Version JWA/2

EGT2
ENGINEERING TRIPOS PART IIA

Tuesday 26 April 2016 14.00 to 15.30

Module 3G1

INTRODUCTION TO MOLECULAR BIOENGINEERING

*Answer not more than **three** questions.*

All questions carry the same number of marks.

*The **approximate** percentage of marks allocated to each part of a question is indicated in the right margin.*

*Write your candidate number **not** your name on the cover sheet.*

STATIONERY REQUIREMENTS
Single-sided script paper

SPECIAL REQUIREMENTS TO BE SUPPLIED FOR THIS EXAM
CUED approved calculator allowed

10 minutes reading time is allowed for this paper.

You may not start to read the questions printed on the subsequent pages of this question paper until instructed to do so.

1 You have been given an agar plate with a very small bacterial colony, composed of only a few cells. These bacteria have been transformed with a plasmid vector containing the gene coding for the green fluorescent protein (GFP), flanked by two restriction sites, *Hin*dIII and *Bam*HI.

(a) What are restriction enzymes, what is their original function in bacteria, and why are they useful in modern bioengineering? [10%]

(b) You are asked to carry out a DNA digestion using these two restriction enzymes (*Hin*dIII and *Bam*HI) to isolate the gene encoding GFP, to be used in subsequent experiments. Knowing that the small colony on your agar plate will not yield enough DNA to carry out the restriction digest, how could you obtain a sufficient amount of DNA to carry out the restriction digest? [10%]

(c) You are also interested to study the cellular localisation of a special type of γ-tubulin from the fruit fly *Drosophila melanogaster*, and decide to make a chimeric DNA molecule by joining your target gene (γ-tubulin) with the reporter gene (the GFP) to make a single open reading frame. First you decide to perform a polymerase chain reaction to amplify the target gene (γ-tubulin), which is 2 kb long. Describe the reagents you should mix in your tube and what happens during each step in the thermo-cycler (the machine performing the amplification reactions). [30%]

(d) Your supervisor then informs you that he wants to use a collaborator's strain that already expresses GFP for the study, so you have to PCR-amplify another reporter gene, a red fluorescent protein (RFP) to tag γ-tubulin. You then decide to use isothermal Gibson assembly to join your gene of interest (γ-tubulin) and the new reporter gene (RFP) in this order (γ-tubulin - RFP) to a plasmid. Describe the necessary PCR reactions, showing diagrams for each (include primers, DNA template and post-PCR fragments). [30%]

(e) You now want to carry out the isothermal Gibson assembly to join up the γ-tubulin gene, the RFP gene and plasmid backbone. Briefly describe which reagents you have to mix, the overall protocol, what happens during the reaction, and why your primers had to be designed the way they did for the reaction to work. [20%]

2 Lactate can be used as a precursor for many useful molecules. Yeast does not naturally produce lactate but can be made to do so by introducing a highly expressed lactate dehydrogenase (LDH) with very high Vmax (the activity of an enzyme at the maximum substrate concentration) and deleting the genes encoding GPD1 and PDC1 that produce glycerol and ethanol, respectively (Figure 1). NDE1 is a cytosolic NADH dehydrogenase that oxidizes NADH, functioning alongside glycolysis and fermentation to provide additional redox balance.

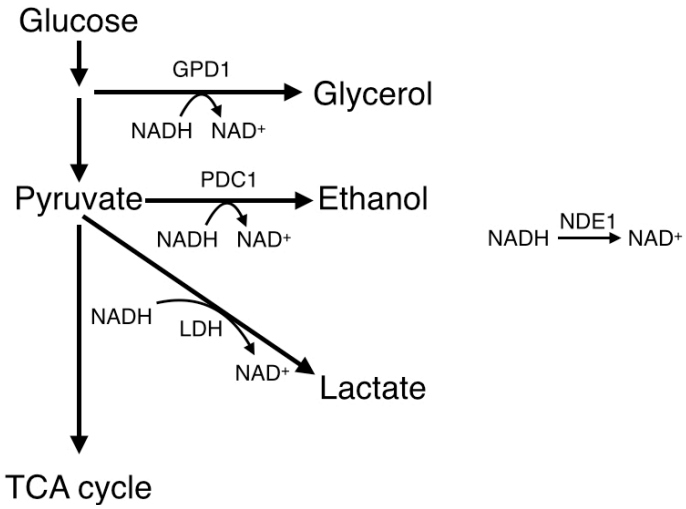

Figure 1.

(a) Why do organisms produce lactate and/or ethanol from pyruvate and what conditions should you grow your yeast in to make sure lactate is produced? [20%]

(b) Why would deleting the genes encoding GPD1 and PDC1 improve lactate production? [10%]

(c) The strategy of simply introducing a gene encoding LDH and deleting the genes encoding GPD1 and PDC1 only produced a small amount of lactate and led to unhealthy yeast. What is a likely cause of this problem and how might you fix it? Assume that the activity of LDH is much higher than the activity of GPD1 or PDC1 would be if they were present. [30%]

(c) What are some other strategies you might use to increase the production of lactate? [40%]

3 The five-gene violacein operon (*vioA-vioE*) from *Chromobacterium violaceum* (a gram negative bacterium) is constitutively expressed under its native $_{P}$vio promoter. Expression of the operon results in the production of a purple pigment, visible to the naked eye with an absorption wavelength of 575 nm. The violacein production pathway is shown in Figure 2.

This entire sequence (shown in Figure 3), was directly inserted into the *Bacillus subtilis* (a gram positive bacterium) genome at the *amyE* locus, a well documented site for gene integration. Although molecular analysis confirmed that the sequence had been integrated successfully with no mutations, there was no violacein pigment expressed.

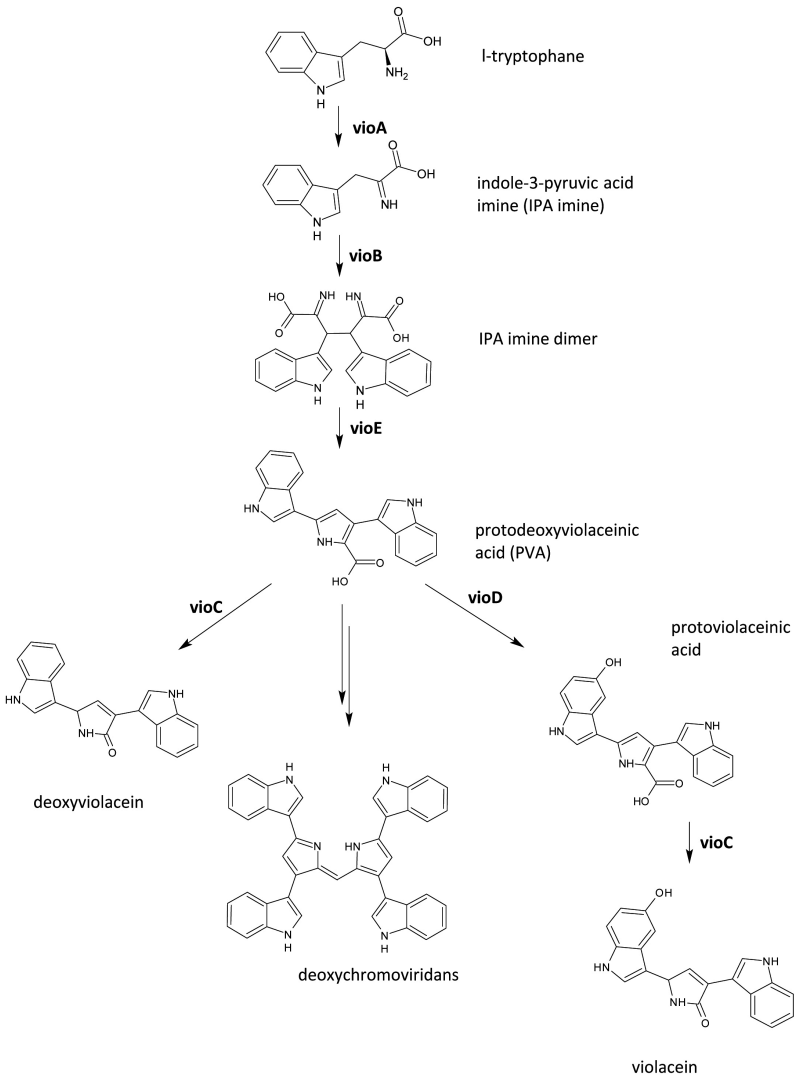

Figure 2.

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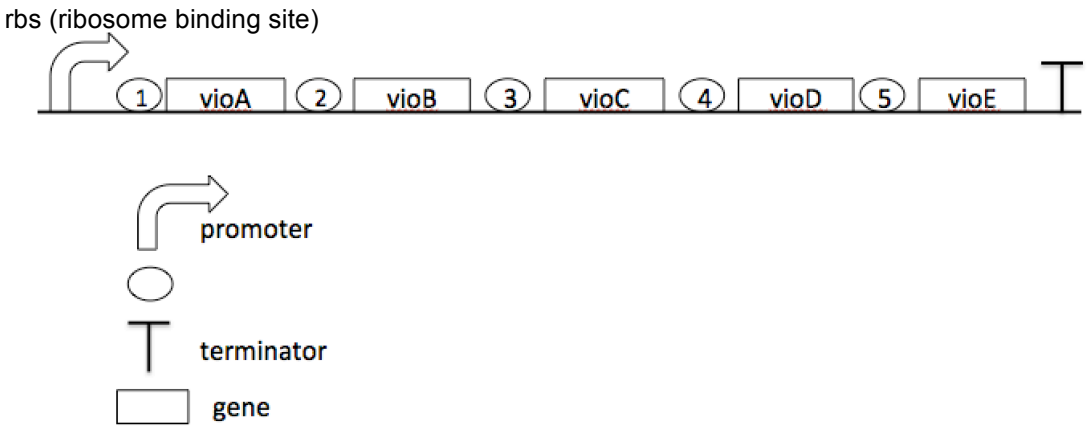

Figure 3.

(a) Describe what mechanisms of gene expression may have contributed to the failure of pigment production in the modified *Bacillus* strain. [15%]

(b) How can we manipulate these mechanisms to fix this problem? [20%]

(c) After optimizing transcription of the operon, you find that you get very little pigment production. The *Bacillus* colonies grow very slowly and are generally smaller than they are when expressing your control constructs which express GFP.

(i) What could explain this phenomenon? [15%]

(ii) How would you test to find what part of the operon is causing the problem? [20%]

(iii) How would you fix the problem so that you get good growth and pigment production? [30%]

4 Figure 4 shows a system you are building that has two fluorescent reporters being induced by two separate inducible promoters. Assume that every open reading frame has an rbs (ribosome binding site) preceding it. The genetic circuit described in Figure 4 was expected to yield a yellow output when both inducers are present at the same concentration (0.1 mM), but the output is orange. Table 1 is a list of genetic parts. Table 2 is a reference list of colours.

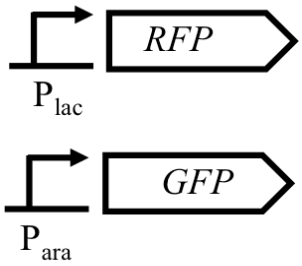

Figure 4.

(a) How would you most effectively fix this system using the parts provided in Tables 1 and 2? Explain why your solution to this problem is appropriate. [20%]

(b) You now want to use your system at different concentrations of inducer, but yield an orange output in the same way that the original system was providing. In this case, your inducers are present at 0.1 mM IPTG (as before) and 0.001 mM of Arabinose.

(i) Using the list of parts in Tables 1 and 2, how would you achieve this? Explain why your solution to this problem is appropriate. [40%]

(c) You want to use your system to produce a single output of red or green when one inducer is present at 0.1 mM and the other inducer is absent. In its current configuration, the system is only giving brown or pink outputs.

(i) Why is the system not performing as it should? [10%]

(ii) How would you redesign the system to give you a single output using parts form Tables 1 and 2? Explain why your solution is appropriate. [30%]

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Table 1.

Part	Function	Description
LacI	Lac repressor	Protein tetramer forms DNA loop to prevent RNA polymerase binding. When IPTG is bound to the repressor protein, the loop is opened, allowing RNA polymerase to bind.
AraC	Arabinose repressor/activator	Protein dimer forms DNA loop to prevent RNA polymerase binding. When arabinose is bound to repressor protein, the dimer changes conformation and stabilises RNA polymerase to behave as a transcriptional activator.
ArsR	Arsenic repressor	Protein dimer is specific for pArsR and covers RNA polymerase binding site, preventing RNA polymerase binding. When arsenic is bound the repressor is released from the DNA allowing RNA polymerase to bind.
T7 polymerase	This protein binds and activates the T7 promoter and works as an RNA polymerase	The protein binds to the T7 promoter (pT7) and acts as a highly active RNA polymerase, yielding 100x mRNA transcripts per T7 polymerase unit compared to native polymerases.
RFP	Fluorescent reporter protein	The protein fluoresces red under blue light
GFP	Fluorescent reporter protein	The protein fluoresces green under blue light
pArsR	Arsenic promoter	Sequence covers RNA polymerase binding site.
pLac	*Lac* promoter	Two LacI binding sites on either side of RNA polymerase binding site, allowing a loop to form preventing RNA polymerase to bind in the absence of IPTG.
pAra	Arabinose promoter	In the absence of arabinose, inhibits transcription by blocking RNA polymerase. In the presence of arabinose, promotes transcription by stabilising RNA polymerase binding.
pLac/Ara	Hybrid *Lac*/*Ara* promoter	The two adjacent upstream sites for AraC remain and the downstream site is eliminated so AraC can only act as an activator. The two sites are in between two LacI binding sites so that RNA polymerase can only bind when AraC is bound and LacI is not bound.
pC1	constitutive promoter	Binds RNA polymerase with high efficiency.
pC2	constitutive promoter	Binds RNA polymerase with high efficiency.
pT7	T7 polymerase promoter	Highly specific for T7 RNA polymerase binding and does not bind cellular RNA polymerase.

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Table 2.

Visible Colour	Ratio (% fluorescence units):
Red	98% FU RFP 2% FU GFP
Pink	85% FU RFP 15% FU GFP
Orange	75% FU RFP 25% FU GFP
Yellow	50% FU RFP 50% FU GFP
Brown	25% FU RFP 75% FU GFP
Green	2% FU RFP 98% FU GFP

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