National Institute for Public Health
and the Environment
Ministry of Health, Welfare and Sport

Educational material for addressing Safe-by-Design in biotechnology: *Cases and guidance*

Case 2: Orthogonality in the genetic code

Content of the case:

2.1 General information

Biotechnology domain and focus

- contained use;
- fundamental research;
- molecular genetics.

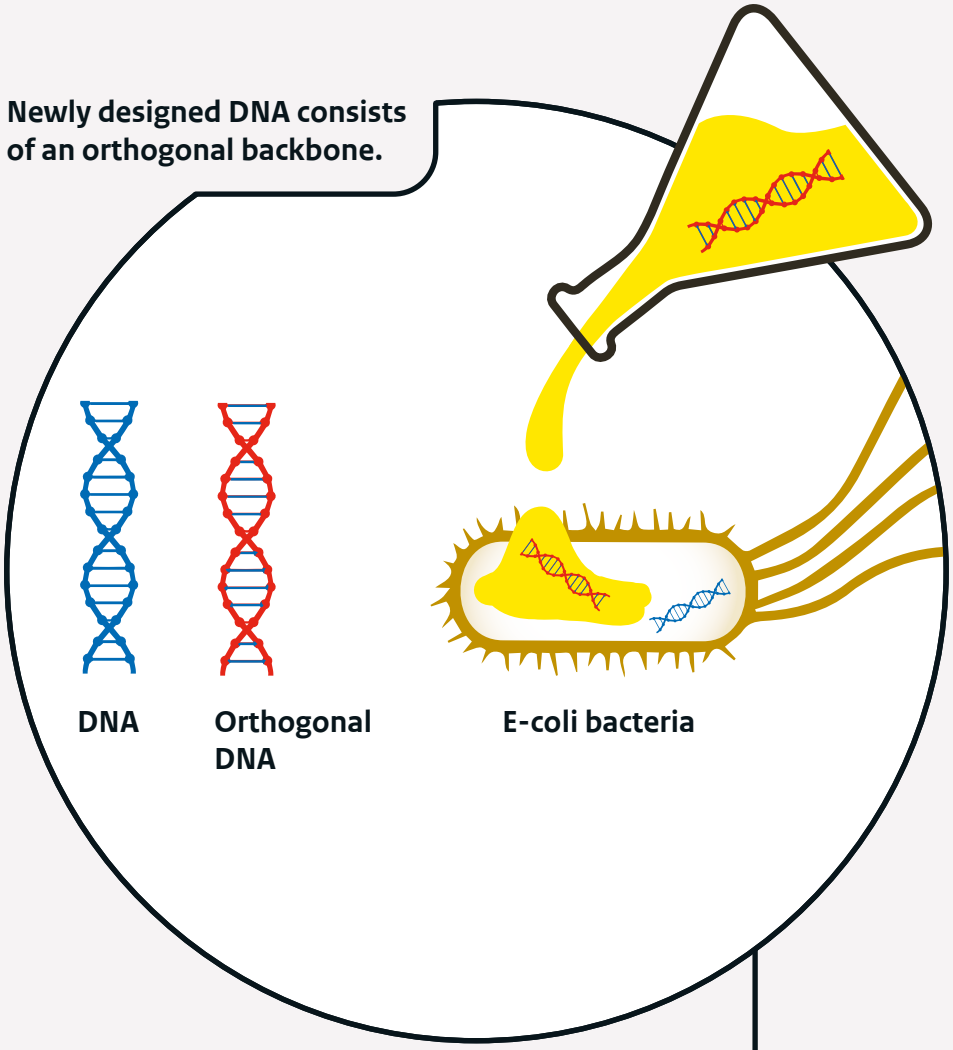
Learning objectives

- understand the risks of orthogonal DNA in living organisms;
- put promising developments into a safety perspective;
- understand dual use research.

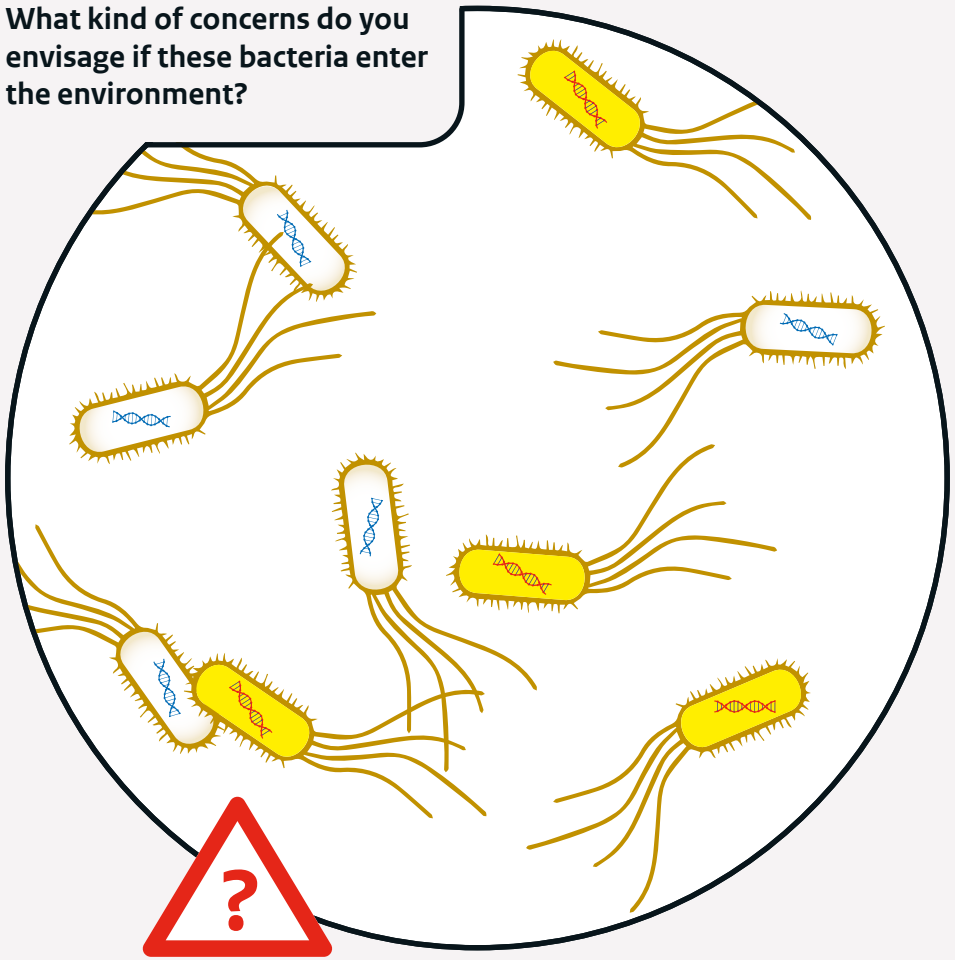
Case specific knowledge required

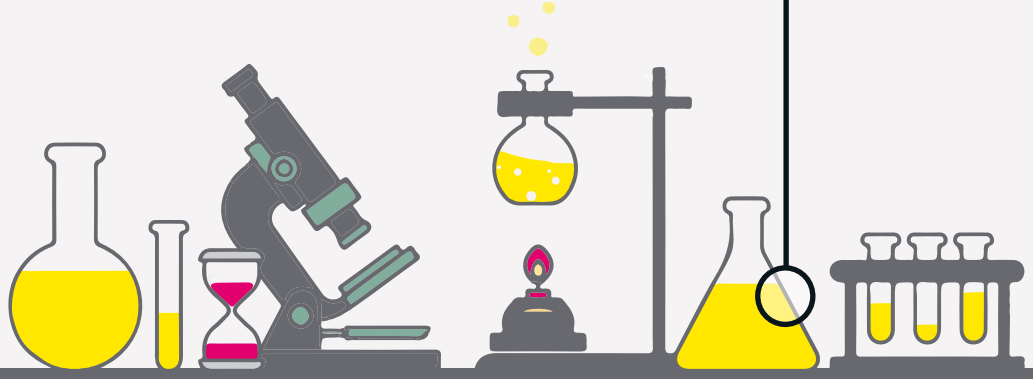
- knowledge of PCR and cloning strategies;
- knowledge of molecular cell biology.

Newly designed DNA consists of an orthogonal backbone.



What kind of concerns do you envisage if these bacteria enter the environment?





Orthogonality in the genetic code

2.2 Case description

Orthogonality refers to biological systems whose basic structure is dissimilar to those occurring in nature in such a way that they can only interact with the natural system to a limited extent, if at all.
This case describes orthogonality with respect to the chemical composition of the DNA helix. The backbone of DNA is redesigned and composed of phosphoramidate (PN) linkages. These linkages are enzymatically incorporated into a functional gene. The resulting PN-DNA is acid-labile and could be completely hydrolysed under mildly acidic conditions. This demonstrates that this genetic material can be specifically degraded.

As well as designing PN-DNA, the scientists also developed a cloning strategy for introducing the orthogonal DNA into *Escherichia coli*. They show the incorporation of the chemically modified gene into an expression vector thereby establishing a hybrid genomic system. The synthetic PN-DNA was transferred to *E. coli* bacteria and the PN-modified fragment was successfully recognised by the *E. coli* replication machinery for it to provide a functional protein in vivo. To demonstrate protein functionality, the PN-DNA gene encoded trimethoprim resistance which enabled the bacteria to grow in the presence of this antibiotic.

Origin of case and background information

This case is based on a scientific paper[1] describing an alternative genetic system carrying 'phosphoramidate' (PN) linkages in the DNA. The authors show that this genetic system was able to replicate and could function in bacterial gene expression.
There are several options for developing orthogonal genetic material such as the use of non-natural nucleobases or sugar and phosphate backbone moieties (see references 4 to 12 in aforementioned paper). The development of orthogonal DNA is interesting and relevant, from a fundamental point of view, to an understanding of the chemical and biological characteristics of DNA. It is also useful for investigating its possibilities as a non-natural building material, an information carrier, or as genetic material in a living cell. In this case, the use of orthogonal PN-DNA as genetic material in a living cell is demonstrated. The paper describes the incorporation and functionality of a PN-DNA gene in a bacterial plasmid. Questions that are raised are 'what are the risks if new genes can be chemically synthesised and if new functionalities can be combined in a living cell?'

[1] https://pubmed.ncbi.nlm.nih.gov/31868979/

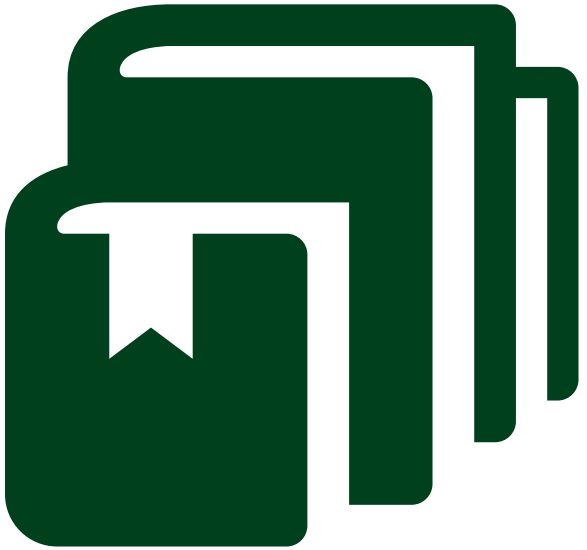
Additional sources

Review of orthogonal genetic polymers
https://pubmed.ncbi.nlm.nih.gov/24745974/

Opinions on the design of artificial biodiversity in a natural world
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2759432/
https://pubmed.ncbi.nlm.nih.gov/27903826/

Review of antibiotic resistance
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1113838/

Background information regarding dual use
https://bureaubiosecurity.nl/beleid/dual-use-research



2.3 The group discussion

Theme 1: Potential hazards and risks of orthogonality

Background information (for discussion leader)
In the paper the researchers describe the use of *E. coli* as a host for PN-DNA. This host can be safely used in an ML-I/BSL-1 laboratory. Safety concerns may arise from the PN-DNA itself. Two aspects need to be considered:

1. *The stability of PN-DNA.*
   The authors explain that the synthetic PN-DNA is acid-labile and that propagation of the genetic information in an open environment is, therefore, prevented. It is unclear what precisely is meant by the authors: is it the instability of PN-DNA itself, or the presence of PN-DNA in the host? The presence of PN-DNA as part of the genetic information in the host must be taken into account to understand whether there are any possible risks for humans and the environment. What is your understanding of what is meant by the authors when they state that 'propagation of the genetic information in [an] open environment is prevented'?
   Is PN-DNA unable to be passed to other organisms when PN-DNA containing organisms are (unintentionally) released into the environment? These aspects are important to consider with regard to the biosafety of PN-DNA and the organism containing it.

2. *The genetic information (the PN-DNA) that encodes an antibiotic resistant (AbR) gene.*
   Several antibiotic resistance genes are commonly used for the selection of recombinants in an R&D setting. The genetic information encoding a resistance gene was also used here to show the functionality of a gene. Many other marker and reporter genes, besides AbR genes, are available for showing genetic functionality. LacZ and GFP are well known examples. Therefore, was it necessary to use an AbR gene in this experiment?

Suggestions for the group discussion

Stability and safety of PN-DNA
What has been shown? What kind of experiments have been done by the authors to prove the instability of PN-DNA in acidic media? Is it possible to conclude that bacteria containing PN-DNA are also instable in the environment? Is it likely that PN-DNA containing bacteria, if (unintentionally) released in the environment, would harm human health and the environment?
Both 'natural DNA' and 'orthogonal DNA' may encode the same gene. Would you therefore regard both DNA templates equally safe? If not, why not?

The use of genetic information encoding an antibiotic resistance gene
Use of 'harmless' AbR genes in a laboratory setting, and in small scale experiments, is accepted, however in light of concerns about antibiotic resistance development, is it still wise to use them? Could you think of any other genes that could be easily used in this experiment for showing functionality?

Theme 2: Expanding the safety perspectives

Background information (for discussion leader)
The group is asked to act as a critical thinker and go beyond the safety aspects discussed under Theme 1. A number of the questions given below relate to the possibilities of dual use: a development that can do good but which can also be used in a wrong, harmful way. This is intended misuse as opposed to the unintended impacts of a development. A group discussion could begin with an explanation of the terminology 'dual use, intended misuse, unintended consequences'.

Suggestions for the group discussion

Dual use, unintended and intended consequences

- The use of PN-DNA could presumably be applied to produce a wide range of chemically redesigned genes, including metabolic pathways which could lead to novel products. What kind of applications do you think are possible and for what purpose would you design such genes? What would be the added benefit of PN-DNA properties in these applications and what might the harmful consequences potentially be? Or do you think the design of PN-DNA is only to be regarded as a proof of concept?

- In this example of orthogonality the PN-DNA is instable under acidic conditions. It can be attractive to design DNA in such a way that it is chemically more stable under different conditions. In this way the resulting DNA could be useful for interesting applications like a chemical polymer to store information, or as building material for DNA-origami structures. Apart from such applications, orthogonal DNA can also be used for harmful applications (dual use). Do you think that the design of a chemically-stable genetic polymer could indeed be harmful and should not be undertaken? Can you think of an example? Whose responsibility is this?

Other future perspectives

- Imagine, you are a designer of PN-DNA or any other form of orthogonal DNA. To what extent do you feel responsible for the safe use and application of such DNA by others in future products?
- Redesigning DNA may lead to the design of new, non-natural organisms. Who needs to be involved in discussions about the desirability of research going in that direction?

2.4 Wrap up

This case describes a successful attempt to design an orthogonal DNA that can be replicated and transferred into a living cell and expressed in a functional protein. While still in a very fundamental, early experimental setting, it is interesting, and necessary, to think about future developments that might occur from designing such a system.

By taking into account the learning objectives (see Section 'learning objectives') you can summarise the collective view on the safety aspects of this orthogonal DNA and what has been identified as relevant in relation to additional safety concerns.

Options for enriching the learning experience

Make an infographic that can be used to involve the interested public in a discussion on the contentious issues around the design and possibilities of PN-DNA.

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