml of acetic buffer at

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pH 5 in the presence of 1 % BSA was placed in the presence of the marker (12) at a final concentration of 0.25 mM. Excess marker was removed by ultrafiltration on a unit Ultrafree 30,000 from Millipore®. The solution was loaded onto streptavidin-agarose. After washing the column with 5 ml of PBS/1 % BSA, the phages were eluted with 500 mM dithiothreitol. An aliquot of eluted phages was plated at various dilutions on LB-tet dishes; the colonies were thus counted. A total of 1.3×10^6 phages were recovered in this manner. The number of phages carrying active β-lactamase was determined by directly measuring the β-lactamase activity of the colonies by the nitrocefine plate test as described above. This number was also verified by plating an aliquot of the eluate on LB-Amp dishes. The number of eluted active phages was 1.1×10^6. The enrichment factor obtained was therefore 5.5.

Example VII: Enrichment, with active phages, of a mixture containing active (fd-bla) and inactive (fd-bla$^-$) phages: elution with factor Xa

An experiment of the same type was carried out with a mixture of active phages and inactive phages to directly determine the enrichment factor; the elution was this time carried out with factor Xa cleaving a peptide linkage between the β-lactamase and the protein III.

A mixture containing 2.8×10^{10} active phages and 2.2×10^{10} inactive phages in 1.5 ml of acetic buffer 1 % BSA was placed in the presence of the marker (12) at a final concentration of 0.75 mM, passed over streptavidin-agarose. After washing the column with 80 ml of PBS, 2 ml of 0.1 M TRIS buffer at pH 7 containing 0.02 % NaN_3 and 2 ml of Tris buffer at pH 7, 1 ml of a solution containing 10 µg of factor Xa (Boeringher) was added to the suspension and it was allowed to stand overnight at room temperature, with gentle stirring. After centrifugation, the supernatant was analyzed by plating at various dilutions on LB-tet dishes; the colonies were thus counted. A total of 6×10^5 phages were recovered in

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this manner. The number of eluted active phages was 5.7×10^5. The enrichment factor obtained was therefore 15.

Example VIII: Enrichment, with active phages, of a mixture containing active (fd-bla) and inactive ($fd\text{-}bla^-$) phages: preimmobilization of the marker

Another experiment was carried out with a mixture of active phages and inactive phages, but with prior immobilization of the marker on the streptavidin-agarose column.

250 µl of streptavidin-agarose suspension are incubated for one hour in an acetic buffer at pH 5 in the presence of 1 % BSA; after washing with 2.5 ml of acetic buffer without BSA, 2 µl of a 50 mM solution of marker (12) in DMSO are added. After 1 minute and 5 cycles of washing and centrifugation with, each time, 1 ml of acetic buffer, 0.8 ml of phages in an acetic buffer 1 % BSA containing 9.6×10^{10} active phages and 5×10^{10} inactive phages were added to the suspension with gentle stirring, overnight at 4°C. After washing with 20 ml of PBS/1 % BSA and 50 ml of solution) 0.1 % Tween 20 in PBS, the phages are eluted with factor Xa and counted as in example VI. 1.15×10^5 active phages and 1.85×10^5 inactive phages were recovered in this manner. The enrichment factor obtained was therefore 3.2.

Example IX: Enrichment, with active phages, of a mixture containing active (fd-bla) and inactive ($fd\text{-}bla^-$) phages: elution by hydrolysis of the acyl-enzyme linkage

An experiment similar to that of example VIII was carried out with a mixture of active phages and inactive phages to verify the possibility of recovering phages by cleaving the linkage between the β-lactamase and the suicide inhibitor, namely the ester linkage of the acyl enzyme. This cleavage was carried out by hydrolysis.

A mixture containing 2.8×10^{10} active phages and 1.05×10^{10} inactive phages were [sic] loaded onto a column where the marker had been immobilized. After

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extensive washing as in example VIII, the suspension was allowed to stand overnight at room temperature and the supernatant from a centrifugation was analyzed. 1.4×10^5 active phages and 2.5×10^5 inactive phages were recovered in this manner. The enrichment factor obtained was therefore 2.1.

In the examples mentioned, the percentage of immobilized phages is low because the size of the arm between the immobilizable ligand and the inhibitor is quite small. In order to avoid this disadvantage, another marker having a larger-sized arm was prepared.

Example X: Synthesis of a marker of active β-lactamase provided with a large-sized arm between suicide inhibitor and ligand

Carbobenzyloxy-2-(2-aminoethoxy)ethanol (15) (figure 7) was prepared by addition of 1 equivalent of benzyl chloroformate (13) to 2-(2-aminoethoxy)ethanol (14) in water saturated by bicarbonate. The product was extracted in organic phase and purified on a silica column (eluant: ethyl acetate-dichloromethane 50:50). The yield was 58 %.

Ethyl vinyl-sulfonate (17) was prepared from 2-chloroethane-sulfonyl chloride (16) according to the method of Whitmore and Landau (J. Am. Chem. Soc., 68, p. 1797-1798 (1946)). It was purified by horizontal distillation at 88°C under 0.6 mm of mercury to give a colorless oil (yield 45 %). One equivalent of the protected amino alcohol (15) was added onto (17) in dry acetonitrile to which finely ground potassium bicarbonate was added. After heating, for 3 days at 80°C, filtration and evaporation of the solvent, the product (18) was purified on silica (eluant: ethyl acetate-dichloromethane 50:50). The yield was 60 %. The sulfone functional group was deprotected according to the method of Tipson et al. (J. Org. Chem., 12, 133-137 (1947)) to give, after 24 hours of reaction, (19) in the form of a white powder after washing with hexane (yield 86 %). This product was converted to an acid chloride (20) according to the method of Fujita (Synthesis, 423-424 (1982)).

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The acid chloride (20) (figure 7) was coupled directly with the ester of of [sic] 6-aminopenicillanic acid (6) in dichloromethane in the presence of triethylamine for one hour. The product obtained (21), purified on silica (eluant: ethyl acetate-dichloromethane 50:50) was obtained with a yield of 34 %. It was oxidized into a sulfone (22) according to the method of Johnson et al. (J. Org. Chem., 28, 1927-28 (1963)), the product obtained was washed with ether to give a white solid which was purified on silica (eluant: ethyl acetate-dichloromethane 50:50). The yield was 72 %.

On deprotection of the amine functional group by hydrogenation on palladium/carbon in ethanol according to the conventional method, the product (23) (figure 8) was obtained which was coupled directly with sulfosuccinimidyl 2-(biotinamido)ethyl-1,3-dithiopropionate (10) (marketed by PIERCE®) in order to obtain the marker precursor (24) which was purified by chromatography on silica (eluant: ethyl acetate-dichloromethane 50:50). The yield was 13 %.

The protection of the carboxylic functional group in position 3 of the penam ring in (24) was removed with the aid of magnesium bromide in dichloromethane according to the method of Kim et al. (Tetrahedron Letters, 32, 3099-3100 (1991)).

Example XI: Enrichment, with active phages, of a mixture containing active (fd-bla) and inactive (fd-bla$^-$) phages: labeling with the inhibitor (24), elution with factor Xa

An enrichment experiment was carried out with labeling with the inhibitor (24) of a mixture of active phages and inactive phages in order to determine the effect of the size of the arm connecting the biotin ligand with the suicide inhibitor module.

A mixture containing 1.2×10^{10} active phages and 3.3×10^{10} inactive phages in 1.5 ml of acetic buffer 1% BSA was placed in the presence of the marker at a final concentration of 0.75 mM, passed over

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streptavidinagarose. After washing the column, elution as described in example VII and analysis, 6.8×10^6 active phages and 1.9×10^5 inactive phages were found. The enrichment factor obtained was therefore 98.

EXAMPLE XII: Synthesis of a second active β-lactamase marker provided with a large-sized arm between suicide inhibitor and ligand

In order to have a general method for the synthesis of a marker provided with a large-sized arm between the ligand and the inhibiting head, a precursor carrying an activated ester functional group and therefore capable of being coupled to numerous inhibitors and containing itself a large-sized arm was prepared. It was coupled to the suicide inhibitor of the β-lactamase.

The activated ester of the N-hydroxysuccinimide with biotin-N-ε-aminocaproic (26) was prepared as described (Wilchek and Bayer, Methods Enz. (1990), 184, 123-160). It was dissolved in DMF and added to a solution of 3 equivalents of cystamine (26) [sic] in DMF (figure 9).

The crude product, isolated by precipitation with ether (10 times the volume of the solution) was purified on a Sepharose S (Pharmacia®) column equilibrated with lithium chloride, using as eluant 25 % ethanol. The addition product (28) was eluted with 50 mM LiCl. The solution was freeze-dried. The yield was 79 %.

Three equivalents of glutaric anhydride (29), dissolved in acetonitrile, were added in portions to the purified product (containing LiCl) dissolved at a concentration of the order of 0.01 M in buffered aqueous medium ($KHCO_3$-K_2CO_3, 50/50, 10 mM at pH 9.6) containing 10 % ethanol. The acid obtained (30) was precipitated by acidification with 1N HCl up to pH 2. The product was filtered and dried, the yield was 43 %.

The acid (30) was converted into an activated ester under standard conditions by coupling with N-hydroxysuccinimide (31) with the aid of dicyclohexylcarbodiimide (DCC) in the DMF. After 72 hours, the reaction medium was precipitated with dry

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ether. The precipitate was dried in a desiccator (yield: 87 %), this product (32) was used as such for the coupling reaction with the deprotected ester of 6-aminopenicillanic acid (6) in dry ethanol.

Example XIII: Enrichment, with active phages, of a mixture containing active (fd-bla) and inactive (fd-bla$^-$) phages: immobilization on paramagnetic particles.

An experiment similar to experiment VI was carried out, the only difference being that the streptavidin is carried by paramagnetic particles. The washes are carried out by resuspension in fresh buffer. The elution of the immobilized phages is carried out with 50 mM dithiothreitol. The enrichment factor is determined as in example VI.

Example XIV: Enrichment, with active phages, of a mixture containing active (fd-bla) and inactive (fd-bla$^-$) phages: immobilization by "panning" in a polystyrene tube

An experiment similar to experiment VII is carried out, the only difference being that the streptavidin is immobilized by "panning" at the surface of a polystyrene tube following a technique conventionally used for ELISA tests. The washes are carried out by resuspension in fresh buffer. The elution of the immobilized phages is carried out with factor Xa. The enrichment factor is determined as in example VII.

Given that in examples XIII and XIV, the washes can be carried out more rapidly than in the examples using the chromatographic technique and the phages less easily entrapped here than in a gel, these variants of the enrichment experiments are more suitable for the observation of high enrichment factors.

Example XIII: [sic] Selection of phages carrying an abzyme

In this case, the covalent inhibitor will consist of a suicide inhibitor characteristic of the reaction to be catalyzed by the abzyme or by an analog of the specific transition state.

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The detection and selection of these phages are then carried out in a manner identical to the detection and selection of the phages which expose active enzymes at their surface.

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Claims

1. Method for selecting recombinant microorganisms containing at their surface at least one molecule having enzymatic activity, characterized in that the recombinant microorganisms are immobilized on a solid support by a covalent inhibitor of the active site of the molecule having enzymatic activity, connected to the solid support by an arm; in that the recombinant microorganisms or all or part of their genome are recovered and in that all or part of their genome is isolated.

2. Method of selection according to claim 1, characterized in that prior to their immobilization on the solid support, the recombinant microorganisms are labeled with the covalent inhibitor of the active site of the molecule having enzymatic activity, connected by an arm to a ligand which can be immobilized on the solid support.

3. Method of selection according to any one of claims 1 or 2, characterized in that the microorganisms are viruses, preferably phages such as filamentous phages.

4. Method of selection according to any one of claims 1 or 2, characterized in that the microorganisms are phagemids.

5. Method of selection according to any one of the preceding claims, characterized in that the solid support consists of a chromatographic column on which avidin, streptavidin and/or an antibody is immobilized.

6. Method of selection according to any one of the preceding claims, characterized in that the molecules having enzymatic activity are chosen from the group consisting of enzymes, abzymes, catalytic peptides or a mixture thereof.

7. Method of selection according to any one of the preceding claims, characterized in that the microorganisms are recovered by detachment from their solid support.

8. Method of selection according to any one of

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the preceding claims, characterized in that the recombinant microorganisms are recovered by cleavage of the arm connecting the covalent inhibitor to the solid support.

9. Method of selection according to any one of claims 1 to 7, characterized in that the recombinant microorganisms are recovered by detaching the ligand from the solid support with an eluting solution, preferably a solution at an acidic pH.

10. Method of selection according to any one of claims 1 to 7, characterized in that the recombinant microorganisms are recovered by cleavage of the bond between the covalent inhibitor and the molecule having enzymatic activity.

11. Method of selection according to any one of claims 1 to 7, characterized in that the recombinant microorganisms are recovered by cleavage of the peptide linkage between the molecule having enzymatic activity and the microorganism.

12. Method of selection according to claim 11, characterized in that the cleavage is performed with a protease.

13. Method of selection according to claim 11, characterized in that the cleavage is performed with cyanogen bromide.

14. Method of selection according to claim 11, characterized in that the cleavage is performed with hydroxylamine.

15. Method of selection according to any one of claims 1 to 7, characterized in that the recombinant microorganisms are destroyed and that all or part of their genome is recovered, isolated and cloned.

16. Marker characterized in that it consists of a covalent inhibitor of the active site of a molecule having enzymatic activity, connected by an arm to a ligand which can be immobilized on a solid support.

17. Marker according to claim 16, characterized in that the covalent inhibitor is an irreversible inhibitor.

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18. Marker according to claim 16 or 17, characterized in that the ligand is a biotin or an iminobiotin.

19. Marker according to any one of claims 16 to 18, characterized in that the arm contains a cleavable functional group, preferably a disulfide functional group.

20. Marker according to any one of the preceding claims 16 to 18, characterized in that the covalent inhibitor is an analog of the specific transition state of the enzymatic reaction performed by the molecule having enzymatic activity.

21. Method for preparing the marker according to any one of claims 16 to 20, characterized in that a covalent inhibitor of the active site of a molecule with enzymatic activity is coupled by an arm to a ligand which can be immobilized on a solid support.

22. Solid support, characterized in that it comprises a covalent inhibitor of the active site of a molecule having enzymatic activity, connected to a solid support by an arm.

23. Solid support according to claim 22, characterized in that the covalent inhibitor connected to the solid support consists of the marker according to any one of claims 17 to 20.

24. Solid support according to claim 22, characterized in that the arm is connected to the solid support by an ester linkage or contains a vicinal diol.

25. Method for preparing a chromatographic column according to any one of claims 22 to 24, characterized in that a covalent inhibitor of the active site of a molecule having enzymatic activity is connected by an arm to a solid support.

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