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Gene regulation in bacteria

Investigating the *Lac* operon

Background information

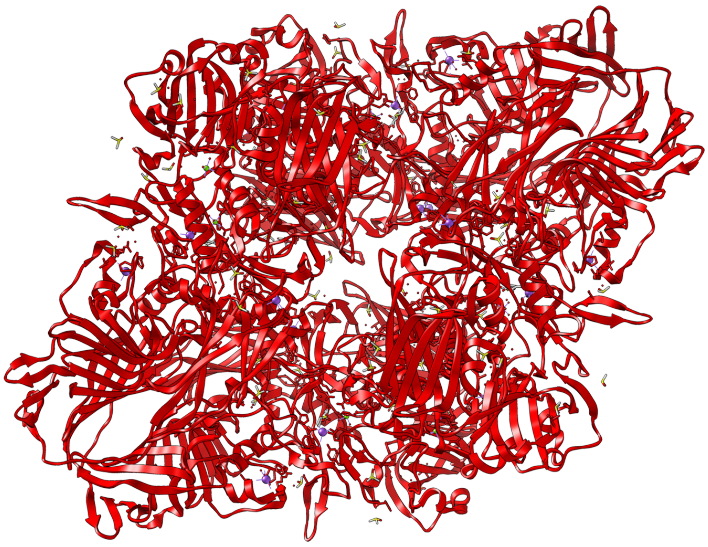
The genome of *E. coli* is about 4.6 million base pairs in length, encoding about 4,300 proteins. At any one time, only a fraction of these proteins are being made. How is the expression of these genes regulated?

Promoters and terminators

In a bacterium such as *E. coli*, each gene is flanked by a *promoter* and a *terminator*. The promoter is simply the site where the enzyme RNA polymerase binds to the DNA and starts making complementary messenger RNA (mRNA). This mRNA carries the instructions required to make a protein and it is later 'read' by the bacterium's ribosomes, where the protein is strung together from amino acids.

The terminator is a similar instruction in the DNA which tells the RNA polymerase to stop transcribing mRNA and to dissociate from the DNA.

It might be tempting to think of the promoter as the 'on' switch and the terminator as the 'off' switch, but this would be wrong. The promoter and terminator just mark the start and end of the DNA sequence that encodes one or more proteins — the promoter and terminator don't tell the cell *when* to start and stop making those proteins.



A molecular model of the enzyme β-galactosidase (lactase). The enzyme breaks down lactose to glucose and galactose.

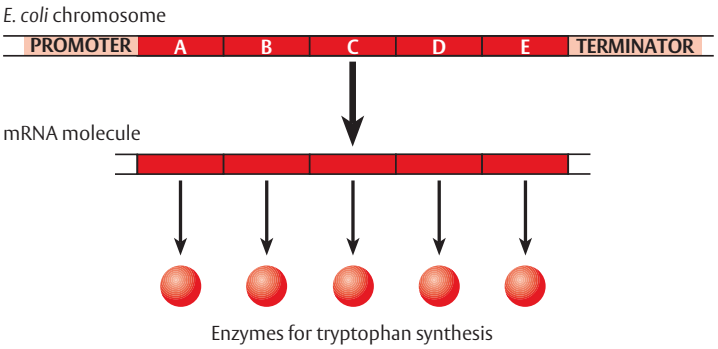
Data from Dugdale, L.M. et al (2010).

Operons

Sometimes, in bacteria, clusters of two or more related genes are under the control of a single promoter and terminator. A single stretch of mRNA is transcribed from them, allowing the different proteins encoded by the genes to be produced at the same time. For example, there is a group of five genes in *E. coli* encoding enzymes that are needed for synthesising the amino acid tryptophan.

A grouping like this is called an 'operon'. There is a similar group of three genes encoding enzymes that are needed to break down the sugar arabinose and another group of three genes that are needed to absorb and metabolise lactose. hence the groups of genes above are called the 'tryptophan operon', the 'arabinose operon' and the 'lactose (or *Lac*) operon'.

In eukaryotes, in contrast, there are no 'operons' — every gene has its own unique promoter and terminator.

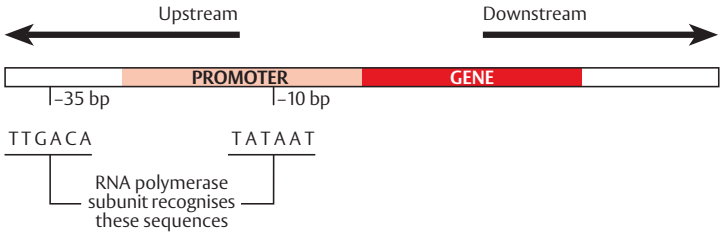


Sometimes a group of genes has just a single promoter and terminator. For example, there are five genes (labelled A to E) that E. coli needs to synthesize the amino acid tryptophan. A group of related genes like this is called an 'operon'.

Constitutive promoters

Some proteins are made continuously by the cell. These are usually essential 'housekeeping' proteins that the cell needs to stay alive. Their genes are switched on all of the time, and they are said to be expressed 'constitutively'. A constitutive promoter is therefore one which keeps the gene (or genes) downstream of it switched on all the time and is under no higher level of control.

In technical terms, constitutive promoters have two sites in the DNA (at 10 and 35 bases before the start of the protein-encoding sequence) that a subunit of the RNA polymerase always recognises and binds to. The sequence of bases there hardly ever varies: at –10 bases it is **TATAAT** and at –35 bases it is **TTGACA**. The strength of a promoter depends upon how closely the DNA matches these two sequences. A strong promoter will have a perfect, or almost perfect, match, and the RNA polymerase will bind to it every time.



Genes that are switched on all the time are under control of so-called constitutive promoters. RNA polymerase binds to two sites before the gene(s) to be transcribed. If the sequences of DNA in the promoter are 'perfect', then the promoter is strong. If the DNA has undergone mutations, however, the RNA polymerase won't bind so well and the promoter will be weaker.

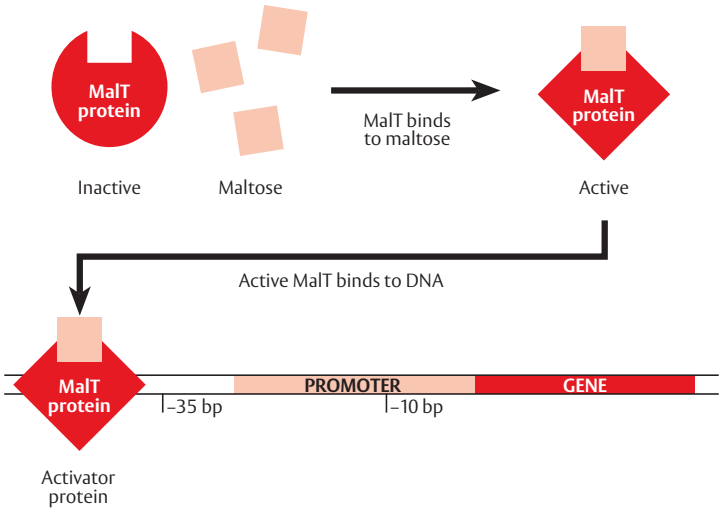
Positive regulation — gene activator proteins

Some proteins are only required at certain times. For instance, it would be wasteful if a bacterium made enzymes for metabolising maltose if this sugar was not present in its surroundings. Gene activator proteins can switch on protein production in such circumstances. How do these proteins work?

The DNA sequences in promoters before genes that are not continuously active are generally more variable than they are in constitutive promoters, so they are less easily recognised by the RNA polymerase subunit. A gene activator protein helps the RNA polymerase bind to the DNA. The gene activator protein itself first binds to the DNA, then it guides the RNA polymerase into place.

The activator protein, however, is not normally able to bind to DNA. Only in the presence of a signal molecule can this be done. When the signal molecule binds to the activator protein, it forces the protein to change shape and this new shape is able to bind to the DNA [proteins whose activity is altered by binding to another molecule like this are called *allosteric*].

The signal molecule is often the sugar, for instance, that is broken down by the enzymes that are encoded downstream of the promoter. The presence of the sugar therefore acts as a switch to trigger the production of the proteins that metabolise it.



In E. coli, an activator protein, MalT, helps RNA polymerase to bind to the promoter upstream of the genes that encode enzymes enabling maltose to be metabolised. The activator protein can only bind to DNA in the presence of maltose, ensuring that the genes are only switched on when necessary.

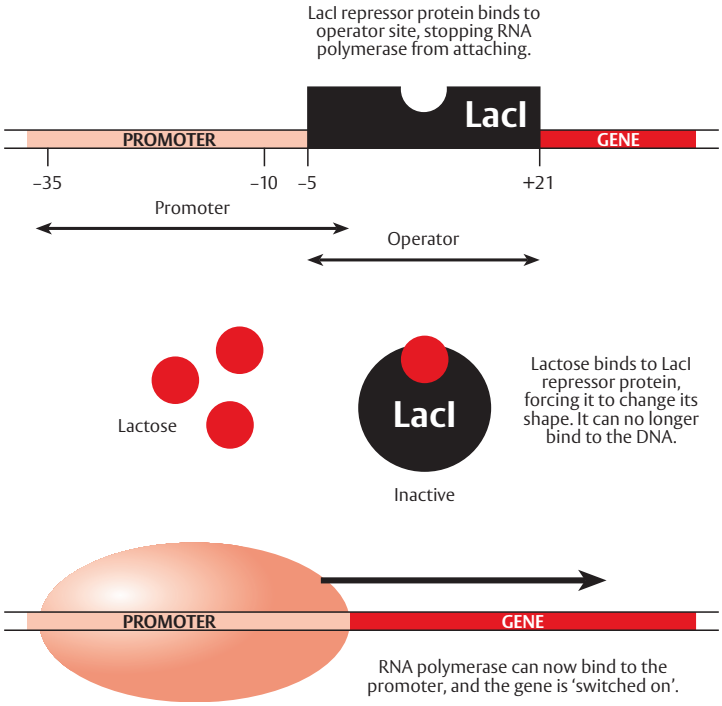
Negative regulation — repressors

In addition to activator proteins that switch genes on, there are also *repressor* proteins that switch genes off. The most famous of these is the Lac repressor protein encoded by the gene *LacI*. It regulates the genes that are needed by *E. coli* to digest the sugar lactose.

When there is no lactose in the growth medium, the Lac repressor protein binds to the DNA just before the genes that are involved in digesting lactose. By so doing, it blocks part of the promoter sequence, so that RNA polymerase cannot bind to the DNA. Consequently, the genes downstream of the promoter are not transcribed into mRNA.

When lactose is present in the medium, however, it interacts with the Lac repressor protein, forcing it to change shape. With its new shape, the protein cannot bind to DNA, so the RNA polymerase binding site is *not* blocked and the lactose-digesting proteins are made.

The tryptophan and L-arabinose operons referred to above are controlled in a similar way by tryptophan and L-arabinose repressor proteins respectively.



The lactase gene in E. coli is only expressed when lactose is present in the surrounding growth medium.

Positive and negative regulation — the *Lac* operon — a logic gate

In fact, the *Lac* operon, like many regulatory mechanisms, is slightly more complex than suggested above and the promoter is controlled by *two* different regulatory molecules.

In response to low levels of its preferred energy source (glucose), *E. coli* produces a molecule called cyclic AMP (cAMP). This signal molecule interacts with the *Cyclic AMP Receptor Protein* (CRP), forcing that protein to change shape so that it can bind to DNA. When it does so (in several places throughout the *E. coli* genome), it activates genes that would allow *E. coli* to utilise alternative sugars such as lactose, maltose or fructose. CRP is thus a general-purpose activator which operates when glucose is not available.

Note: CRP was formerly known as CAP (the Catabolite Activator Protein) so you may see it referred to by this name in some books.

It would, however, be wasteful of *E. coli* to start producing for example, the three enzymes needed to take up and utilise lactose if there was none of that sugar available either. The process of switching on the genes that encode the lactose-utilising enzymes is therefore as follows:

- a general signal is sent by cAMP ('Help! The glucose has run out');
- cAMP binds to the activator protein, CRP;
- CRP guides the RNA polymerase so that it can bind to DNA at the promoter site...
- ...but it can only do so if another signal molecule, lactose, is present and interacts with the Lac repressor protein, unblocking the promoter site.

This can be compared to an AND logic gate: only when glucose is absent AND lactose is present are the relevant genes switched on. Many genes are regulated in this way.

CRP protein	Repressor protein	RNA polymerase	Switch ON or OFF
Present	Absent	Binds	On
Absent	Present	Cannot bind	Off
Present	Present	Cannot bind	Off
Absent	Absent	Cannot bind	Off

JARGON BOX

Promoter
Site where RNA polymerase binds to DNA

Terminator
Site where RNA polymerase dissociates from DNA

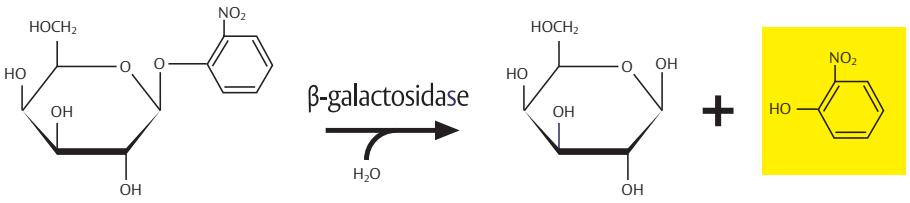
Operator
Site where a repressor protein binds to DNA

Repressor
A protein which prevents RNA polymerase from binding to DNA

Activator
A protein which helps RNA polymerase to bind to DNA

Aim

The *Lac* operon is the classic example of gene regulation in *E. coli*, in which the production of the enzyme β-galactosidase (lactase) is induced by the presence of lactose in the growth medium. In this practical investigation, ONPG, rather than lactose, is used as a substrate for the enzyme. ONPG is colourless, but when it is broken down by lactase, a yellow-coloured product (ONP) is formed. The intensity of the yellow colour can be measured using a colorimeter, allowing the action of the lactase to be measured.



ONPG Galactose ONP

Equipment and materials

Needed by each person or working group

Equipment

- test tubes, caps or *Parafilm®*, rack and marker pen
- a micropipette (*e.g.*, 40–200 µL) and sterile tips or a Pasteur pipette attached by a short length of tubing to a 1 cm^3 syringe
- 5 cm^3 syringe, for transferring ONPG solution
- stopclock or timer
- cuvettes for spectrophotometer or colorimeter
- eye protection *e.g.*, safety spectacles or goggles

Materials

- waste container of disinfectant
- broth cultures of *E. coli*
- ONPGdissolved in Z-buffer, 2 cm^3 per test sample
- methylbenzene (toluene), 1 drop per test sample
- waste container of disinfectant

You will also need access to a

- water bath maintained at 37 °C
- fume cupboard
- hair drier
- spectrophotometer or colorimeter to measure optical density at 420 nm (a 440 nm filter or similar can be used if a 420 nm filter is not available)

Procedure

This method is used to determine how much β-galactosidase is produced by a culture.

1 Transfer 0.1 cm^3 of each microbial suspension to be tested into a separate test tube.

2 Transfer 0.1 cm^3 of LB broth to a 'control' tube.

3 Label the tubes appropriately.

4 Add a drop of methylbenzene to each tube, cap the tubes and shake well to mix. *Methylbenzene kills the cells and partially disrupts the cell membrane, allowing the ONPG to diffuse into the cells.*

You must perform the next operation in a fume cupboard.

5 Use a hair drier to evaporate the methylbenzene. *Methylbenzene is lighter than water and will appear as a 'greasy' film on the surface. You must wait until all of this solvent has evaporated before proceeding to the next step.*

6 Transfer the samples to a water bath maintained at 35–37 °C. Add 2 cm^3 of ONPG in Z-buffer to each sample. Record the time.

7 Measure the optical density (OD) of the samples at 420 nm (or 440 nm) at 10 minute intervals until there is no further colour change.

8 Plot a graph of the results (OD_{420} *vs.* Time).

9 The enzyme units* in a given sample can be calculated as follows:

$$\frac{1000 \times (OD_{420} - 1.75 \times OD_{550})}{t \times v \times OD_{600}} = \text{Units of } \beta\text{-galactosidase}$$

The optical density (OD) at 420 and 550 nm is read from the sample.

The $1.75 \times OD_{550}$ is an optional correction factor which allows for light-scattering by *E. coli* cell debris, *etc*.

OD_{660} reflects the cell density at the start of the reaction
t = time of the reaction in minutes
v = the volume of the culture in cm^3

* A fully-induced culture of *E .coli* has an activity of about 1000 units, whereas an uninduced culture has an activity of 1 unit. 1 unit (U) of enzyme activity is the amount of enzyme needed to break down 1 micromole (µmol) of ONPG per minute at 37 °C.

The enzyme 'unit' is an old measure of enzyme activity that was officially replaced by the katal (kat) in 1999. One katal is the amount of enzyme that converts 1 mole of substrate per second, so 1 U = 1/60 microkatals (µkat).

Teacher's and technician's notes

Preparation and timing

You will need a *Lac*+ strain of *E.coli* that possess the *LacZ* (β-galactosidase) gene. It must be grown in LB or nutrient broth for 24–48 hours in advance. To induce the production of β-galactosidase, lactose must be added to the growth medium (1% w/v). The ONPG solution should be prepared a day before at the earliest. Please refer also to the safety notes below.

The activity itself takes about 60 minutes, including an incubation period of 10 minutes.

Safety

Methylbenzene (toluene)

Methylbenzene is flammable and produces harmful vapour. It must be kept away from naked flames and larger volumes should therefore be handled in a fume cupboard. The small amounts used in the protocol (1 drop per sample) can safely be handled at the bench, but keep the methylbenzene away from naked flames. Skin and eye contact should be avoided and eye protection must be worn.

Microbiology

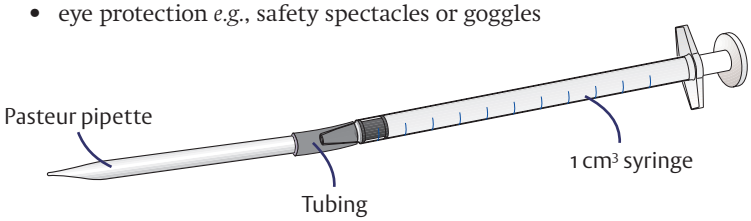
Good microbiological laboratory practice must be observed when handling microorganisms.

Equipment and materials

Needed by each student or working group

Equipment

- test tubes, caps or *Parafilm*®, rack and marker pen
- inoculation loop
- a micropipette (*e.g.*, 40–200 µL) and tips or a Pasteur pipette attached by a short length of tubing to a 1 cm^3 syringe
- 5 cm^3 syringe, without a needle, for transferring ONPG solution
- stopclock or timer
- cuvettes for the colorimeter
- eye protection *e.g.*, safety spectacles or goggles



If you don't have suitable micropipettes, a 1 cm^3 syringe can be attached with rubber tubing to a Pasteur pipette to dispense 0.1 cm^3 volumes.

Materials

- waste container of disinfectant *e.g.*, freshly made-up *Virkon*® solution (1% w/v) or *Biocleanse*® solution (5% v/v)
- broth cultures of *E. coli e.g.*, a culture of a *Lac*+ strain in nutrient or LB broth with added lactose (1% w/v) and another culture in broth without added lactose. (Additional controls could be *Lac*– cultures with and without lactose added to the broth.)
- ONPG (ortho-Nitrophenyl-β-D-galactoside) dissolved in Z-buffer, 2 cm^3 per test sample
- methylbenzene (toluene), 1 drop (about 50 µL) per test sample

The following items are also needed per class

- water bath maintained at 37 °C
- access to a fume cupboard
- hair drier
- spectrophotometer or colorimeter to measure optical density at 420 nm (a 440 nm filter or similar can be used if a 420 nm filter is not available)

Recipes

Z-buffer

Used for making up ONPG reagent. Makes 100 cm^3
Do not autoclave. Store in a refrigerator, for up to several months.

Ingredients
- $Na_2HPO_4.7H_2O$, 1.6 g
- $NaH_2PO4.H_2O$, 0.55 g
- KCl, 0.075 g
- $MgSO_4.7H_2O$, 0.025 g
- beta-mercaptoethanol, 0.27 cm^3

Instructions
1 Add the ingredients listed above to 80 cm^3 of deionised or distilled water.
2 Stir to dissolve.
3 Adjust the pH to 7.0 with 1 or 2M NaOH.

ONPG reagent

Used for detecting and measuring β-galactosidase activity
Makes 100 cm^3. Store in a refrigerator, for no more than 24 hours.

Ingredients
- Z-Buffer, 100 cm^3
- Ortho-Nitrophenyl-β-D-galactoside (ONPG), 0.55 g

Instructions
1 Dissolve the ONPG in the buffer.
2 Store in a bottle, wrapped in foil to exclude light.

Suppliers

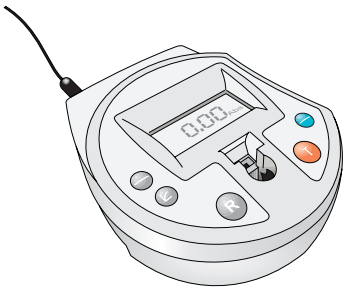
E. coli cultures

Suitable *E. coli* cultures, both *Lac*+ and *Lac*– K-12 strains, are available from the NCBE.

ONPG

ONPG is very costly. It is available from *Sigma-Aldrich* or *Thermo Fisher Scientific*. Note that 0.55 g of ONPG will make up 100 cm^3 of solution, of which 2 cm^3 is needed per test sample. [0.5 g of ONPG will cost about £21 (October 2016 prices)]

Colorimeters



Biochrom
A colorimeter suitable for school use is the WPA CO7500 from Biochrom Ltd, 22 Cambridge Science Park, Milton Road, Cambridge CB4 0FJ. **www.biochrom.co.uk/product/19/biochrom-wpa-co7500-colorimeter.html** This colorimeter has a digital readout, it is simple to use, reliable, robust and the results are repeatable.

Mystrica
An inexpensive alternative is the *Mystrica* colorimeter, available from Mystrica Ltd, 39 Charterhall Road, Edinburgh, EH9 3HS. **www.mystrica.com**

Both colorimeters are supplied with software and can be linked to PC computers. Additionally, the *Mystrica* colorimeter can link to an iPhone or iPad or similar Android device *via* Bluetooth 4.0.

Further reading

A short course in bacterial genetics: Laboratory manual and handbook by Jeffrey Miller (1992) Cold Spring Harbor Laboratory Press, New York. ISBN: 0 87969 349 5

A comprehensive review of β-galactosidase structure and function is available in: Juers, D. H., Matthews, B. W. and Huber, R. E. (2012) *LacZ* β-galactosidase: Structure and function of an enzyme of historical and molecular biological importance. *Protein Science* 21: 1792–1807.

The lac operon. A short history of a genetic paradigm by Benno Müller-Hill (1996) Walter de Gruyter, Berlin. ISBN: 3 11014830 7.

Molecular modelling

The molecular image on page 1 was created using data from the Protein Data Bank: **www.rcsb.org/pdb**. The data for β-galactosidase molecule was published in: Dugdale, M.L. *et al* (2010) Importance of Arg-599 of β-galactosidase (*Escherichia coli*) as an anchor for the open conformations of Phe-601 and the active-site loop. *Biochemistry and Cell Biology* 88: 969–979 [PDB ID: 3MUY].
The software used to produce this image was UCSF Chimera, which can be obtained free-of-charge from: **www.cgl.ucsf. edu/chimera/**

