

EXPRESSION OF cDNA FOR MARINE ANNELID METALLOPROTEIN IN ESCHERICHIA COLI

Reçu le 15/03/1999 – Accepté le 28/05/2002

Abstract

A cDNA library of *Nereis diversicolor* coelomocytes was constructed in expression vector λ gt 11. Selection of clones of recombinant phages was performed using the immunoreactivity of an anti-metalloprotein (anti-MPII) polyclonal antibody. It is of importance to note that this selection was confirmed with the use of two anti-MPII monoclonal antibodies. Conditions for strong expression of fusion proteins by bacteria which had integrated the recombinant phages, and conditions for detection of these proteins by host cells. Nevertheless, it must be mentioned that these proteins, though altered, retained their antibacterial activity.

Key words: cDNA, Fusion protein, Marine worm, Metalloprotein, Polychaeta, *E. coli*.

Résumé

La banque d'ADNc des coelomocytes de *N. diversicolor* a été construite dans le vecteur d'expression λ gt 11. La sélection des clones de phages recombinés est obtenu par réaction immunologique d'un anticorps polyclonal anti-métalloprotéine (anti-MPII). Il est important de noter que cette sélection a été confirmée par l'utilisation de deux anticorps monoclonaux anti-MPII. Les conditions d'une forte expression des protéines de fusion par les bactéries qui ont intégré les phages recombinés, ainsi que les conditions de détection de ces protéines par l'utilisation des anticorps anti-MPII, nous conduit à évoquer une dégradation de ces protéines de fusion par les cellules hôtes. Cependant, il est à signaler que ces protéines, même altérées, conservent leur activité antibactérienne.

Mots clés: ADNc, Protéine de fusion, Ver marin, Métalloprotéine, Polychaeta, *E. coli*.

D. BENOURETH
Département de Biochimie
Faculté des Sciences
Université Badji-Mokhtar
B.P.12 - El-Hadjar
23000 Annaba, Algérie

ملخص

بنك الحمض النووي المنقوص الأكسجين المكمل لخلايا الفراغات الداخلية لـ *N. diversicolor* أنشأت و حملت على الحامض الفاجي λ gt11. اختبر نسخ الفاجات الحاملة لهذا الحمض تمت باستعمال القدرة المناعية للجسم المضاد عديد النسيالات موجه ضد البروتين المعدنية MPII. للعلم هذا الاختبار أكد باستعمال جسمين مضادين احادي النسيالات موجهين ضد نفس البروتين. الطرق المستعملة من أجل تعبير قوي للبروتينات المرتبطة من طرف البكتيريا التي تلقت الفاجات الحاملة لهذا الحمض النووي المنقوص الأكسجين المكمل و الطرق المستعملة للتعرف على هذه البروتينات باستعمال الأجسام المضادة الموجه ضد MPII. أظهرت تغيرات لهذه البروتينات من طرف الخلايا المظيفة للإشارة، فإن هذه البروتينات و رغم التغيرات المحدثة عليها فهي تحتفظ بنشاط ضد بكتيري.

الكلمات المفتاحية: الحمض النووي المنقوص الأكسجين المكمل (cDNA), البروتينات المرتبطة, دودة البحر, البروتين المعدنية, (*Polychaeta*), *E. coli*.

Metalloproteins comprise a family low molecular mass, cysteine-rich protein, which have high affinity for heavy metals [1]. Recent, it has been established in both vertebrates and invertebrates that low molecular mass proteins with different properties than those of metallothioneins are involved in the fixation of heavy metals. *Nereis diversicolor* (*Annelida*, *Polychaeta*) is a euryhaline marine worm particularly well adapted to the mud of polluted estuaries. Recent studies demonstrated that this species withstands cadmium and mercury exposure (Cd: 22ppm, Hg : 0.075ppm) [2]. It has been concluded that the defense mechanism (detoxification) is the result of binding of heavy metals to a protein named metalloprotein II (MPII) because of its location in a second peak, with metalloproteins, followed gel filtration chromatography (Sephadex G 75) [2]. After passing fractions of this second peak through a DEAE-cellulose column, the chromatography pattern exhibited only one protein peak, superimposed on that of cadmium. The amino acids composition and ultraviolet absorption gave evidence that the MPII molecular mass (about 10 kDa) was different from that of metalloproteins [3].

In order to confirm that coelomocytes (free cells in the coelomocytes fluid of worm) are sites of strong synthesis of MPII, Benouareth *et al.* [4] isolated mRNA from coelomocytes and the total poly(A)⁺ mRNA was translated in a cell-free system and in *Xenopus laevis* oocytes. The monoclonal antibodies raised against MPII recognized a polypeptide with an apparent molecular mass of 11-13 kDa among *in vitro* and *in vivo* translated products.

In the present paper, we report the synthesis and molecular cloning of cDNA and selection of desired clones by screening the original library with poly- and monoclonal anti-MPII antibodies. In addition, we induced synthesis of the fusion protein by lytic bacteria infected with recombinant phages, and characterized these proteins with anti-MPII antibodies. Furthermore, we present evidence for the antibacterial activity of these fusion proteins.

MATERIALS AND METHODS

cDNA synthesis and cloning

Total coelomocytes RNA was obtained as previously described [4], followed by selection of poly (A)⁺ mRNA by the oligo(dT)-cellulose affinity chromatography method [5, 6]. cDNA was synthesized by the method of Guber and Hoffman [7]. Insertion of the cDNA at the *EcoRI* site of λ gt11 vector (Amersham) was performed after addition of *EcoRI* linkers (Amersham). Bacteria used for transformation was *E. coli* (strain Y1090, Institut Pasteur, Lille).

Screening of the cDNA library

The λ gt11 library was plated on a lawn of *E. coli* (strain Y1090) and screened by a modification of the method of Young and Davis [8]. The antigen-producing clones were identified by incubating nitrocellulose filters with defined poly- or monoclonal antibodies raised against MPII [9, 10] previously preabsorbed with *E. coli* (strain Y1090) lysate and then with sheep anti-rabbit or anti-mouse IgG conjugated to peroxidase (Institut Pasteur, Paris). Recombinant immune complexes were detected using chromogenic substrates of peroxidase.

Preparation and characterization of fusion proteins

The preparation of fusion proteins was performed according to the modified Villarreal-Ramos technique [11]. We have improved Villarreal-Ramos technique as follows: 1) IPTG (IsoPropyl-3-D Thio-Galactopyranoside) induction was reduced to 1h at 37° C instead of 2h, 2) the buffer (10 mM Tris-HCl, pH 8, 10 mM MgCl₂, 0.5% Triton X100, 1mg/ml lysozyme, 10 mg/ml DNase I) was added to the bacteria culture and incubation was performed for 15 min, 3) 1mM PMSF (PhenylMethyl-Sulfonyl Fluoride) was added to the mixture, 4) the latter was centrifuged 10 min at 10.000 g, 5) the pellet was resuspended in buffer (60 mM Tris-HCl, pH 7.5, 2% SDS, 10% glycerol, 3% β -mercaptoethanol), 6) samples were then used for Dot-Immunobinding or electrophoretic separation. Dot-Immunobinding, as described by Hawkes *et al.* [12] and Western-blot as described by Towbin *et al.* [13] after electrophoretic separation on 10% polyacrylamide gel [14], were used for characterization of fusion proteins using poly- and monoclonal anti-MPII antibodies.

Antibacterial activity test

The sensitivity of the bacteria (*Micrococcus luteus*) was determined in nutrient agar according to the antibiogram method, using sterilized disks impregnated with 20 μ l of fusion protein solution.

RESULTS AND DISCUSSION

Isolation of MPII-cDNA clones

About 10⁶ phages per ml of the original library were obtained, 67% of these were recombinants (*lac Z*). After amplification, the strength of the library rose to 10⁸ phages per ml, and more than 72% were recombinants. Approximately 30.000 clones in the library were screened with polyclonal anti-MPII antibody. After 3 successive screening, 12 phages corresponding to positive clones giving strong signals were chosen (Fig. 1, line A). From these clones, and using a monoclonal anti-MPII antibody, 6 clones were purified after 3 successive screening (Fig. 1, line B). The latter were chosen and amplified and then were used for infection of bacteria in order to produce fusion proteins by host cells.

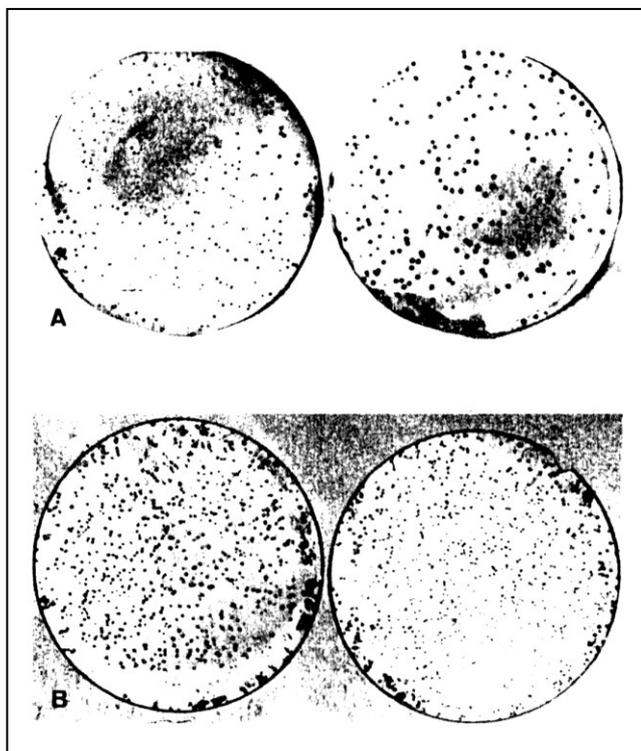


Figure 1: Recombinant clones selected with polyclonal anti-MPII (line A) and with monoclonal anti-MPII (line B) antibodies. After final screening, all phages of lysis immunoreacted with both batches of antibodies. The confirmation of selection with previously well defined monoclonal antibodies enhanced the positive selection of recombinant clones.

Characterization of fusion proteins

The research for possible synthesis of fusion proteins was performed on pelleted fragments of bacteria transformed with recombinant phages, frequently, these proteins are immobilized in an insoluble form, i.e., in cytoplasmic inclusions [15]. Using poly- or monoclonal anti-MPII antibodies for Dot-Immunobinding tests, we obtained positive immunoreactivity for all tested clones. Intensity varied with the clones (Fig. 2, line A). However, constant low reactivity was noted when anti-MPII monoclonal antibodies were used (Fig. 2, line B).

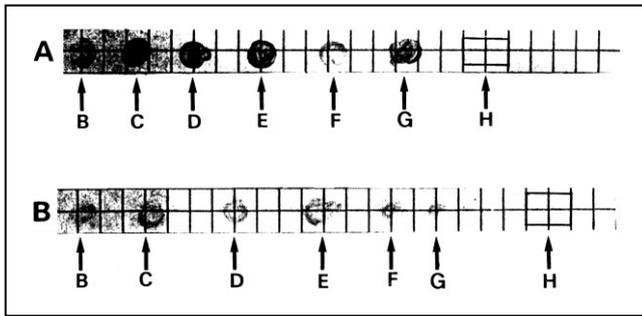


Figure 2: Dot-immunoblots of polyclonal anti-MPII (line A) and monoclonal anti-MPII (line B) antibodies to fusion proteins. B to G pelleted fragments of bacteria transformed with recombinant phages. H (control) pelleted fragments of bacteria transformed with non-recombinant phages. Bacteria fragments in B to G exhibited positive immunoreactions, but those obtained with monoclonal anti-MPII antibodies were weak. Controls (H) were clearly negative.

Evidence of an antibacterial activity of the fusion protein

In order to ensure the validity of the actual selection of clones, and consequently of the true synthesis of fusion proteins, we investigated the antibacterial activity of fusion proteins, a property these proteins may share with original MPII synthesized by live worms. Such a hypothesis stems from results of Dhainaut *et al.* [16] who demonstrated that the anti bacterial activity of the coelomic fluid of *N. diversicolor* is the consequence of the presence of proteins the molecular mass of which was on the average 10 kDa and which cross-reacted with anti-MPII antibodies.

Results obtained from biological test showed inhibition of bacterial growth by bacterial lysates produced by cells infected with recombined phages studied (Fig. 3, B and G). In contrast no antibacterial activity was detectable with lysate from cells infected with non-recombined phages (Fig. 3, H). Thus, these data lent strong support to our selection performed with both batches (poly- and monoclonal antibodies).

The actual presence of biologically active fusion proteins led us to inquire as to its apparent molecular mass. After electrophoretic separation of proteins, analysis of bands colored with Coomassie blue focused upon the area of migration of proteins the molecular mass of which was comprised between 116 and 130 kDa {(total of the β -galactosidase molecular mass (116 kDa) and the MPII molecular mass (average 14 kDa)}. Two bands of 128 and 129 kDa were detected in lanes corresponding to proteins extracted from bacteria infected with recombinant phages (Fig. 4, lanes B to G). The presence of the same bands, less well defined, in lines of proteins from bacteria infected with non-recombined phages (Fig. 4, lane H), suggested that the proteins of 128 and 129 kDa were not the fusion proteins we expected.

We then proceeded with immunodetection of antigens following protein transfer from the gel to a nitrocellulose sheet, using anti-MPII antibodies. Following the Western-bolt technique, anti-MPII antibodies cross-reacted with polypeptides of 24 and 34 kDa for the 6 tested clones

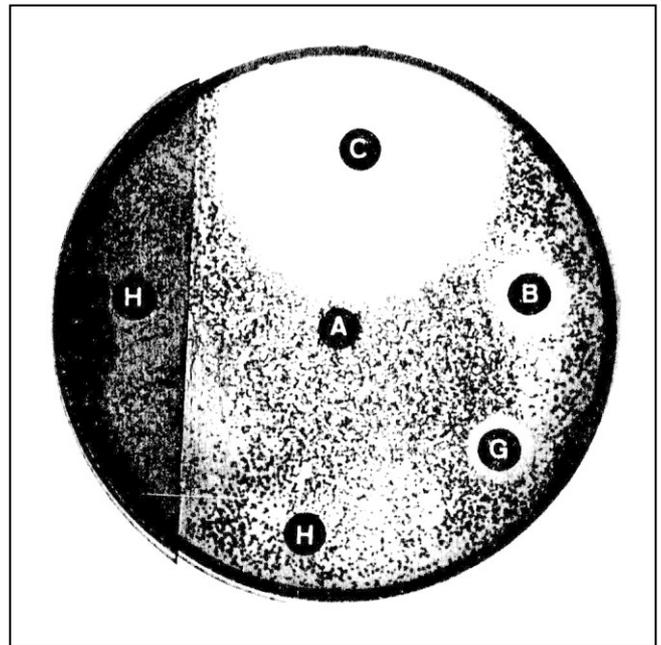


Figure 3: Biological test : antibacterial activity of the pelleted fragments of bacteria transformed with recombinant phages (clones B and G). H (negative control) : pelleted fragments of bacteria transformed with non-recombinant phages. C (positive control) : disk impregnated with ampicillin (35 μ g/ml). A (another negative control) : disk impregnated with distilled water on agar plate inoculated with *Micrococcus luteus*. Fragments, which originated in bacteria transformed with recombinant phages clearly inhibited bacterial growth (clones B and G). Such inhibition could be compared to that obtained with pure ampicillin (positive control). On the contrary, neither negative control (A and H) showed any effect upon growth of bacteria.

selected (Fig. 5, lane B, C, E, F and G). Neither of these two bands was detectable in the control lane (Fig. 5, lane H). It appeared that polypeptides of 24 and 34 kDa were the more constant molecules, recognized by antibodies, synthesized by bacteria infected with recombined λ gt11 phage. Less constant other products were also detected.

These results led us to postulate that the fusion protein, though biologically active, was degraded in several protein species, at least in two constant polypeptides of 24 and 34 kDa by the host bacteria, and not during the extraction procedure that we have modified from Villarreal-Ramos [11]. Degradation of fusion protein have already been proposed by some authors indeed; it has been noted that the production of some eukaryotic products and their accumulation are frequently limited in *E. coli*. These alterations are result of the instability of these products in host cells [17-19]. These proteins are probably considered as foreign product, abnormal, possibly toxic and consequently modified by host cells [20-22]. Some authors have proposed the evaluation of the half-life of these macromolecules. Thus, Berka *et al.* [23] calculated a half-life of 15 min for a yeast metallothionein and Romeyer *et al.* [24] estimated a 50 min half-life for the human metallothionein. Our results are thus in good agreement with those of several authors who report the instability of

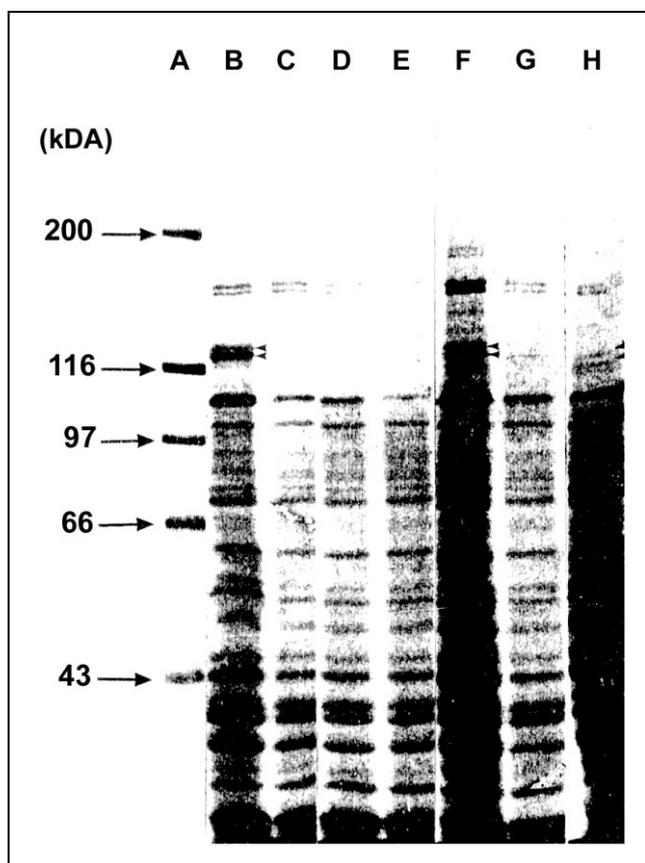


Figure 4: Coomassie blue stained SDS-PAGE profile of total pelleted transformed *E. coli* proteins. Lane A : molecular mass of standards from Pharmacia. Lanes B to G : proteins extracted from bacteria infected with the respective recombinant phages. Lane H (control) : proteins extracted from bacteria infected with non-recombinant phages. Two bands of 128 and 129 kDa (arrows) are more or less well defined in lanes B to G, but these two bands are visible in the control (H), though always tiny. Thus, we postulate that these proteins cannot represent fusion proteins.

some fusion proteins; but conservation of the bacteriostatic activity MPII fusion proteins seems to be a strong argument in the demonstration of the genuine synthesis of this protein of interest.

CONCLUSION

In this work, we have contributed to the recognition of metalloprotein (MPII) of *Annelida Polychaeta Nereis diversicolor* by a molecular approach. After isolation and purification of mRNA, the availability of poly- or monoclonal anti-MPII antibodies have led us to elaborate an expression cDNA library. The title of the library and the percentage of recombined clones may follow the grading and the selection of the interest recombined clones. The monoclonal anti-MPII antibodies, a probe of choice, very specific, were used to confirm the positive character of clones which were preselected with the help of polyclonal anti-MPII antibodies. The expression of fusion proteins by host bacteria of recombined bacteriophages and the difficulties to detect these proteins have led us to evoke a

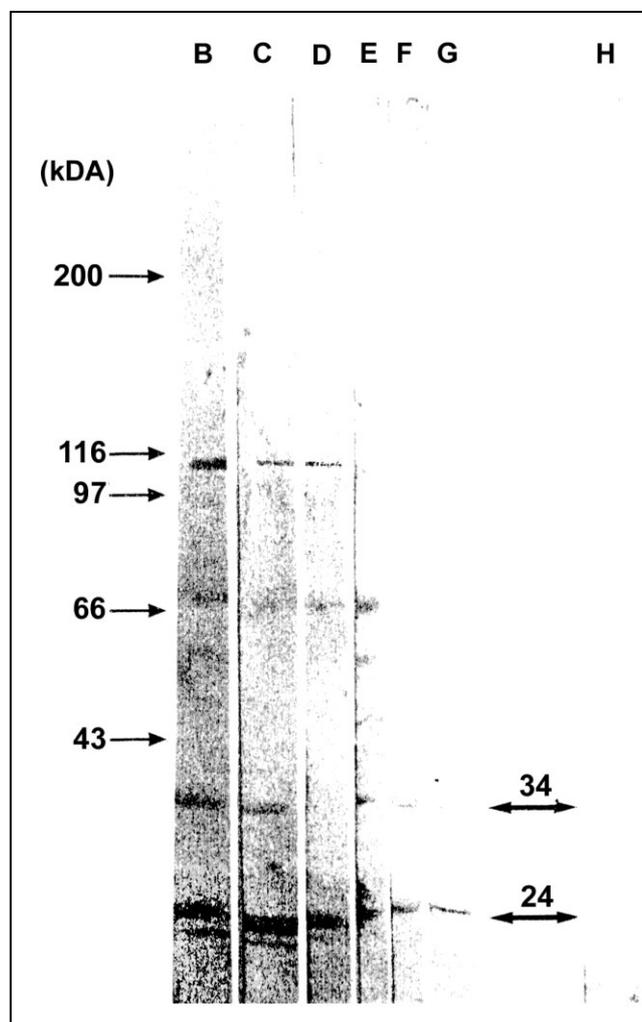


Figure 5: Western-blot analysis performed with anti-MPII antibodies confronted with transformed *E. coli* proteins. Lanes B to G : proteins extracted from bacteria infected with the respective recombinant phages. Lane H (control) : proteins extracted from bacteria infected with non-recombinant phages. Number on the left are molecular mass markers from Pharmacia. No immunoreaction appeared in the area of proteins the molecular mass of which was comprised between 116 and 130 kDa. But for all experiments, including those of recombinant phages, an immunopositive reaction was obvious at 24 kDa. For 5 of the 6 recombinant clones, another immunopositive reaction occurred at 34 kDa (clones B, C, E, F and G). Several proteins were less constantly recognized by antibodies. No immunoreaction appeared at 24 kDa, nor at 34 kDa in the control (H). Thus, we postulate that immunoreaction polypeptides correspond to fusion proteins modified by host bacteria.

damage of these macromolecules. This phenomenon doesn't seem exceptional; it has already been stated by several authors.

REFERENCES

- [1]- Kåji J.H.R. and Nordberg M., "Metallothionein and other low molecular weight metal-binding proteins", *Experientia Suppl.*, 34, (1979), pp. 41-124.
- [2]- Dennaï N., Dhainaut-Courtois N., Bouquegneau J.M. and

- Nejmeddine A., "Effets du cadmium et du mercure sur un ver marin (*Nereis diversicolor* O.F. Müller). Mécanismes de détoxification", *C.R. Acad. Sci.*, Paris, 302, (1986), pp. 489-494.
- [3]- Nejmeddine A., Dhainaut-Courtois N., Baert J.L., Sautière P., Fournet B.L. and Boulenguer P., "Purification and characterization of a cadmium-binding protein from *Nereis diversicolor* (Annelida, Polychaeta)", *Comp. Biochem. Physiol.*, 89C, (1988), pp. 321-326.
- [4]- Benouareth D.E., Dhainaut-Courtois N., Porchet-Henneré E. and Cury J.J., "Traslation in a cell-free system, and in *Xenopus* oocytes, of mRNA which specify a cadmium-binding protein in *Nereis diversicolor* (Annelida, Polychaeta)", *Biol. Cell.*, 67, (1989), pp. 167-171.
- [5]- Aviv H. and Leder P., "Purification of biologically active globulin messenger RNA by chromatography of oligothymidylic acid cellulose", *Proc. Natl. Acad. Sci.*, USA, 69, (1972), pp. 1408-1417.
- [6]- Maniatis T., Fritsh E.F. and Sambrook J., "Molecular cloning: a laboratory manual", Cold Spring Harbor, New York, (1982), p. 545.
- [7]- Gubler U. and Hoffman B.J., "A simple and very efficient method for generating cDNA libraries", *Gene*, 25, (1983), pp. 263-269.
- [8]- Young R.A. and Davis R.W., "Yeast RNA polymerase II genes: isolation with antibody probes", *Science*, 222, (1983), pp. 778-782.
- [9]- Dhainaut-Courtois N., Nejmeddine A., Baert J.L., Dhainaut A., "Localisation immunocytochimique en microscopie électronique d'une protéine complexant le cadmium chez un ver marin (*Nereis diversicolor* O.F. Müller)", *C.R. Acad. Sci.*, Paris, 305, (1987), pp. 237-241.
- [10]- Porchet-Henneré E., Nejmeddine A., Baert J.L. and Dhainaut-Courtois N., "Selective immunostaining of type I granulocytes of polychaeta annelid *Nereis diversicolor* by a monoclonal antibody against a cadmium-binding protein (MPII)", *Biol. Cell.*, 60, (1987), pp. 259-261.
- [11]- Villareal-Ramos B., "A method for the screening of fusion protein expression by λ gt11 recombinant clones without the preparation of lysogens", *Nucleic Acids Res.*, 17, (1989), p. 6421.
- [12]- Hawkes R., Niday E. and Gorden J., "A dot-immunobinding assay for monoclonal and other antibodies", *Anal. Biochem.*, 119, (1982), pp. 142-147.
- [13]- Towbin H., Staehelin T. and Gordon J., Electrophoretic transfer of proteins from acrylamide gels to nitrocellulose sheets: procedure and some application", *Proc. Natl. Sci.*, USA, 76, (1979), pp. 4350-4354.
- [14]- Laemmli U.K., Cleavage of structural proteins during assembly of the head of bacteriophage T4", *Nature*, 227, (1970), pp. 680-685.
- [15]- Martson F.A.O., "The purification of eukaryotic polypeptides synthesized in *Escherichia coli*", *Biochem. J.*, 240, (1986), pp. 1-12.
- [16]- Dhainaut A., Raveillon B., M'Béri M., Porchet-Henneré E. and Demuynck S., "Purification of an antibacterial protein in the coelomic fluid of *Nereis diversicolor* (Annelida, Polychaeta). Similitude with a cadmium-binding protein", *Comp. Biochem. Physiol.*, 94C, (1989), pp. 555-560.
- [17]- Davis A.R., Bos T.M., Veda D.P., Nayak D. and Dowbenko R.W., "Compan: Immune response to human influenza virus hemagglutinin expressed in *Escherichia coli*", *Gene*, 21, (1983), pp. 273-284.
- [18]- Itakura K., Hirose T., Crea R. and Riggs A.D., "Expression in *Escherichia coli* of a chemically synthesized gene for the hormone somatostatin", *Science*, 198, (1977), pp. 1056-1063.
- [19]- Taniguchi T., Guarente L., Roberts T.M., Kimelman D., Douhan J. and Ptashne M., Expression of the human fibroblast interferon in *Escherichia coli*", *Proc. Natl. Sci.*, USA, 77, (1980), pp. 5230-5233.
- [20]- Goldschmidt R., "In vivo degradation of nonsense fragments in *E. coli*", *Nature*, 228, (1970), pp. 1151-1154.
- [21]- Jacobs F.A., Romeyer F.M., Beauchemin M. and Brousseau R., "Human metallothionein-II is synthesized as a stable membrane-localized fusion protein in *Escherichia coli*", *Gene*, 83, (1989), pp. 95-103.
- [22]- Lin Z. and Zabin I., " β -galactosidase. Rates of synthesis and degradation of incomplete chains", *J. Biol. Chem.*, 247, (1972), pp. 2205-2211.
- [23]- Berka T., Shatzman A., Zimmerman J., Strickler J. and Rosenberg M., "Efficient expression of the yeast metallothionein gene in *Escherichia coli*", *J. Bact.*, 170, (1983), pp. 21-26.
- [24]- Romeyer F.M., Jacobs F.A., Masson L., Haana Z. and Brousseau R., Bioaccumulation of heavy metals in *Escherichia coli* expression an inducible synthetic human metallothionein gene", *J. Biotech.*, 8, (1988), pp. 207-220. □