Devoir n°3
Le génome et son expression

1. Réplication et transcription de l'ADN (25 points)

1.1. La réplication (9 points)

En s'aidant d'un schéma commenté, résumer la succession des événements marquant la croissance des molécules d'ADN à la fourche de réplication : préciser en particulier le nom des enzymes impliquées ainsi que leurs rôles respectifs.
Décrire la finition des brins d'un chromosome bactérien et celle d'un chromosome eucaryote.

1.2. La transcription (11 points)

a) Initiation de la transcription

Nommer en précisant leurs rôles, tous les facteurs nécessaires pour cette étape. Décrire la structure d'un et le rôle d'un promoteur et du terminateur. Définir brin sens et antisens.

b) Régulation de la transcription

Chez les bactéries, la régulation transcriptionnelle est particulièrement efficace en raison de la brièveté de la demi vie de l'ARNm : l'opéron lactose est un exemple de ce type de contrôle.
Définir: Ooéron, monocistronique, polycistronique, induction, répression catabolique.
En s'aidant du document n°1 décrire :

- comment la cellule annule la répression en présence de lactose ?
- comment la levée de la répression ne peut s'annuler en présence de glucose et malgré la présence de lactose ?

1.3. Expression des gènes (5 points)

Le brin antisens d'un segment d'ADN bicaténaire contient la séquence :

AGCCTACTAGGTAGCATGCATGC

a) Quelle séquence d'ARNm donne la transcription de ce brin ?

b) A l'aide du document n°2, écrire la séquence peptidique codée par le transcrit obtenu en a)

2. Diagnostic bactérien par PCR (25 points)

Le document n°3 présente une méthode alternative de détection de Vibrio cholerae dans l'eau et les aliments lors d'épidémie. L'intérêt majeur est le gain de temps fondamental par rapport aux méthodes classiques afin de mettre en place rapidement un traitement efficace auprès des populations atteintes.

2.1. Quelle séquence amplifie-t-on afin de réaliser ce diagnostic? Justifier la réponse.
Préciser la taille de la séquence recherchée.

2.2. Réaliser un organigramme simplifié des différentes phases de cette recherche. Préciser les témoins nécessaires à la validation de la méthode.

2.3. Préparation de la PCR (12 points)

a) Définir cet acronyme.

b) Rappeler les grandes étapes d'une PCR. Expliquer comment on amplifie spécifiquement la séquence d'intérêt en fin de processus.

c) A partir du document, dégager quelques précautions techniques nécessaires au bon déroulement de l'amplification.

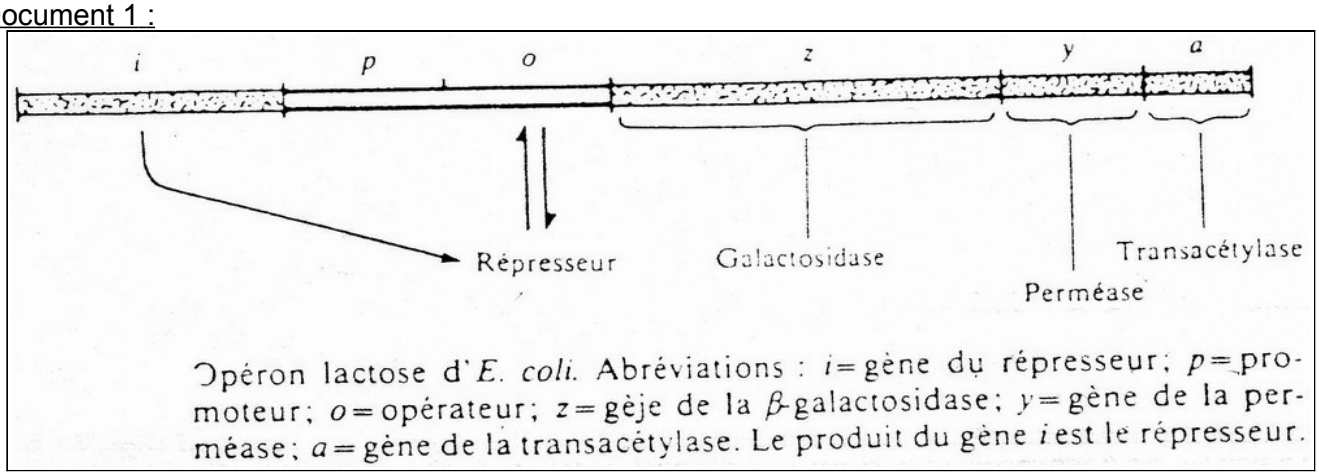
3.4. Analyse pratique (6 points)

a) On peut estimer le Tm d'une amorce par la relation suivante : **Tm = 2°C x (A+T) + 4°C x (C+G)** Calculer pour chaque amorce son Tm. Que représente cette valeur? Justifier alors la température d'hybridation (primer annealing) utilisée dans le programme du thermocycleur.

b) A l'aide des données fournies dans le document n°3 :

- calculer les volumes des différents composants d'un tube de PCR pour un volume final de 100µL,
- calculer la quantité d'Agarose, de tampon TBE et de BET afin de préparer un gel de 150mL à 1,5% pour analyser les produits de PCR.

Document 1 :



Opéron lactose d'*E. coli*. Abréviations : *i* = gène du répresseur; *p* = promoteur; *o* = opérateur; *z* = gène de la β-galactosidase; *y* = gène de la perméase; *a* = gène de la transacétylase. Le produit du gène *i* est le répresseur.

Document 2 :

Code génétique : correspondance codons-acides aminés

Position 1	Position 2				Position 3
	U	C	A	G	
U	Phe	Ser	Tyr	Cys	U
	Phe	Ser	Tyr	Cys	C
	Leu	Ser	(TC)*	(TC)*	A
	Leu	Ser	(TC)*	Trp	G
C	Leu	Pro	His	Arg	U
	Leu	Pro	His	Arg	C
	Leu	Pro	Gln	Arg	A
	Leu	Pro	Gln	Arg	G
A	Ile	Thr	Asn	Ser	U
	Ile	Thr	Asn	Ser	C
	Ile	Thr	Lys	Arg	A
	Met(IC)**	Thr	Lys	Arg	G
G	Val	Ala	Asp	Gly	U
	Val	Ala	Asp	Gly	C
	Val	Ala	Glu	Gly	A
	Val(IC)**	Ala	Glu	Gly	G

* TC = terminaison de chaine
** IC = initiation de chaine

Document n°3 :

Detection of Enterotoxïgenîc Vibrio cholerae in Foods by the Polyrnerase Chain Reaction

U.S. Food & Drug Administration Center for Food Safety & Applied Nutrition 2001-OCT-24

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Recent epidemics of cholera in various parts of the world have emphasized the urgent need for rapid and reliable detection methods for Vibrio cholerae, especially in food and water. Classical microbiological methods are sensitive and specific; however, they require several days to complete and may result in considerable loss of perishable foods. Since cholera toxin production (encoded by the ctxAB genes) is the major factor in the pathogenesis of cholera, a polymerase chain reaction (PCR) method that selectively amplifies a DMA fragment within the ctxAB operon of V. cholerae has been developed and applied to various foods.

Many of these PCR methods have relied on extraction of DNA from contaminated foods, an additional step which adds several hours to the procedure and often requires modification of each diverse food matrix tested. An advantage of PCR is that the amplification reaction often proceeds well with crude lysates of cells, in some cases requiring only brief boiling of a bacterial suspension.

V. cholerae of the Inaba and Ogawa serotypes which lack the cholera toxin genes have been isolated; however, such strains are generally nonpathogenic. Since the presence of the cholera toxin operon is a prerequisite for pathogenicity, various PCR methods for the detection of pathogenic V. cholerae have all used the ctxAB genes as a target for amplification; these and the PCR method described here, will not detect nontoxigenic V. cholerae. As a practical matter, this PCR detection method allows one to define food samples as negative for the presence of toxigenic V. cholerae much more quickly than by following the complete microbiological identification scheme. However, it is recommended than alkaline peptone water (APW) enrichment broths used for PCR analysis also be plated onto selective thiosulfate-citrate-bile salts-sucrose (TCBS) agar for isolation and direct confirmation of the presence of V. cholerae in samples that give positive PCR results.

A. Equipment and materials

1. For APW enrichment of V. cholerae.
2. Programmable automatic thermocyler
3. Horizontal gel electrophoresis apparatus
4. Electrophoresis constant-voltage powersupply
5. Heating plate
6. Microcentrifuge tubes, 1,5 and 0,6 mL
7. Variable digital micropipettors (e.g., 0,5-20µL, 20-200µL)
8. Aérosol-résistant pipet tips
9. Microcentrifuge
10. UV transilluminator
11. Polaroid camera
12. Polaroid film

B. Media and reagents

1. Alkaline peptone water (APW)
2. Cholera toxin PCR primers, 10pmol/µL stock solutions : (5'-TGAAATAAAGCAGTCAGGTG-3'; 5'-GGTATTCTGCACACAAATCAG-3')
3. Taq DNA polymerase (native available from various vendors) 5 U/µL
4. 2'-deoxynucleoside-5'-triphosphates (dATP, dCTP, dGTP, dTTP); stock solution 1,25mM of each dNTP
5. 10X PCR buffer (100mM Tris-HCI, pH 8.3, 500mM KCI, 15mM $MgCI_2$)
6. Sterile deionized water
7. 10X TBE (0,9M Tris-borate, 0,02M EDTA, pH 8.3)
8. Agarose (nucleic acid electrophoresis grade)
9. Ethidium bromide solution, 10mg/ml
10. 6X sample loading buffer
11. DNA molecular weight markers (e.g., 123bp ladder, Bethesda Research Laboratories, Gaithersburg, MD)

C. Procedure for amplification of cholera toxin gene sequences from V. cholerae using APW enrichment broth

Food sample preparation and APW enrichment

APW enrichment lysate preparation.

Prepare APW washes or blends. Sample and freeze immediately (about 1mL) After enrichment (6-24 h), prepare crude APW lysates for PCR by boiling 1mL samples in 1,5mL microcenlrifuge tubes for approximately 5min. Lysates may be used for PCR immediately or stored in a -20°C freezer until use.

NOTE: Due to the enormous amplification possible with the PCR, minute levels of contamination can result in false positives. It is recommended that sample preparation, PCR reaction set-up, and PCR product analysis be physically separated from one another to minimise contamination.

Minimally, use of aseptic technique in handling ail PCR reagents and solutions is absolutely necessary. Use aerosol-resistant pipet tips for preparing samples and reagents for PCR reactions, and, if possible, a separate set of pipettors for analysis of PCR reaction products.

PCR reaction preparation.

To minimize cross contamination of PCR reagents, it is recommended that master mix solutions be prepared, aliquoted, and stored frozen. Master mixes contain ail necessary reagents except Taq polymerase and the lysates being amplified. The final reaction contains 10mM Tris-HCI, pH 8.3; 50mM KCI, 1.5mM $MgCl_2$, 200mM each dATP, dCTP, dGTP, and dTTP; 2 to 5% (v/v) APW lysate; 0.5µM of each primer and 2.5U Taq polymerase per 100µL; reaction volumes of 25-100µL may be used. Add Taq polymerase to the master mix and add APW lysate upon distribution to 0,6mL microcentrifuge tube reaction vessels.

Temperature cycling.

While there is some variability in the heating and cooling dynamics of thermocylers from different manufacturera, use of the following temperature cycling regimen should yield efficient amplification of the cfxgene fragment: Denaturation for 1min at 94°C, primer annealing for 1min at 55°C, and primer extension 72°C for 1min, repeated for no more than 35 cycles. Increasing the cycle number beyond 35 cycles often leads to the formation of nonspecific amplification products, including primer dimers.

Agarose gel analysis of PCR products.

Mix 10-20 pi portions of PCR reactions with 6X gel loading buffer (choose one of four common buffers from Molecular Cloning: A Laboratory Manual by by Sambrook et al. (27) and load into sample wells of 1,5-1,8% agarose gel submerged in 1X TBE containing 1µg/ml ethidium bromide. After appropriate migration with a constant voltage of 5-10 V/cm, illuminate the agarose gel with a UV transilluminator and visualize bands relative to molecular weight marker migration. The primers listed above give rise to a 777 bp fragment (19). Take Polaroid photographs of gels to document results. Further details regarding gel electrophoresis analyses may be found in the above-mentioned Molecular Cloning Lahoratory Manual (27).

Proper controls.

The need for a number of control reactions to ensure accurate interpretation of PCR results cannot be overemphasized. Minimally, for PCR analysis of food types previously optimized for this method (e.g , vegetable washes, oyster, crab and shrimp blends), include a master mix contamination control containing no lysate and a toxigenic V. cholerae APW positive control in every analysis. For every new food blend to be analyzed by this PCR method, determine the potential inhibitory effects of that food. Minimally, this entails spiking 1 ml of a 1:10 and 1:100 APW food blend post-enrichment with about 5 x 10^6 organisms per ml (or an equivalent amount of positive control lysate). A direct comparison of these spiked samples with the APW (no food) lysate containing identical numbers of cfx^+ cells, allows one to determine if any inhibition occurs at either of the two food concentrations and prevents the occurrence of false negatives. It is unlikely that food washes (e.g., fruits and vegetables) will inhibit the PCR reaction unless the fruits are bruised and washing releases excessive acidity to the APW wash.

For additional information on this PCR method, contact Walter H. Koch at FDA, CFSAN, Division of Molecular Biological Research and Evaluation, 200 C St., S.W., Washington, DC 20204. Téléphone: (202) 205-4172 or (202) 205-5060; FAX: 205-4183: E-Mail: WHK@VAX8.CFSAN.FDA.GOV.