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Chapter 20

HOW TO MAKE GOOD USE OF A "BAD" ENZYME: UTILIZATION OF EFFICIENT β-LACTAMASES FOR THE BENEFITS OF BIOCHEMICAL RESEARCH

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ABSTRACT

The activity of β-lactamases is easy to estimate, they often exhibit very high turn-over numbers and many of them are quite stable. For these reasons they have emerged as promising reporter enzymes to study gene expression and as models to analyse protein-ligand interactions. They also accept the insertion of rather large peptides without losing their activity. This has allowed the development of practically useful technologies which are described in this chapter.

INTRODUCTION

β-lactamases have been extensively used as biochemical markers for identification of β-lactam antibiotics which are active against bacterial pathogens. But during the last decade, class A β-lactamases have emerged as promising reporter enzymes to study gene expression and to study protein/ligand interactions. In this context these enzymes are very attractive because they are monomeric, very stable and of small size [1]. Their three-dimensional structures are well-defined and they are considered as very efficient enzymes [2]. Among the various reporter enzymes described in the literature, β-lactamases are particularly interesting because they are not expected to interfere with the function of most of the proteins to which they might be fused and the absence of endogenous counterparts in mammalian cells [3].

They have also been successfully expressed in prokaryotic and eukaryotic cells allowing their utilizations for *in vitro* as well as *in vivo* applications [4]. They can be overexpressed with prokaryotic expression systems so that their production times and costs are drastically reduced [5]. Their success is also partly due to the development of a variety of β-lactam substrates including chromogenic β-lactams for immunoassays, fluorescent membrane-permeant β-lactams which allow cell imagery, flow cytometry and gene expression assay, and non-toxic β-lactam prodrugs for antibody-directed enzyme prodrug therapy [3, 6-8].

Thanks to this uniqueness they have been extensively employed as "reporters" or "sensors" for monitoring various biological processes and interactions. Sensitive and rapid detection of β-lactamase activity in biological samples is thus of importance in both research and clinical applications [3]. This chapter is dedicated to the many practical utilisations of β-lactamases.

1. β-LACTAMASES AS REPORTERS OF GENE EXPRESSION

Biological specificity is mediated by the precise and selective regulation of gene expression in response to intrinsic developmental programs and extrinsic signals. To understand the regulation of gene expression, it is essential to use an assay of high sensitivity and fidelity that reports expression at the level of a single cell [9]. Correlation of gene expression with physiological responses and developmental fates would be facilitated by non-destructive transcriptional assays compatible with high-throughput methods such as fluorescence-activated cell sorting (FACS). Such assays are currently based on reporter genes such as those of chloramphenicol acetyltransferase, secreted alkaline phosphatase, β-galactosidase, and firefly luciferase [10] which typically require cell permeabilization or lack single-cell resolution, whereas the green fluorescent protein (GFP) is a relatively insensitive reporter due to the lack of enzymatic amplification [9, 11]. In this context, Zlokarnik and coworkers demonstrated the use of the 29 kDa isoform of the TEM-1 β-lactamase and a membrane-permeant, fluorogenic ester substrate to measure gene expression in single live mammalian cells with high sensitivity and real-time responses. To limit interference from variations in cell size, probe concentration, excitation intensity, and emission sensitivity, they constructed a new substrate molecule presented in figure 1 based on the following strategy: since cleavage of cephalosporins by a β-lactamase triggers the spontaneous elimination of any leaving group in the C3' position [9], they attached two fluorophores with overlapping spectra on the 7' and 3' positions of a cephalosporin. When on the same molecule, these exhibit efficient fluorescence energy transfer (FRET). The β-lactamase action allows the release of the C3' fluorophore, disrupting FRET and reestablishing fluorescence emission from the 7' donor. For that purpose they used a membrane-permeant ester derivative of a cephalosporin CCF2/AM. The donor fluorophore was 7-hydroxycoumarin and the 3'-acceptor fluorophore was fluorescein. They validated this substrate with several receptor-regulated gene expressions under the control of adequate promoters in living mammalian cells by visualizing the β-lactamase-catalysed hydrolysis of CCF2/AM. This was done in both transitory expression systems and also in stable cell lines.

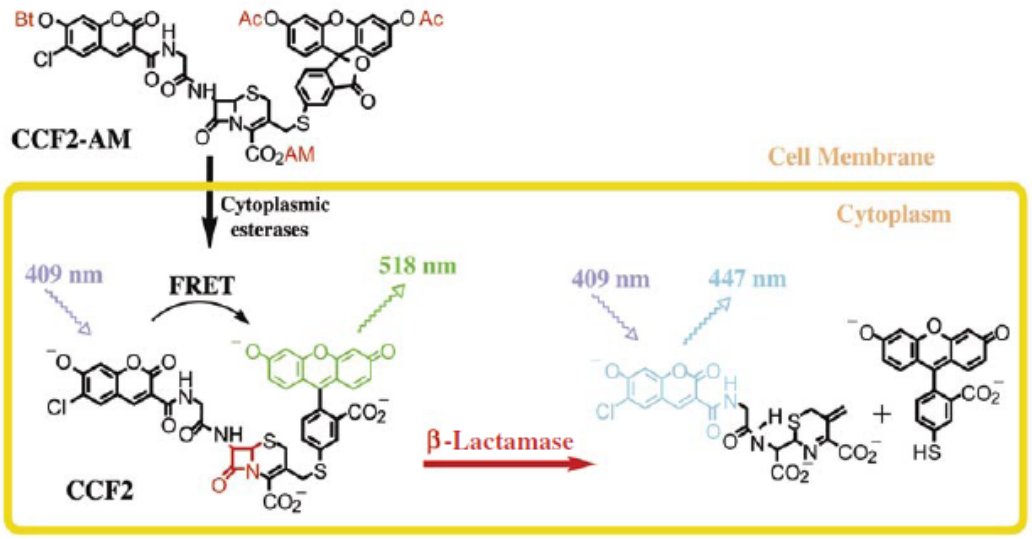


Figure 1. Schematic representation of the CCF2/4-AM substrate molecule loading into cultured cells [12]. The esterified (acetoxymethylated: AM) form of the CCF2/4 substrate, called CCF2/4-AM, diffuses across the cell plasma membrane where endogenous esterases convert it to CCF2/4, thereby trapping it inside the cell. Exciting CCF2/4 at 408 nm leads to efficient FRET from the coumarin moiety to the fluorescein derivative and produces green fluorescence detectable at 530 nm. After CCF2/4 hydrolysis by the β-lactamase, the two fluorophores separate causing loss of FRET. Excitation at 408 nm now results in blue fluorescence detectable at 460 nm.

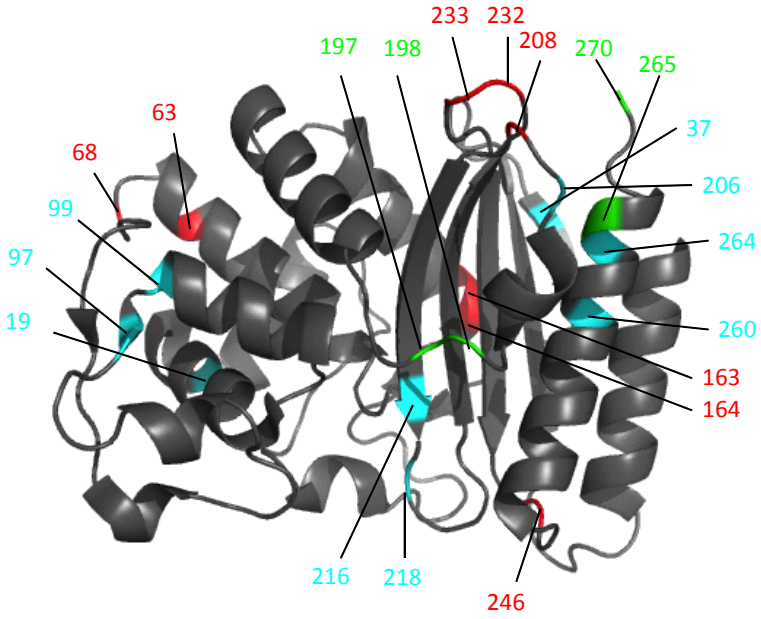


Figure 2. Sites of pentapeptides insertions in the three-dimensional structure of TEM-1 β-lactamase [25]. Numbers identify the insertion positions. Green, cyan and red residues indicate pentapeptide insertions which result in high-level, intermediate-level and loss of ampicillin hydrolysis activity, respectively.

β-Lactamase reporter gene assays have also been successfully used as readouts for ligand-induced activation of G protein-coupled receptors [13], for selecting high-producing clones in the yeast *Pichia pastoris* [14], to identify inhibitors of the hepatitis C virus (HCV) replication [15], and to monitor spatially restricted patterns of genes expression in the early zebrafish embryos [16].

1. β-LACTAMASE AS A REPORTER ACTIVITY

The β-lactamase reporter activity has also been extensively used to study biochemical processes, especially protein/ligand interactions. For this purpose, three different possibilities detailed in this section were developed.

The Use of β-lactamase Fusion Proteins

An interesting application of β-lactamase fusion proteins is the use of this reporter system to study exported proteins. For example McCann and coworkers identified proteins exported by *Mycobacterium tuberculosis* growing in host cells with the assumption that, when used in protein fusions, a β-lactamase can report on the subcellular location of another protein as measured by the protection from β-lactam antibiotics afforded to a mutant strain of *M.tuberculosis* lacking its major β-lactamase [17]. The exported proteins of *M.tuberculosis* have received attention for a long time because of the well-established fact that the majority of bacterial virulence factors and antigens are proteins exported to the bacterial cell wall or secreted [18]. Proteomic and genetic methods were used to identify proteins exported from the cytoplasm especially enzymatic reporter systems [19-21]. Among these reporter systems, β-lactamases are exported proteins that were not initially employed in *M.tuberculosis* because of endogenous β-lactam resistance. But the construction of a β-lactam-sensitive mutant of this bacterium has opened the door to the utilization of β-lactamases as reporters of protein export directly in *M.tuberculosis* with the β-lactam-resistance as a powerful indicator of export. This reporter enzyme presents the advantage of using a selection system instead of a more labor-intensive screening process. This study demonstrated that the TEM-1 β-lactamase lacking its export signal sequence conferred β-lactam resistance when fused to the major *M.tuberculosis* endogenous signal sequences. They further showed that β-lactamase fusion proteins report on protein export even while the bacterium is growing in its host cell namely human macrophages.

In another interesting study, Simonen and coworkers [22] investigated the relationship between folding and secretion competence of a *Saccharomyces cerevisiae* secretory protein: hsp150. They fused the *E.coli* TEM-1 β-lactamase, deprived of its own signal sequence, to the C-termini of various portions of hsp150. The evaluation of the β-lactamase activity of the different chimeric proteins was used to study the effect of the hsp150-carrier sequence on the folding of the β-lactamase portion and the effect of folding on the secretion competence of the fusion proteins. With this reporter system they identified the hsp150 subunits responsible for both the folding and the secretion of the fused protein.

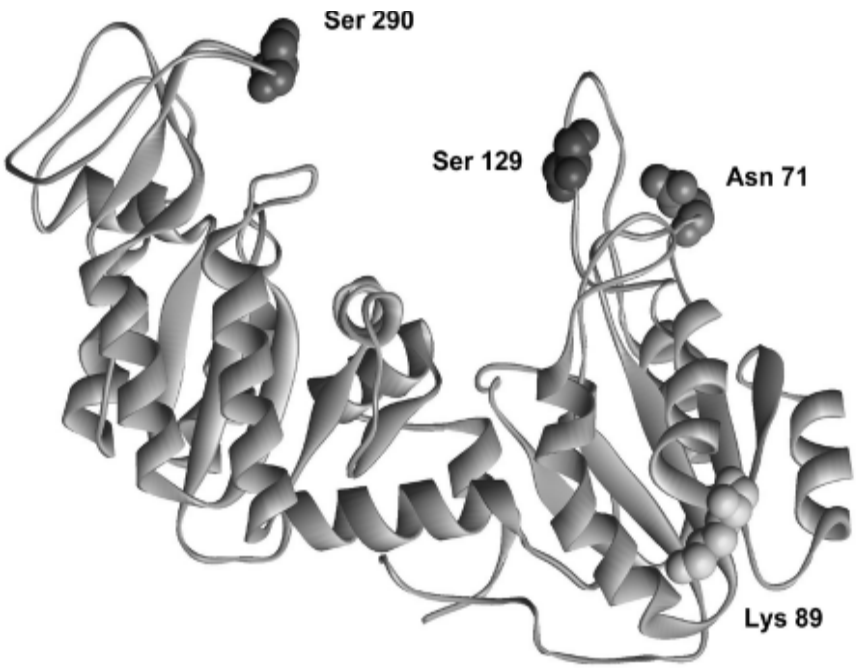
The TEM β-lactamse was also used to probe the topology of membrane proteins [23]. When the β-lactamase gene (devoid of signal peptide) is fused to DNA fragments encoding portions of increasing lengths of the target protein, starting from the N-terminus, the β-lactamase can localise into the periplasm or the cytoplasm. Resistance of single-cell colonies to ampicillin is increased in the first case, not in the second. This technique was used to elucidate the transmembrane organisation of the *Bacillus licheniformis* BlaR sensory transducer (see chapter 17) involved in the induction of β-lactamase in this bacterium [24].

The Use of β-lactamases as Carrier Proteins

Besides the use of β-lactamases as fusion proteins, protein and or peptide insertions were also demonstrated to be efficient tools especially to study protein/ligand interactions. Indeed, class A β-lactamases were demonstrated to accept insertions of both random peptides as well as structured protein/domain. Hallet and coworkers determined the tolerance of the *E.coli* TEM-1 β-lactamase to 23 randomly inserted pentapeptides (Figure 2). They associated the phenotypes of the mutated β-lactamases to the local peptide structures in which the pentapeptide insertions occurred and to their positions in the three-dimensional structure of the enzyme [25].

In the case of complete domains or proteins, the location of the insertion site was demonstrated to strongly influence the tolerance of the inserted polypeptide. For example Betton and coworkers inserted the TEM-1 β-lactamase into the maltose-binding protein MalE. This resulted in a chimeric protein retaining the activity of both parental proteins [26]. They showed that this bifunctionality was dependent on the insertion site and that the activity of one of the chimeric protein partners could be modulated by an independent ligand specific for the other partner of the macromolecule. Similarly, Collinet and coworkers inserted TEM-1 into four surface loops of the phosphoglycerate kinase (PGK) and showed that the chosen insertion site strongly influenced the tolerance to insertions [27] (Figure 3).

The class A BlaP β-lactamase produced by *Bacillus licheniformis* was used as a carrier protein to study the function of heterologous protein fragments [28]. The chitin-binding domain (ChBD) of the human macrophage chitotriosidase was inserted into a solvent exposed loop of BlaP far away from its active site (position 198) (Figure 4). The product of this construction behaved as a soluble chimeric protein that conserved both the capacity to bind chitin and to hydrolyze β-lactams. Using the β-lactamase reporter activity, the relative association constant (K_r) between the chimeric protein and insoluble chitin was determined. Furthermore, this hybrid protein allowed the detection of chitin in fungal cell wall and to measure the amount of protein bound to various insoluble polysaccharides with a good accuracy. This is a major advantage of this system when compared to the techniques already described in the literature attempting to determine the affinity parameters of carbohydrate binding domains. Indeed, in these studies, protein concentration determination assays or intrinsic fluorescence measurements used to characterize the interaction between the protein domains and the insoluble polysaccharides resulted in large standard deviations [29-30].



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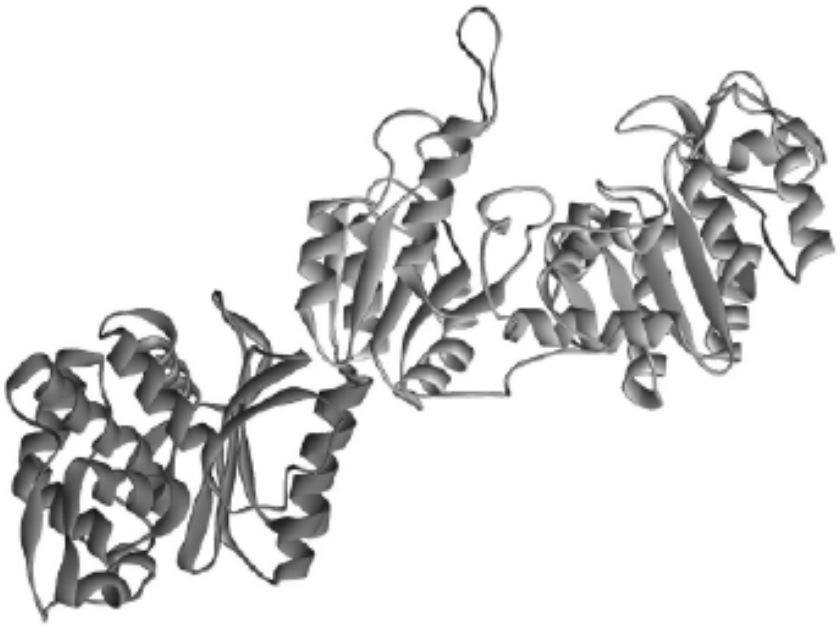

B

Figure 3. A. Structure of the yeast PGK. The four tested insertion sites are indicated. B. Illustrative scheme of the chimeric protein in which the *E.coli* TEM-1 β-lactamase was inserted into the yeast PGK at position 89 [27].

The Use of β-lactamase Fragments Complementation

The complementation of β-lactamase fragments fused to interacting polypeptides that regenerate enzymatic activity was also used as a reporter system in assays monitoring inducible protein-protein interactions in eukaryotic cells (Figure 5) [4, 12, 31]. This strategy, called protein fragment complementation assay (PCA), is based on protein interaction assisted folding of rationally designed fragments of enzymes. It has also been used with other reporter proteins such as luciferase, β-galactosidase, the green fluorescent protein and the dihydrofolate reductase. However the advantage of the β-lactamase activity is that no ortholog exists in eukaryotes, and thus PCA based on β-lactamase activity can be used universally in eukaryotic cells and many prokaryotes with no intrinsic background activity. β-lactamase also has the desirable features of enzymatic amplification and facile *in vivo* and *in vitro* assays. Finally as mentioned above, it has the advantage that the versatile membrane-permeable fluorescent β-lactamase substrate, CCF2/AM, is available which allows ratiometric fluorescence detection and thus superior reproducibility and excellent quantification of the results in intact cells [9]. β-lactamase complementation also exhibits an extremely high signal-to noise ratio [31].

The strategy for the selection and design of fragments in the *E.coli* TEM-1 β-lactamase was based on the analysis of its structure and consists in the cleavage of the enzyme between Glu 197 and Leu198, because this site is located on a surface loop opposite to the active site (Figure 6) and produces fragments of similar lengths. It contains no periodic secondary structure, and the fragments can fold independently into native-like structures [4]. This β-lactamase complementation assay has been validated with several known protein-protein interactions, including the homodimerizing GCN4 parallel coiled-coil leucine zipper, the heterodimerizing soluble Bad and truncated (Bcl2T) pair [4] and even the interaction between a transmembrane receptor and cytoplasmic protein as in the case of the epidermal growth factor receptor and the FKBP12 cytoplasmic protein [31].

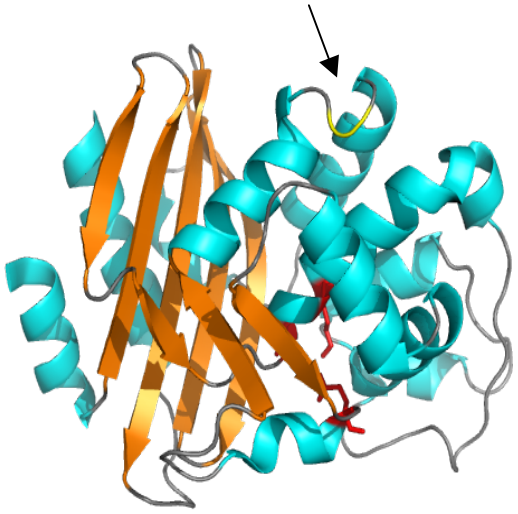

Figure 4. Ribbon diagram of the three dimensional structure of the *Bacillus licheniformis* BlaP β-lactamase. α-helices and β-sheets are represented in blue and orange respectively. The insertion site is

indicated by an arrow. It is located in a loop connecting helix 9 to helix 10 between residues Lys 197 and Leu 198. Residues involved in the enzymatic catalysis are shown in red.

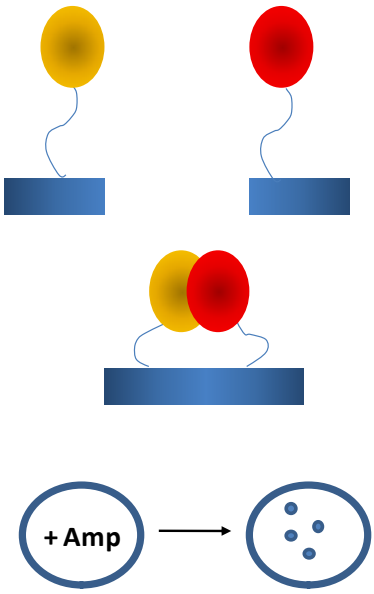

Figure 5. Schematic representation of the ß-lactamase based protein fragment complementation assay (PCA) [32]. The β-lactamase is split into two inactive fragments of similar lengths. Interacting fusion proteins attached to the fragments allow fragment reassembly, which can be detected by growing cells on media containing ampicillin or alternatively by using a chromogenic substrate.

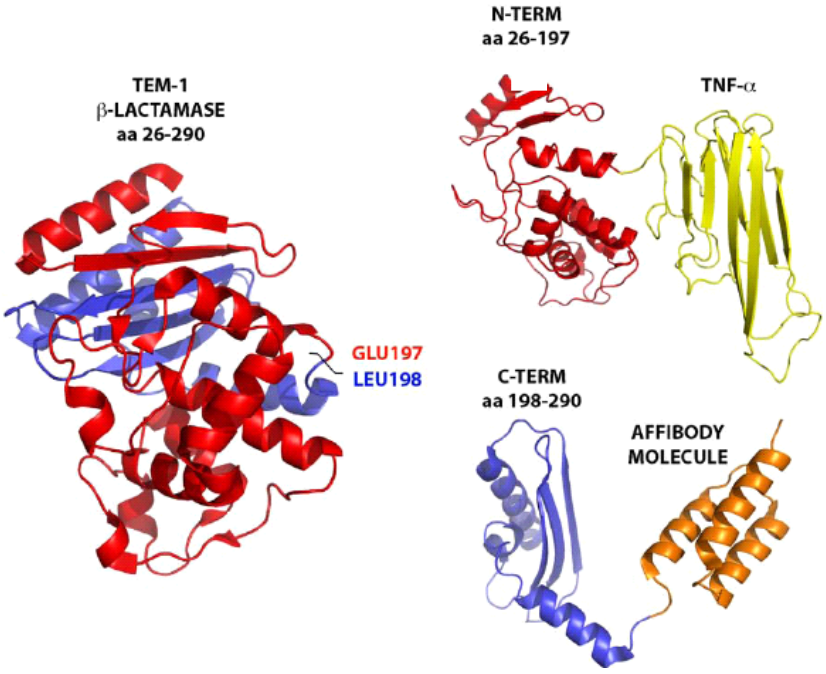

Figure 6. Representation of the *E.coli* TEM-1 β-lactamase with the N- and C- terminal subfragments highlighted in red and blue respectively [33]. An example of two co-expressed constructs whose

complementation is monitored in the PCA system is also represented: the TNF-α target (yellow) is fused to the N-terminal fragment and an affibody protein library member (orange) to the C-terminal fragment.

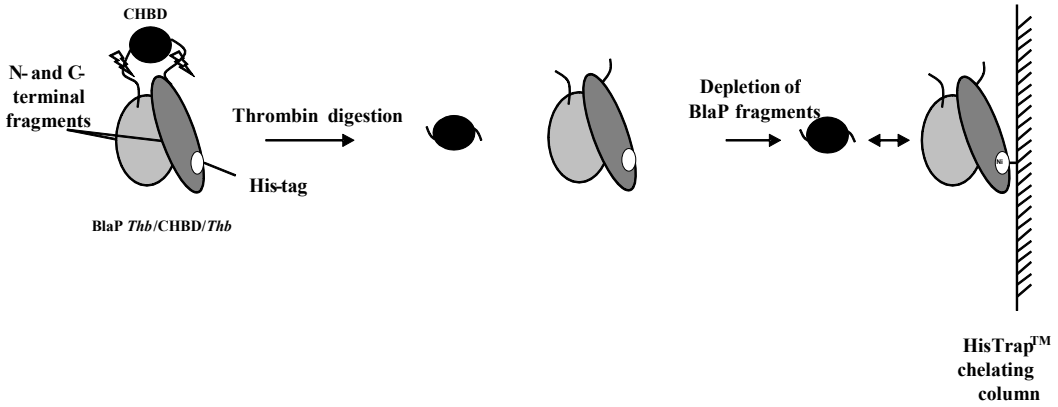

Figure 7. Schematic representation of the 2-step protocol used to prepare purified ChBD. Purified ChBD is collected in the flow through after loading the digested chimeric protein on the HisTrap™ chelating HP [37].

More recently, Löfdah and coworkers applied this TEM-1 β-lactamase based PCA system to select affibody molecules binding the human tumor necrosis factor-α (TNF-α) from a combinatorial library [33]. This system has also been used for the high throughput screening of toll-like receptor signaling inhibitors [34].

3. β-LACTAMASE AS AN ALTERNATIVE FOR THE PRODUCTION OF DIFFICULT TO EXPRESS PROTEIN DOMAINS

Many *Escherichia coli* expression vectors are available to produce and to purify proteins fused to affinity tags, such as the *Schistosoma japonicum* glutathione S-transferase (GST), the *E.coli* maltose binding protein and thioredoxin [35-36]. All these systems allow the subsequent proteolytic cleavage of the fusion protein to separate the protein of interest from the affinity tags. But a common feature of these systems is the limited utility of the affinity tag in the biochemical characterization of the studied protein. Recently, Vandevenne and coworkers [37] described a system able to elicit the expression of protein fragments inserted into a solvent exposed loop of the *Bacillus licheniformis* BlaP class A β-lactamase. Two thrombine (*thb*) cleavage sites were engineered on both sides of the β-lactamase permissive loop in order to cleave off the exogenous protein fragment from the carrier protein by an original two-step procedure [37]. This system has been used to express the chitin binding domain of the human macrophage chitotriosidase (ChBD) inserted between the two protease cleavage sites. This 73 residues polypeptide contains 3 disulfide bridges reported as essential to its chitin binding activity [38-39]. Recent studies showed that ChBD expression in *E.coli* can be achieved by fusing the domain to the *glutathion-S-transferase* (GST) [39] but with very limited yields which, combined with its low solubility and stability, strongly limited the characterization of ChBD. Upon insertion into the carrier β-lactamase, the resulting chimeric

protein named BlaP *Thb*/ChBD/*Thb* was successfully produced in *E.coli* in a soluble form with a very good yield (≈ 20 mg of pure protein per liter of culture) and purified to homogeneity. The separation of ChBD from its carrier protein did alter neither the biochemical activity nor the native disulphide bridge formation of the inserted ChBD. Pure ChBD poplypeptides were prepared based on both the mutual affinity of the N- and C-terminal fragments of BlaP after thrombin cleavage and the presence of a poly-histidine tag at the C-terminal end of BlaP. The two-step purification procedure presented in figure 7 consists in a cleavage of BlaP *Thb*/ChBD/*Thb* with thrombin in solution followed by the elimination of BlaP fragments on a HisTrap Chelating HP column. This step can be repeated until the desired level of purity is obtained. This is a good example of the utilization of the β-lactamase carrier protein to increase the production and the solubility of difficult to express protein fragments and facilitate their purifications and studies.

4. β-LACTAMASE AS A BIOSENSING MOLECULE

Biosensors are based on induced conformational changes of proteins to detect small ligands and ions [40]. The proteins selected for biosensor construction are usually allosteric enzymes which upon ligand binding exhibit altered catalytic efficiencies. Some hydrolytic enzymes have been widely used [41-42]. However, despite all the advantages exhibited by β-lactamases, the rational design of biosensors based on these enzymes remains a great challenge since neither substrate binding nor catalysis has been reported to induce large conformational changes in these proteins. The Ω-loop, a loosely packed 17-residue segment (residues 163-179) adjacent to the active site of class A β-lactamases, appears to be flexible and its motion essential to the enzymatic reaction [43]. This Ω-loop contains a key active-site residue Glu-166, whose substitution impairs but still allows acylation of the enzyme by β-lactam antibiotics. By contrast, the deacylation step is much more affected [44]. Moreover, crystal structures of the enzyme show that the Glu-166 side chain is pointing toward the active site. Based on these results Chan and coworkers hypothesized that, if an environment-sensitive fluorescent probe is placed specifically at the 166 position, an enzyme with drastically suppressed hydrolytic activity that can function as a biosensor can be constructed. To demonstrate this concept, they constructed a mutant of the β-lactamase I from *Bacillus cereus* 569/H, E166C, in which the Glu-166 residue on the Ω-loop was replaced by a cysteine residue. The E166C mutant exhibiting a 1800-fold lowered efficiency was labeled with the thiol-specific fluorophore (fluorescein-5-maleimide) to give the labeled E166Cf mutant. As the wild-type β-lactamase I contains no cysteine residue, this single-point mutation allows the specific attachment of a thiol-reactive fluorophore. In aqueous buffers, E166Cf is only weakly fluorescent but when β-lactam antibiotics are added, the fluorescence intensity at 515 nm increases as a function of the antibiotic concentration (Figure 8). The increase in fluorescence intensity can be observed even when the antibiotic concentration is as low as 0.05 μM, indicating that an extremely sensitive biosensor for β-lactam antibiotics has been obtained [45]. Indeed the detection of antibiotics present at trace levels in food (e.g. milk) is an important application since the ubiquitous presence of antibiotics can have serious health and economical consequences ranging from allergic reactions and the evolution of antibiotic-resistant bacteria [41] to serious problems in the dairy industry.

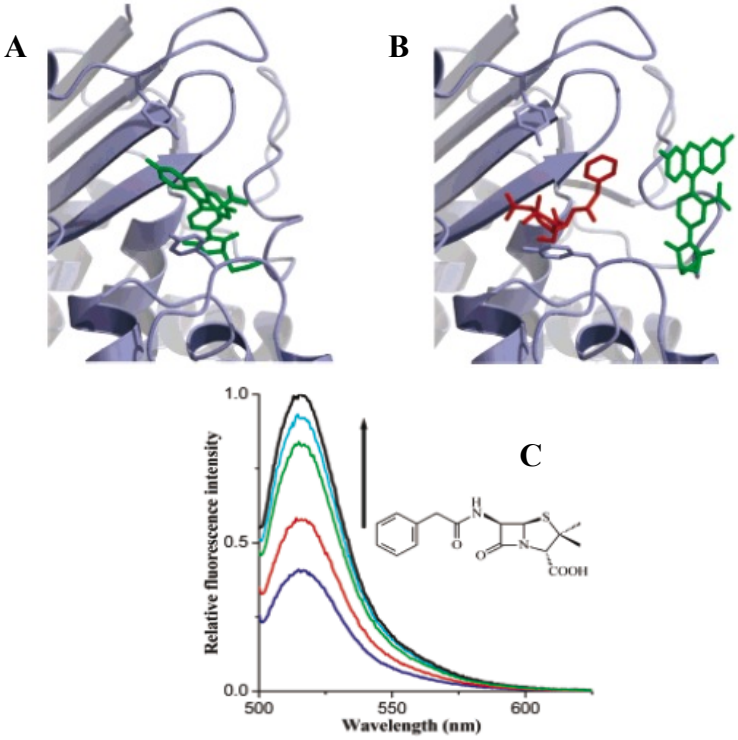

Figure 8. (A) Molecular models of the fluorescein label (green) on E166Cf before and (B) after binding with penicillin G (red). (C) Fluorescence spectra of E166Cf in the presence of penicillin G. Red, green, light blue, and black, after addition of 0.1, 1.0, 10, 100 μM antibiotics respectively [41].

In another interesting work, Legendre and coworkers attempted to induce conformational changes in β-lactamases to study protein interactions [46]. Their strategy was to chemically modify the TEM-1 β-lactamase by coupling of a hapten molecule in order to create a conjugate whose activity is regulated by the binding of an antihapten antibody. Peptide mimotopes were chosen as haptens. They are nonlinear protein epitopes selected from phage display libraries [47]. These peptides bind to antibodies or to other proteins with high affinity. This technology was used to insert random peptides libraries near the active site of TEM-1 displayed on the phage fd in fusion to the coat protein pIII and to select variants in which the engineered mimotopes serve as binding sites for antibodies directed against antigens of interest. Combination of both elements (insertion of degenerate sequences and *in vivo* selection of active clones) permitted the production of enzyme libraries in which a large variety of antigen epitopes or mimotopes were perfectly integrated within the protein structure. The binding of antibodies to the antigen molecule was demonstrated to affect the β-lactamase activity.

5. THE USE OF β-LACTAMASES IN IMMUNOASSAYS

Utilization of enzyme immunoassays (EIAs) is widespread for the detection and/or quantification of small organic analytes (e.g. drugs, hormones) [46] or for the recognition of microbial antigens in clinical specimens. EIA systems utilize enzyme-catalysed reactions to measure the binding of antigens to specific antibodies [48-49]. The performance characteristics of EIAs are thus determined to a great extent by the reaction kinetics of the enzyme-substrate system used as the indicator [50]. Enzymes which are usually used in these assays include alkaline phosphatise [51], horseradish peroxidase [52], and β-galactosidase [53]. These enzymes have been chosen because they can be linked to immunoglobulin molecules to yield stable antibody-enzyme conjugates which retain a substantial portion of the enzymatic and antigen-binding activities of the original reagents [54]. β-lactamases represent another group of enzymes useful for EIA systems and present several advantages. They are easily available and exhibit favourable reaction kinetics with a wide range of available substrates devoid of mutagenic or carcinogenic potential [55-56]. In addition, β-lactamases can be linked to biotin, thus allowing their use in avidin-biotin systems [48, 57-58]. A few examples in which β-lactamases have been successfully implemented into EIAs are described.

β-lactamase enzyme-linked immunosorbent assays (ELISA) for various reproductive hormones have been developed and were found to be quite useful in fertility related problems. This system has now replaced radioimmunoassay (RIAs) which use radioactive labels. The short half-life of I^{125}-labeled hormones and the potential of health hazards in handling and disposing of gamma ray emitters limit the useful lifetime of a kit and increase its cost, thereby hindering its general application [59]. In this context, Shrivastav and co-workers used β-lactamase EIAs to detect prolactin in plasma at concentrations as low as 2,5 ng/ml [60]. This assay has been used to detect and estimate a wide range of β-lactamase labelled hormones and steroids in plasma such as testosterone derivatives [61-62], cortisol [59], human chorionic gonadotropin [63], estradiol, lutropin, follitropin [60] with a high sensitivity and a high specificity. Furthermore a prototype of a ready-to-use β-lactamase based ELISA kit has been developed by Khatkhatay and co-workers [64].

As mentioned above, the noncovalent but exceptionally strong avidin-biotin interaction has been implemented in β-lactamase based immunoassays. In such an indirect procedure, biotin labelled antibodies were used [49] to quantify and specifically detect antigens of the human adenoviruses and the rotavirus [54].

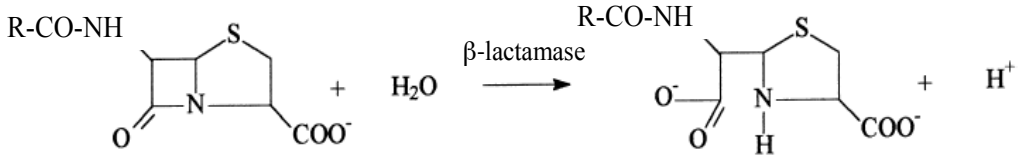

Figure 9. Schematic representation of a β-lactam antibiotic hydrolysis by a β-lactamase accompanied by the release of a proton.

Other interesting β-lactamase-based immunoassays are those implementing the biosensor technology. The enzyme-based biosensors technology relies on two operational mechanisms. The first one involves the catalytic transformation of a non-detectable molecule into a detectable form. The second mechanism involves the detection of molecules that modulate the enzyme activity. Among the various detection methods, the potentiometric measurements rely on the development of charges related to the reporter enzyme activity. Most potentiometric biosensors for detection of environmental analytes have used enzymes that catalyze the appearance or disappearance of protons. For instance, phosphoric and carbamic pesticides can be evaluated through the use of a pH electrode that measures the activity of acetylcholinesterase [65-66]. In this approach, polyalanine (PANI) is an efficient polymer for developing biosensors because its redox potential, conductivity and adsorption wavelength are modified in response to a pH variation [67-69]. In this context, since the hydrolysis of β-lactams generates protons, β-lactamases are good candidates for the development of enzyme-based biosensors (Figure 9).

A good example of application of these biosensors has been described by Sergeyeva and coworkers in which a β-lactamase label-based potentiometric biosensor for the detection of α-2 interferon was used consisting of a highly specific, stable and miniature electrochemical biosensor based on pH-sensitive field effect transistors (pH-FET) [70]. A specific sensing element was built by immobilizing α-2 interferon on the gate of a pH-FET. The interaction between anti-interferon antibodies labeled β-lactamase and interferon-pH-FET (in the presence of a specific enzyme substrate) leads to a local pH-change at the surface of the transducer and produces an electrochemical signal which is proportional to the conjugate concentration.

Gaudin and coworkers also used a similar β-lactamase-based biosensor immunoassay for the screening of penicillins residues in milk [71].

6. IMPLEMENTATION OF β-LACTAMASES INTO THE PHAGE DISPLAY TECHNOLOGY

Phage display is a powerful technology to study protein/ligand interactions. In this technique, peptides, proteins or protein fragments are expressed on the surface of phage particles in fusion with one of their coat proteins [72]. The advantage of this system rests on the physical link between the heterologous polypeptide expressed at the surface of the phage and the corresponding nucleotide sequence cloned into the phagemid genome. The ability of each phage to individually replicate and to display a specific polypeptide on its surface allows a high throughput screening of libraries of variant nucleotide sequences with diversities from 10^6 to 10^{10}. This technology is of particular interest to study protein interactions and offers many advantages: (i) the peptides, proteins, or protein fragments expressed at the surface of phages particles are solvent-exposed and thus accessible for interaction with their targets; (ii) the bacteriophage can easily be purified; and (iii) there is a physical link between the phenotype and the corresponding genotype. However, phage display presents a few bottlenecks: (i) the lack of an easy and rapid quality control of the representativeness of the initial library; (ii) the lack of a direct assessment of the enrichment level of libraries during the successive rounds of panning without interfering with the phage elution step and finally

(iii) the large nucleotidic sequencing efforts necessary to characterize the selected epitope. This can be time-consuming and laborious.

To overcome these difficulties, Legendre and co-workers proposed to combine the phage display technology with the enzymatic activity of the *E.coli* TEM-1 β-lactamase. They identified two loops near the active site of the enzyme in which small peptides could be inserted, replacing one or two codons of the TEM-1 gene [73]. They displayed libraries of hybrid β-lactamases on the surface of phages and selected them for affinity towards specific targets. Allosteric regulations (inhibition or activation) of the enzyme activity were observed for some hybrid proteins upon interaction with their specific targets [46]. This coupling between the phage display technology and the enzymatic β-lactamase activity presents several advantages: (i) it decreases the conformational freedom of the displayed inserted protein fragment; (ii) it protects the inserted polypeptide from proteolytic cleavage; (iii) it allows to directly assess the interaction of the inserted fragments using the β-lactamase as a reporter activity; (iv) the enzymatic activity can also be used to monitor the enrichment of the libraries during the successive selection rounds; and finally (v) it facilitates the determination of the best parameters of the assay as well as the optimization of the experimental procedure. Recently Filée and co-workers have implemented this coupling between phage display and the so-called "bifunctional hybrid β-lactamase technology" (BHP) to perform epitope mapping. Furthermore, they validated the use of these combined technologies to methods relying on both potentiometric biosensors and DNA-arrays (publication in preparation).

Epitope Mapping

In immunology, epitope mapping is used to study antigen/antibody (B-cell epitope) or antigen/T cell receptor (T cell epitope) interactions, develop immunoassays, identify neutralising sites, produce protective peptide vaccines, develop hypoallergens, investigate the pathogenesis of autoimmune diseases, etc. Chevigné and co-workers demonstrated that using the hybrid β-lactamase technology coupled with phage display could not only identify protein regions involved in protein-protein interactions but also supply useful molecules in which these regions are linked to an enzymatic activity [74]. They validated this approach by preparing an influenza virus hemagglutinin (HA1) gene fragment library inserted into the solvent-exposed permissive loop of the *Bacillus licheniformis* BlaP β-lactamase (position 198). Hybrid β-lactamases, fused to protein pIII of the fdTet phage, were exposed on the surface of phage particles. This library was successfully used to select a linear epitope recognised by a monoclonal antibody. The procedure alternating successive affinity selection of phages and growth of phage infected cells in the presence of β-lactams allows the selection of bifunctional hybrid β-lactamases with high affinity for the target ligand (figure 10). Furthermore they demonstrated that modifying the β-lactam concentration in the culture medium results in a positive selection of hybrid β-lactamases that retain a native-like catalytic efficiency. This is a useful tool to accelerate the convergent selection of bifunctional β-lactamases.

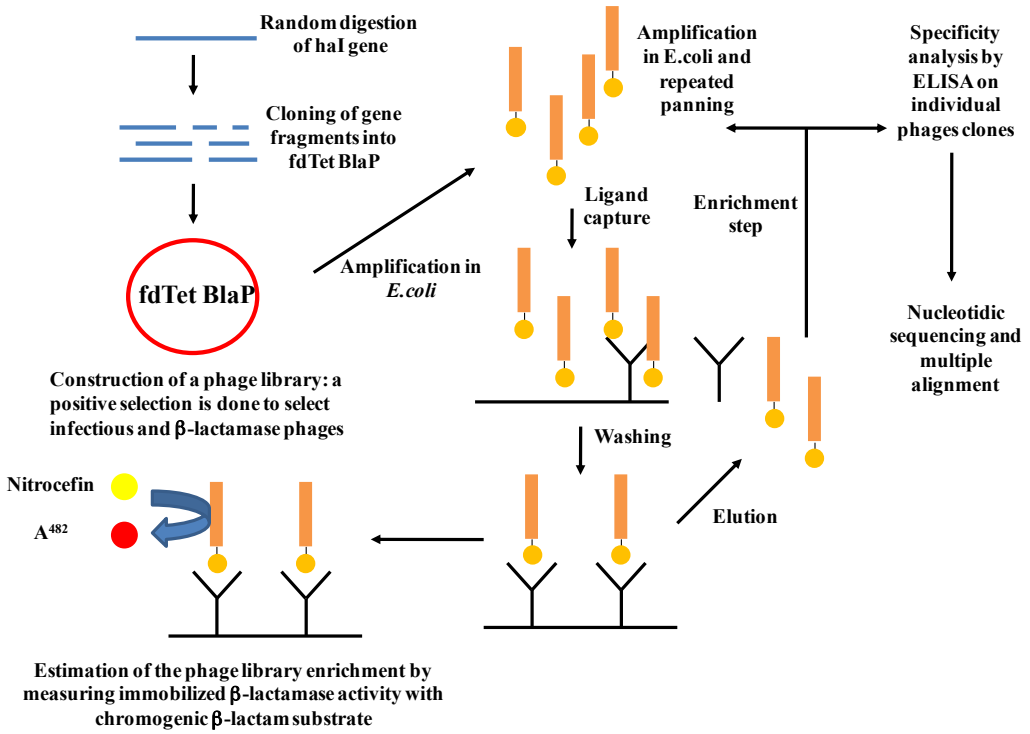

Figure 10. Schematic representation of the epitope mapping procedure [74].

Biosensor

As mentioned above, since the hydrolysis of β-lactams generates protons, β-lactamases are good candidates for the development of enzyme-based biosensors. Recently, Ruth and co-workers combined the phage display-BHP technology with potentiometric biosensor and validated this system for the epitope mapping of the human epidermal growth factor receptor (he*gfr*) (submitted publication). The h*egfr* cDNA was randomly digested and used to generate a random epitope library within the BlaP β-lactamase. This library, exposed on the surface of the fdTet phage, was screened by affinity selection against mouse monoclonal anti-human EGFr using phage display. The biopanning was successfully achieved by coating the anti-EGFr antibodies on a biosensor platform composed of a carbon electrode coated with a thin layer of polyalanine (PANI) (Figure 11). They demonstrated that the release of protons during benzylpenicillin hydrolysis induces a detectable change of electrode potential between the PANI-coated carbon and calomel reference electrodes upon specific binding of various monoclonal antibodies to phages harbouring the antibodies related epitopes. This indicated that biofunctionnalized PANI-electrode constitutes a suitable platform for performing biological screening with the advantage that this type of measurement is less time consuming than the colorimetric assay.

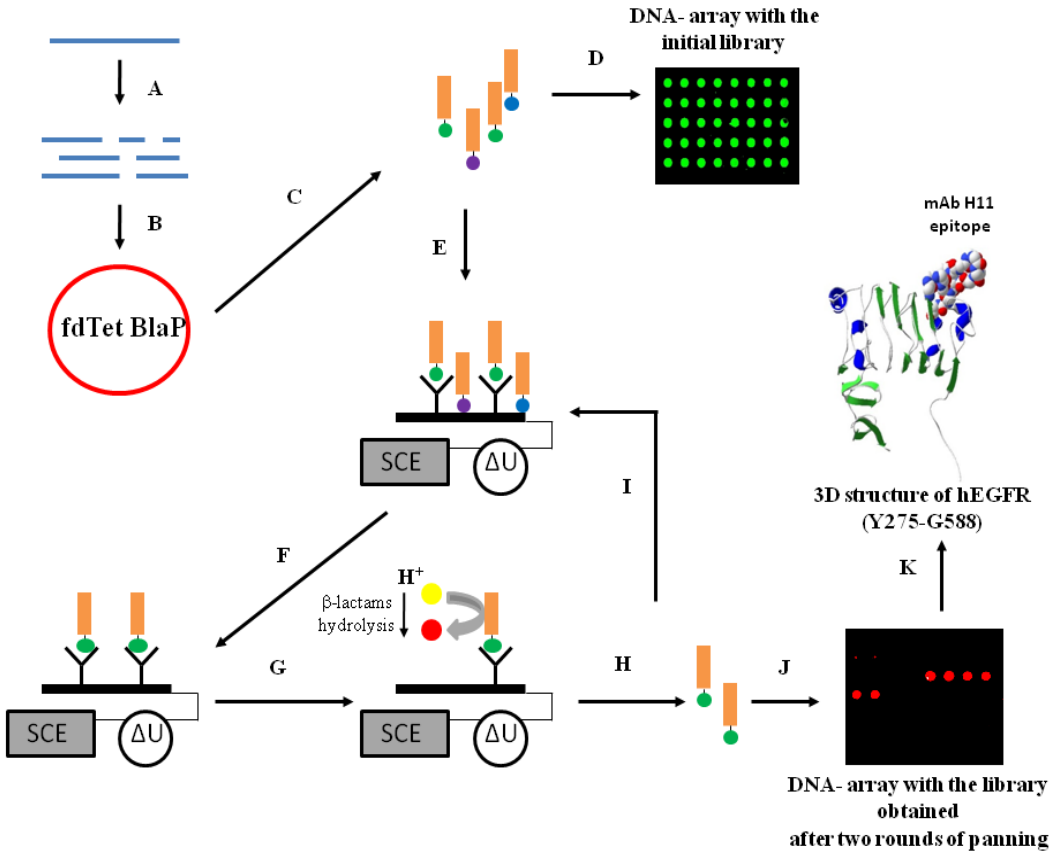

Figure 11. Schematic representation of the epitope mapping procedure combining the combinatorial search for target domains based on hybrid protein synthesis, Phage display, Potentiometric biosensor and DNA-array. SCE: saturated calomel electrode. ΔU: electrical potential variation. A: PCR amplification of the gene coding for the target protein (cDNA encoding the hEGFR) and random digestion of the PCR product with DNaseI. B: cloning of the gene fragments into the fdTet BlaP phagemide. A positive selection is performed to select infectious and β-lactamase positive phages. C: amplification of phage particles in *E. coli*. D: DNA-array analysis to verify the representativeness of the initial library. E: Capture of the phages with specific antibodies (mab H11). F: washing step. G: measurement of the immobilized β-lactamase activity on the potentiometric biosensor using benzylpenicillin as substrate. H: Elution step. I: amplification of eluted phages particles in *E. coli* and performance of a new round of biopanning. J: DNA-array analysis to identify the mAb H11 epitope. K: Assignment of the mAb H11 epitope into the 3D structure of the hEGFR (Y275-G585).

DNA Arrays

The DNA-array technology is used for expression profiling, to monitor expression levels of thousands of genes simultaneously, for comparative genomic hybridization, for identification of protein binding sites or for the identification of single nucleotide polymorphism in the genome of populations. The nucleotidic material spotted on microarrays can be oligonucleotides, cDNA or PCR products. Consequently the broad diversity of nucleotidic material that can be used offers many possibilities of detection in various biological contexts [75-76]. Ruth and co-workers took advantage of the phage-display-BHP

technology to develop a new generation of DNA-array (Figure 11). Indeed, in addition to the complementation of the β-lactamase based potentiometric biosensor used to map epitope of the human epidermal growth factor receptor (h*egfr*), they constructed a so called "epitope array" also based on the combination of phage-display-BHP technologies. In practice, the "epitope-array" was created by spotting short adjacent oligonucleotides covering the coding sequence of hEGFR. Labelled probes were prepared from h*egfr* gene fragments cloned into the BlaP gene by PCR amplification incorporating dCTP-cy5 or cy-3. By combining the array results obtained with both the non-coding and coding strands of *hegfr*, they concluded that the different hEGFR protein regions displayed on phages from the initial library entirely covered the protein sequence and gave rise to β-lactamase positive and infectious phages. Moreover, the array experiments performed with phages obtained after two rounds of screening confirmed that fluorescence signals were only observed with oligonucleotides that encoded a protein region which overlapped that mapped with the multiple sequence alignment. Taking advantage of fluorescence intensities obtained on each spotted oligonucleotide, they reduced the epitope to a 28 residues polypeptide. Altogether, this indicates that the implementation of the phage display-BHP technology into DNA array presents the advantage to control the quality of the initial library and can be successfully used to identify the nucleotide sequence that encodes the polypeptide selected during the phage display procedure.

7. THE USE OF β-LACTAMASE IN DNA/PROTEIN VACCINATION

Another interesting application field of β-lactamases is their use as hapten-carrier proteins to develop vaccines against non-immunogenic proteins. A good example is the work described by Ruth and co-workers who developed a DNA vaccination system against the heat-stable enterotoxin STa from *Escherichia coli* [77]. This toxin is a major cause of enteric colibacillosis, characterized by profuse fluid diarrhoea leading to dehydration and even death of newborn calves and piglets [78-79]. Because of its small size, STa is poorly antigenic and not immunogenic in its native form. Several approaches were explored to obtain non-toxic immunogenic molecules for safe vaccine design. Several proteins were chemically coupled to STa in order to increase the immunogenicity of the enterotoxin, including bovine serum albumin [80], the B-subunit of cholera toxin (CTB) [81] and the heat-labile enterotoxin [81-82]. Some hybrid fusion proteins were constructed between STa and the major subunit ClpG of the *E.coli* surface antigen (ClpG-ST) [83], between STa and one or more subunits of the cholera toxin [84-85] and between STa and LT (labile toxin) [86-87]. The OmpC outer-membrane protein and the flagellin have also been used as carriers for STa [88-89]. However, in general, these constructions failed to elicit the production of neutralizing antibodies or retained some degree of STa-associated toxicity, probably because the immunogenic properties of the toxin are influenced by the conformation associated with toxicity. In consequence, Ruth and co-workers decided to insert the STa toxin in the solvent-exposed permissive loop (position 195) of the *E.coli* TEM-1 β-lactamase in order to enhance its immunogenic properties. Furthermore, since the production and purification of hybrid protein vaccines is labour intensive and expensive, they choose to validate this system using the DNA vaccine technology. Indeed, DNA vaccines are stable, easy to produce, and potent tools for inducing both humoral and cell-mediated immune responses [90-91]. Moreover, immune

responses induced by DNA vaccines may display a broader epitopic repertoire than immune responses induced by traditional peptide or protein vaccines [92-93]. In this context, Ruth et al genetically inserted the nucleotide sequence of STa into the TEM-1 gene and cloned the constructed hybrid gene into an appropriate plasmid DNA expression vector (V1Jns.tPA vector). The plasmids were tested for their capacity to induce a TEM-1 and STa specific immune response in a mice model. No STa specific IgG could be detected after three DNA immunizations. But a boost with the oligopeptide toxin, which is not immunogenic in its native form, induced high STa specific antibody titres. This demonstrated that immunization with plasmid DNA encoding hybrid hapten-carrier proteins, in this case a β-lactamase with the *E.coli* STa insert, can be used as a priming stimulus to provide an antibody response against a non-immunogenic peptide.

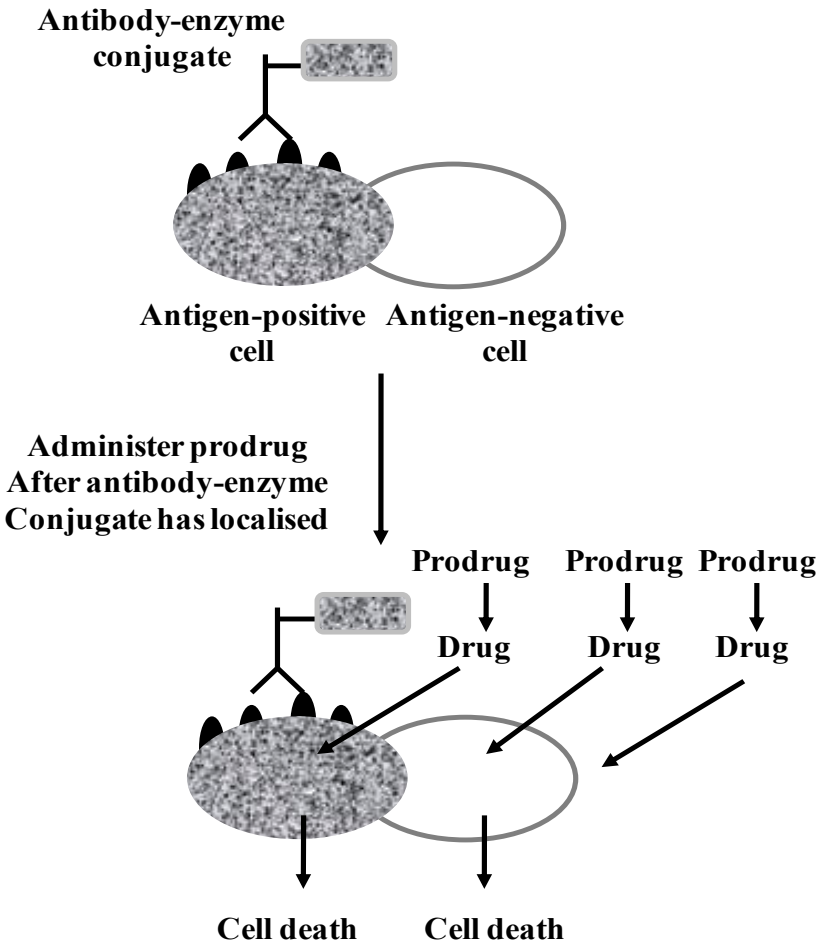

Figure 12. Schematic representation of the antibody-directed enzyme prodrug therapy (ADEPT) in which enzymes are localized to the surface of the target cell where they activate systemically administered prodrugs. The toxic drug can then diffuse inside the tumor [94].

8. β-LACTAMASES AS AN ANTIBODY DIRECTED ENZYME IN PRODRUG THERAPY

The toxicities associated with conventional cancer chemotherapy arise primarily from the lack of specificity for tumour cells [94]. Most of the presently available drugs are designed to be selectively toxic for rapidly dividing cells [95]. A variety of approaches are under development to improve the efficency and tumor cell specificity of cancer treatments. Many of these involve monoclonal antibodies and offer attractive means of directing toxic agents such as drugs, radioisotopes, protein cytotoxins, cytokines, effector cells of the immune system and enzymes to tumor cells. In one approach, known as antibody-directed enzyme prodrug therapy (ADEPT) (Figure 12), enzymes are delivered to tumor cells via the recognition of tumor associated antigens (by a specific antibody conjugated to the enzyme) where they catalyse the activation of a pro-drug exactly at the site of tumor [94].

This method is now well advanced, with many examples of systems already tested in preclinical models and some entering clinical trials. Enzymes usually utilized in ADEPT include alkaline phosphatase, carboxypeptidases, β-glucosidases and β-lactamases. Many β-lactamases hydrolyse a wide range of cephalosporins with 3' leaving groups which are released upon hydrolysis of the β-lactam ring. Various toxic compounds can thus be released from cephalosporin prodrugs, including nitrogen mustard and vinca alkaloid derivatives [96]. Moreover, eukaryotic cells do not produce β-lactamases related enzymes which could cause unwanted activation of prodrugs *in vivo*. A cocktail of different prodrugs can be activated by β-lactamases to release a panel of active drugs which can act independently or synergistically. These reasons explain why β-lactamases are good candidates for this particular application, and examples of their *in vitro* utilization, with the L6 monoclonal antibody, have demonstrated good targeted prodrug activation [97]. The prodrug tested was cephalosporin mustard (CM) which was designed as an anticancer prodrug that can be activated by a site-specific L6 monoclonal antibody-β-lactamase conjugates targeted to antigens present on tumour cell surfaces. The antibody-β-lactamase conjugate catalyzes the release of the active drug phenyl mustard (PDM). Similar antibodies - β - lactamase conjugates have also been assayed with different prodrugs [98]. More recently, Roberge and co-workers described the construction of a novel antibody-enzyme fusion protein for use in ADEPT [99]. The fusion protein combines the proven tumour specificity of the CC49 antibody [100] with β-lactamase. But this first generation of fusion proteins is rapidly degraded when expressed in *E.coli*. Optimization of the antibody fragment by combinatorial consensus mutagenesis (CCM), yielded an improved variant exhibiting significant increased stability allowing its use for ADEPT.

REFERENCES

[1] Philippon, A., J. Dusart, B. Joris, and J.M. Frere, *The diversity, structure and regulation of beta-lactamases.* Cell Mol Life Sci, 1998. 54: 341-6.

[2] Matagne, A., A. Dubus, M. Galleni, and J.M. Frere, *The beta-lactamase cycle: a tale of selective pressure and bacterial ingenuity.* Nat Prod Rep, 1999. 16: 1-19.

[3] Naqvi, T. and R. Singh, *A novel chemiluminescent substrate for detecting lactamase.* Mol Biosyst, 2007. 3: 431-8.

[4] Galarneau, A., M. Primeau, L.E. Trudeau, and S.W. Michnick, *Beta-lactamase protein fragment complementation assays as in vivo and in vitro sensors of protein protein interactions.* Nat Biotechnol, 2002. 20: 619-22.

[5] Frate, M.C., E.J. Lietz, J. Santos, J.P. Rossi, A.L. Fink, and M.R. Ermacora, *Export and folding of signal-sequenceless Bacillus licheniformis beta-lactamase in Escherichia coli.* Eur J Biochem, 2000. 267: 3836-47.

[6] Bebrone, C., C. Moali, F. Mahy, S. Rival, J.D. Docquier, G.M. Rossolini, J. Fastrez, R.F. Pratt, J.M. Frere, and M. Galleni, *CENTA as a chromogenic substrate for studying beta-lactamases.* Antimicrob Agents Chemother, 2001. 45: 1868-71.

[7] Vrudhula, V.M., D.E. Kerr, N.O. Siemers, G.M. Dubowchik, and P.D. Senter, *Cephalosporin prodrugs of paclitaxel for immunologically specific activation by L-49-sFv-beta-lactamase fusion protein.* Bioorg Med Chem Lett, 2003. 13: 539-42.

[8] Lee, M., D. Hesek, and S. Mobashery, *A practical synthesis of nitrocefin.* J Org Chem, 2005. 70: 367-9.

[9] Zlokarnik, G., P.A. Negulescu, T.E. Knapp, L. Mere, N. Burres, L. Feng, M. Whitney, K. Roemer, and R.Y. Tsien, *Quantitation of transcription and clonal selection of single living cells with beta-lactamase as reporter.* Science, 1998. 279: 84-8.

[10] Alam, J. and J.L. Cook, *Reporter genes: application to the study of mammalian gene transcription.* Anal Biochem, 1990. 188: 245-54.

[11] Cubitt, A.B., R. Heim, S.R. Adams, A.E. Boyd, L.A. Gross, and R.Y. Tsien, *Understanding, improving and using green fluorescent proteins.* Trends Biochem Sci, 1995. 20: 448-55.

[12] Qureshi, S.A., *Beta-lactamase: an ideal reporter system for monitoring gene expression in live eukaryotic cells.* Biotechniques, 2007. 42: 91-6.

[13] Kunapuli, P., R. Ransom, K.L. Murphy, D. Pettibone, J. Kerby, S. Grimwood, P. Zuck, P. Hodder, R. Lacson, I. Hoffman, J. Inglese, and B. Strulovici, *Development of an intact cell reporter gene beta-lactamase assay for G protein-coupled receptors for high-throughput screening.* Anal Biochem, 2003. 314: 16-29.

[14] Hribar, G., V. Smilovic, A.L. Zupan, and V. Gaberc-Porekar, *Beta-lactamase reporter system for selecting high-producing yeast clones.* Biotechniques, 2008. 44: 477-8, 480, 482 passim.

[15] Zuck, P., E.M. Murray, E. Stec, J.A. Grobler, A.J. Simon, B. Strulovici, J. Inglese, O.A. Flores, and M. Ferrer, *A cell-based beta-lactamase reporter gene assay for the identification of inhibitors of hepatitis C virus replication.* Anal Biochem, 2004. 334: 344-55.

[16] Raz, E., G. Zlokarnik, R.Y. Tsien, and W. Driever, *beta-lactamase as a marker for gene expression in live zebrafish embryos.* Dev Biol, 1998. 203: 290-4.

[17] McCann, J.R., J.A. McDonough, M.S. Pavelka, and M. Braunstein, *Beta-lactamase can function as a reporter of bacterial protein export during Mycobacterium tuberculosis infection of host cells.* Microbiology, 2007. 153: 3350-9.

[18] Finlay, B.B. and S. Falkow, *Common themes in microbial pathogenicity revisited.* Microbiol Mol Biol Rev, 1997. 61: 136-69.

[19] Braunstein, M., T.I. Griffin, J.I. Kriakov, S.T. Friedman, N.D. Grindley, and W.R. Jacobs, Jr., *Identification of genes encoding exported Mycobacterium tuberculosis*

proteins using a Tn552'phoA in vitro transposition system. J Bacteriol, 2000. 182: 2732-40.

[20] Chubb, A.J., Z.L. Woodman, F.M. da Silva Tatley, H.J. Hoffmann, R.R. Scholle, and M.R. Ehlers, *Identification of Mycobacterium tuberculosis signal sequences that direct the export of a leaderless beta-lactamase gene product in Escherichia coli.* Microbiology, 1998. 144 (Pt 6): 1619-29.

[21] Downing, K.J., R.A. McAdam, and V. Mizrahi, *Staphylococcus aureus nuclease is a useful secretion reporter for mycobacteria.* Gene, 1999. 239: 293-9.

[22] Simonen, M., E. Jamsa, and M. Makarow, *The role of the carrier protein and disulfide formation in the folding of beta-lactamase fusion proteins in the endoplasmic reticulum of yeast.* J Biol Chem, 1994. 269: 13887-92.

[23] Broome-Smith, J.K., M. Tadayyon, and Y. Zhang, *Beta-lactamase as a probe of membrane protein assembly and protein export.* Mol Microbiol, 1990. 4: 1637-44.

[24] Hardt, K., B. Joris, S. Lepage, R. Brasseur, J.O. Lampen, J.M. Frere, A.L. Fink, and J.M. Ghuysen, *The penicillin sensory transducer, BlaR, involved in the inducibility of beta-lactamase synthesis in Bacillus licheniformis is embedded in the plasma membrane via a four-alpha-helix bundle.* Mol Microbiol, 1997. 23: 935-44.

[25] Hallet, B., D.J. Sherratt, and F. Hayes, *Pentapeptide scanning mutagenesis: random insertion of a variable five amino acid cassette in a target protein.* Nucleic Acids Res, 1997. 25: 1866-7.

[26] Betton, J.M., J.P. Jacob, M. Hofnung, and J.K. Broome-Smith, *Creating a bifunctional protein by insertion of beta-lactamase into the maltodextrin-binding protein.* Nat Biotechnol, 1997. 15: 1276-9.

[27] Collinet, B., M. Herve, F. Pecorari, P. Minard, O. Eder, and M. Desmadril, *Functionally accepted insertions of proteins within protein domains.* J Biol Chem, 2000. 275: 17428-33.

[28] Vandevenne, M., P. Filee, N. Scarafone, B. Cloes, G. Gaspard, N. Yilmaz, M. Dumoulin, J.M. Francois, J.M. Frere, and M. Galleni, *The Bacillus licheniformis BlaP beta-lactamase as a model protein scaffold to study the insertion of protein fragments.* Protein Sci, 2007. 16: 2260-71.

[29] Hashimoto, M., T. Ikegami, S. Seino, N. Ohuchi, H. Fukada, J. Sugiyama, M. Shirakawa, and T. Watanabe, *Expression and characterization of the chitin-binding domain of chitinase A1 from Bacillus circulans WL-12.* J Bacteriol, 2000. 182: 3045-54.

[30] Tomme, P., A.L. Creagh, D.G. Kilburn, and C.A. Haynes, *Interaction of polysaccharides with the N-terminal cellulose-binding domain of Cellulomonas fimi CenC. 1. Binding specificity and calorimetric analysis.* Biochemistry, 1996. 35: 13885-94.

[31] Wehrman, T., B. Kleaveland, J.H. Her, R.F. Balint, and H.M. Blau, *Protein-protein interactions monitored in mammalian cells via complementation of beta -lactamase enzyme fragments.* Proc Natl Acad Sci U S A, 2002. 99: 3469-74.

[32] Barnard, E., McFerran, N.V., Nelson, J. and Timson, D.J., *Detection of Protein-Protein Interactions Using Protein-Fragment Complementation Assays (PCA).* Current Proteomics, 2007. 4: 17-27.

[33] Lofdahl, P.A., O. Nord, L. Janzon, and P.A. Nygren, *Selection of TNF-alpha binding affibody molecules using a beta-lactamase protein fragment complementation assay.* N Biotechnol, 2009. 26: 251-9.

[34] Lee, H.K., S.J. Brown, H. Rosen, and P.S. Tobias, *Application of beta-lactamase enzyme complementation to the high-throughput screening of toll-like receptor signaling inhibitors.* Mol Pharmacol, 2007. 72: 868-75.

[35] LaVallie, E.R. and J.M. McCoy, *Gene fusion expression systems in Escherichia coli.* Curr Opin Biotechnol, 1995. 6: 501-6.

[36] Tsunoda, Y., N. Sakai, K. Kikuchi, S. Katoh, K. Akagi, J. Miura-Ohnuma, Y. Tashiro, K. Murata, N. Shibuya, and E. Katoh, *Improving expression and solubility of rice proteins produced as fusion proteins in Escherichia coli.* Protein Expr Purif, 2005. 42: 268-77.

[37] Vandevenne, M., G. Gaspard, N. Yilmaz, F. Giannotta, J.M. Frere, M. Galleni, and P. Filee, *Rapid and easy development of versatile tools to study protein/ligand interactions.* Protein Eng Des Sel, 2008. 21: 443-51.

[38] Tjoelker, L.W., L. Gosting, S. Frey, C.L. Hunter, H.L. Trong, B. Steiner, H. Brammer, and P.W. Gray, *Structural and functional definition of the human chitinase chitin-binding domain.* J Biol Chem, 2000. 275: 514-20.

[39] Ujita, M., K. Sakai, K. Hamazaki, M. Yoneda, S. Isomura, and A. Hara, *Carbohydrate binding specificity of the recombinant chitin-binding domain of human macrophage chitinase.* Biosci Biotechnol Biochem, 2003. 67: 2402-7.

[40] Doi, N. and H. Yanagawa, *Design of generic biosensors based on green fluorescent proteins with allosteric sites by directed evolution.* FEBS Lett, 1999. 453: 305-7.

[41] Chan, P.H., H.B. Liu, Y.W. Chen, K.C. Chan, C.W. Tsang, Y.C. Leung, and K.Y. Wong, *Rational design of a novel fluorescent biosensor for beta-lactam antibiotics from a class A beta-lactamase.* J Am Chem Soc, 2004. 126: 4074-5.

[42] de Lorimier, R.M., J.J. Smith, M.A. Dwyer, L.L. Looger, K.M. Sali, C.D. Paavola, S.S. Rizk, S. Sadigov, D.W. Conrad, L. Loew, and H.W. Hellinga, *Construction of a fluorescent biosensor family.* Protein Sci, 2002. 11: 2655-75.

[43] Banerjee, S., U. Pieper, G. Kapadia, L.K. Pannell, and O. Herzberg, *Role of the omega-loop in the activity, substrate specificity, and structure of class A beta-lactamase.* Biochemistry, 1998. 37: 3286-96.

[44] Guillaume, G., M. Vanhove, J. Lamotte-Brasseur, P. Ledent, M. Jamin, B. Joris, and J.M. Frere, *Site-directed mutagenesis of glutamate 166 in two beta-lactamases. Kinetic and molecular modeling studies.* J Biol Chem, 1997. 272: 5438-44.

[45] Chan, P.H., P.K. So, D.L. Ma, Y. Zhao, T.S. Lai, W.H. Chung, K.C. Chan, K.F. Yiu, H.W. Chan, F.M. Siu, C.W. Tsang, Y.C. Leung, and K.Y. Wong, *Fluorophore-labeled beta-lactamase as a biosensor for beta-lactam antibiotics: a study of the biosensing process.* J Am Chem Soc, 2008. 130: 6351-61.

[46] Legendre, D., P. Soumillion, and J. Fastrez, *Engineering a regulatable enzyme for homogeneous immunoassays.* Nat Biotechnol, 1999. 17: 67-72.

[47] Parmley, S.F. and G.P. Smith, *Antibody-selectable filamentous fd phage vectors: affinity purification of target genes.* Gene, 1988. 73: 305-18.

[48] Yolken, R.H., S.B. Wee, and M. Van Regenmortel, *The use of beta-lactamase in enzyme immunoassays for detection of microbial antigens.* J Immunol Methods, 1984. 73: 109-23.

[49] Guesdon, J.L., T. Ternynck, and S. Avrameas, *The use of avidin-biotin interaction in immunoenzymatic techniques.* J Histochem Cytochem, 1979. 27: 1131-9.
[50] Wisdom, G.B., *Enzyme-immunoassay.* Clin Chem, 1976. 22: 1243-55.
[51] Yolken, R.H.S., P.J. and Harris, C.C., ed. *Manual of clinical immunology*. Rose and H. Friedman ed., ed. W. American Society for Microbiology, D.C. 1980. 692-699.
[52] Avrameas, S., and Ternynck, T., *Peroxidase-labbeled antibody and Fab conjugates with enhanced intracellular penetration.* Immunochemistry, 1969. 8: 1175-1179.
[53] Imagawa, M., S. Yoshitake, E. Ishikawa, Y. Endo, S. Ohtaki, E. Kano, and Y. Tsunetoshi, *Highly sensitive sandwich enzyme immunoassay of human IgE with beta-D-galactosidase from Escherichia coli.* Clin Chim Acta, 1981. 117: 199-207.
[54] Yolken, R.H. and S.B. Wee, *Enzyme immunoassays in which biotinillated beta-lactamase is used for the detection of microbial antigens.* J Clin Microbiol, 1984. 19: 356-60.
[55] Citri, N., ed. *Penicillinase and other b-lactamases*. Academic Press, Inc., New York ed. The enzymes, ed. P.D. Boyer. Vol. 4. 1971. 23-46.
[56] Voogd, C.E., J.J. Van der Stel, and J.J. Jacobs, *On the mutagenic action of some enzyme immunoassay substrates.* J Immunol Methods, 1980. 36: 55-61.
[57] Yolken, R.H., F.J. Leister, L.S. Whitcomb, and M. Santosham, *Enzyme immunoassays for the detection of bacterial antigens utilizing biotin-labeled antibody and peroxidase biotin--avidin complex.* J Immunol Methods, 1983. 56: 319-27.
[58] Yolken, R.H., *Avidin-biotin radioimmunoassay for human rotavirus.* J Infect Dis, 1983. 148: 942.
[59] Shrivastav, T.G., G.L. Kumari, and P.N. Rao, *Enzyme immunoassay of cortisol in human plasma using penicillinase as label.* Clin Chim Acta, 1988. 174: 83-91.
[60] Shrivastav, T.G., P.K. Pandey, and G.L. Kumari, *Enzyme immunosorbent assay of prolactin with penicillinase as label.* Clin Chem, 1988. 34: 2205-7.
[61] Rassaie, M.J., G.L. Kumari, P.N. Rao, T.G. Shrivastav, and H.P. Pandey, *Influence of different combinations of antibodies and penicillinase-labeled testosterone derivatives on sensitivity and specificity of immunoassays.* Steroids, 1992. 57: 112-8.
[62] He, J., Y. Li, and X. Gao, *[Studies of enzyme-linked immunoassay for testosterone using penicillinase as label]*. Hua Xi Yi Ke Da Xue Xue Bao, 1994. 25: 452-5.
[63] Prasad, P.V., S.K. Chaube, T.G. Shrivastav, and G.L. Kumari, *Development of colorimetric enzyme-linked immunosorbent assay for human chorionic gonadotropin.* J Immunoassay Immunochem, 2006. 27: 15-30.
[64] Khatkhatay, M.I., M.P. Desai, G.M. Sankolli, D.K. Pardhe, and U.M. Joshi, *Stability studies of the components of a prototype penicillinase (beta-lactamase)-linked ELISA kit.* J Clin Lab Anal, 1993. 7: 95-9.
[65] Budnikov M.C., a.E., G.A., ed. *Biosensors for direct monitoring of environmental pollutants in field*. Academic publishers, London ed., ed. D. Nikolelis, Krull, U.,Wang J. and Mascini M., Kluwer. Vol. 239. 1998.
[66] Koncki R., H., A., and Glab, S., *Biochemical modifications of membrane ion-selective sensors.* Trends Anal. Chem. , 1997. 16: 528-537.
[67] Karyakin, A.A., Vuki, M., Lukachova, L.V., Karyakina, E., Orlov, L.V., Karpachova, G.P., and Wang, J.W., *Processible polyalanine as an advanced potentiometric pH transducer application to biosensors.* Anal. Chem., 1999. 71: 2534–2540.

[68] Matharu, Z., G. Sumana, S.K. Arya, S.P. Singh, V. Gupta, and B.D. Malhotra, *Polyaniline Langmuir-Blodgett film based cholesterol biosensor.* Langmuir, 2007. 23: 13188-92.

[69] Pandey, P., S.P. Singh, S.K. Arya, A. Sharma, M. Datta, and B.D. Malhotra, *Gold nanoparticle-polyaniline composite films for glucose sensing.* J Nanosci Nanotechnol, 2008. 8: 3158-63.

[70] Sergeyeva, T.A., Soldatkin, A.P., Rachkov, A.E., Tereschenko, S.A., Piletsky, S.A., El skaya, A.V., *b-lactamase label-based potentiometric biosensor for a-2 interferon detection.* Analytica Chimica Acta, 1999. 390: 73-81.

[71] Gaudin, V.a.M., P., *Development of a biosensor-based immunoassay for the screening of penicillins residues in milk.* Food and Agricultural Immunology, 2001. 13: 77-86.

[72] Smith, G.P., *Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface.* Science, 1985. 228: 1315-7.

[73] Legendre, D., B. Vucic, V. Hougardy, A.L. Girboux, C. Henrioul, J. Van Haute, P. Soumillion, and J. Fastrez, *TEM-1 beta-lactamase as a scaffold for protein recognition and assay.* Protein Sci, 2002. 11: 1506-18.

[74] Chevigne, A., N. Yilmaz, G. Gaspard, F. Giannotta, J.M. Francois, J.M. Frere, M. Galleni, and P. Filee, *Use of bifunctional hybrid beta-lactamases for epitope mapping and immunoassay development.* J Immunol Methods, 2007. 320: 81-93.

[75] Kulesh, D.A., D.R. Clive, D.S. Zarlenga, and J.J. Greene, *Identification of interferon-modulated proliferation-related cDNA sequences.* Proc Natl Acad Sci U S A, 1987. 84: 8453-7.

[76] Schena, M., D. Shalon, R.W. Davis, and P.O. Brown, *Quantitative monitoring of gene expression patterns with a complementary DNA microarray.* Science, 1995. 270: 467-70.

[77] Ruth, N., J. Mainil, V. Roupie, J.M. Frere, M. Galleni, and K. Huygen, *DNA vaccination for the priming of neutralizing antibodies against non-immunogenic STa enterotoxin from enterotoxigenic Escherichia coli.* Vaccine, 2005. 23: 3618-27.

[78] Holland, R.E., *Some infectious causes of diarrhea in young farm animals.* Clin Microbiol Rev, 1990. 3: 345-75.

[79] Nagy, B. and P.Z. Fekete, *Enterotoxigenic Escherichia coli (ETEC) in farm animals.* Vet Res, 1999. 30: 259-84.

[80] Svennerholm, A.M., M. Wikstrom, M. Lindblad, and J. Holmgren, *Monoclonal antibodies against Escherichia coli heat-stable toxin (STa) and their use in a diagnostic ST ganglioside GM1-enzyme-linked immunosorbent assay.* J Clin Microbiol, 1986. 24: 585-90.

[81] Klipstein, F.A., R.F. Engert, and R.A. Houghten, *Properties of cross-linked toxoid vaccines made with hyperantigenic forms of synthetic Escherichia coli heat-stable toxin.* Infect Immun, 1984. 44: 268-73.

[82] Klipstein, F.A., R.F. Engert, J.D. Clements, and R.A. Houghten, *Vaccine for enterotoxigenic Escherichia coli based on synthetic heat-stable toxin crossed-linked to the B subunit of heat-labile toxin.* J Infect Dis, 1983. 147: 318-26.

[83] Batisson, I. and M. Der Vartanian, *Contribution of defined amino acid residues to the immunogenicity of recombinant Escherichia coli heat-stable enterotoxin fusion proteins.* FEMS Microbiol Lett, 2000. 192: 223-9.

[84] Sanchez, J., A.M. Svennerholm, and J. Holmgren, *Genetic fusion of a non-toxic heat-stable enterotoxin-related decapeptide antigen to cholera toxin B-subunit.* FEBS Lett, 1988. 241: 110-4.

[85] Sanchez, J., R. Argotte, and A. Buelna, *Engineering of cholera toxin A-subunit for carriage of epitopes at its amino end.* FEBS Lett, 1997. 401: 95-7.

[86] Sanchez, J., B.E. Uhlin, T. Grundstrom, J. Holmgren, and T.R. Hirst, *Immunoactive chimeric ST-LT enterotoxins of Escherichia coli generated by in vitro gene fusion.* FEBS Lett, 1986. 208: 194-8.

[87] Aitken, R. and T.R. Hirst, *Recombinant enterotoxins as vaccines against Escherichia coli-mediated diarrhoea.* Vaccine, 1993. 11: 227-33.

[88] Saarilahti, H.T., E.T. Palva, J. Holmgren, and J. Sanchez, *Fusion of genes encoding Escherichia coli heat-stable enterotoxin and outer membrane protein OmpC.* Infect Immun, 1989. 57: 3663-5.

[89] Pereira, C.M., B.E. Guth, M.E. Sbrogio-Almeida, and B.A. Castilho, *Antibody response against Escherichia coli heat-stable enterotoxin expressed as fusions to flagellin.* Microbiology, 2001. 147: 861-7.

[90] Gurunathan, S., D.M. Klinman, and R.A. Seder, *DNA vaccines: immunology, application, and optimization*.* Annu Rev Immunol, 2000. 18: 927-74.

[91] Donnelly, J.J., J.B. Ulmer, and M.A. Liu, *DNA vaccines.* Life Sci, 1997. 60: 163-72.

[92] Denis, O., A. Tanghe, K. Palfliet, F. Jurion, T.P. van den Berg, A. Vanonckelen, J. Ooms, E. Saman, J.B. Ulmer, J. Content, and K. Huygen, *Vaccination with plasmid DNA encoding mycobacterial antigen 85A stimulates a CD4+ and CD8+ T-cell epitopic repertoire broader than that stimulated by Mycobacterium tuberculosis H37Rv infection.* Infect Immun, 1998. 66: 1527-33.

[93] Chen, Y., R.G. Webster, and D.L. Woodland, *Induction of CD8+ T cell responses to dominant and subdominant epitopes and protective immunity to Sendai virus infection by DNA vaccination.* J Immunol, 1998. 160: 2425-32.

[94] Deonarain, M.P. and A.A. Epenetos, *Targeting enzymes for cancer therapy: old enzymes in new roles.* Br J Cancer, 1994. 70: 786-94.

[95] Valeriote, F. and L. van Putten, *Proliferation-dependent cytotoxicity of anticancer agents: a review.* Cancer Res, 1975. 35: 2619-30.

[96] Alexander, R.P., Beeley, N., Odriscoll, M., Oneill, F.P., Pratt, A.T. and Willenbrock, F.W., *Cephalosporin nitrogen mustard carbamate prodrugs.* Tetrahedron. Lett., 1991. 32: 3269-3272.

[97] Svensson, H.P., J.F. Kadow, V.M. Vrudhula, P.M. Wallace, and P.D. Senter, *Monoclonal antibody-beta-lactamase conjugates for the activation of a cephalosporin mustard prodrug.* Bioconjug Chem, 1992. 3: 176-81.

[98] Meyer, D.L., L.N. Jungheim, S.D. Mikolajczyk, T.A. Shepherd, J.J. Starling, and C.N. Ahlem, *Preparation and characterization of a beta-lactamase-Fab' conjugate for the site-specific activation of oncolytic agents.* Bioconjug Chem, 1992. 3: 42-8.

[99] Roberge, M., M. Estabrook, J. Basler, R. Chin, P. Gualfetti, A. Liu, S.B. Wong, M.H. Rashid, T. Graycar, L. Babe, and V. Schellenberger, *Construction and optimization of a CC49-based scFv-beta-lactamase fusion protein for ADEPT.* Protein Eng Des Sel, 2006. 19: 141-5.

[100] 1Muraro, R., M. Kuroki, D. Wunderlich, D.J. Poole, D. Colcher, A. Thor, J.W. Greiner, J.F. Simpson, A. Molinolo, P. Noguchi, and et al., *Generation and*

characterization of B72.3 second generation monoclonal antibodies reactive with the tumor-associated glycoprotein 72 antigen. Cancer Res, 1988. 48: 4588-96.

INDEX