Application title:

Genetic modifications of *Escherichia coli* to investigate evolutionary processes in aerobic and anaerobic environments

Applicant organisation:

AgResearch Grasslands Palmerston North

Considered by:

IBSC	ERMA
	✓

Please clearly identify any confidential information and attach as a separate appendix.

Please complete the following before submitting your application:

All sections completed	**Yes**
Appendices enclosed	N/A
Confidential information identified and enclosed separately	N/A
Copies of references attached	N/A
Application signed and dated	**Yes**
Electronic copy of application e-mailed to ERMA New Zealand	**Yes**

Signed: _____ **Date:** _____

(Christina Moon)

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Section One – Applicant details

Refer to page 9 of the user guide.

Name and details of the organisation making the application:	
Name:	**AgResearch Ltd**
Postal Address:	**AgResearch Grasslands** **Private Bag 11008** **Palmerston North**
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Name and details of the key contact person *(if different from above)***:**	
Name:	**Christina Moon**
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Name and details of a contact person in New Zealand, if the applicant is overseas:	
Name:	**Richard Scott**
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Note: The key contact person should have sufficient knowledge of the application to respond to queries from ERMA New Zealand staff.

Section Two: Lay summary and scientific project description

Refer to page 9 of the user guide.

Lay summary of the application

Approximately 200 words. This summary should describe the genetically modified organism(s) being developed, the purpose of the application or what you want to do with the organisms(s). Use simple non-technical language.

In this project, we seek to gain a more detailed understanding of the basic processes involved in evolution. In particular, we are interested in how different concentrations of oxygen affect the evolution of a common laboratory bacterium, *Escherichia coli*, where oxygenic conditions are known to impose cellular stress through damage to cellular components. Here, we will investigate the processes responsible for generating genetic variation, and also, which inherited qualities are favourable (and unfavourable) for allowing the bacteria to adapt to living with and without oxygen. We will grow cultures of *E. coli* in the laboratory under highly controlled conditions, and then monitor them for any genetic changes.

We will need to genetically modify *E. coli* to help determine the mechanisms that contribute to the faithful replication of genetic material and for adapting to living with and without oxygen, to confirm the effects of genes that are suspected to have important roles in these processes. Furthermore, to increase the chances of discovering genetic changes, the bacteria may be genetically modified to compromise the faithful reproduction of genetic material, and hence speed up the rate at which errors occur.

Scientific project description (describe the project, including the background, aims and a description of the wider project)

Refer to page 10 of the user guide. This section is intended to put the genetically modified organism(s) being developed in perspective of the wider project(s) that they will be used in. You may use more technical language but make sure that any technical words are included in the Glossary.

This project sets out to understand the effects of aerobic and anaerobic environments on evolutionary processes in bacteria. These processes include the mechanisms by which mutations arise, the types (spectra) of mutations that arise, and the rate at which they arise. Furthermore, we will determine which genetic loci are important for adaptation to aerobic and anaerobic environments. A suite of genetically modified *Escherichia coli* strains will be developed to address the specific areas being investigated around the effects of aerobic and anaerobic environments on evolutionary processes in bacteria.

A. **Parallel lineages of *E. coli* will be propagated in the presence and absence of O_2, from which, the range of mutations, and rates at which they arise will be determined from full genome sequencing. In addition, to "speed up" the rate at which mutations arise, and determine the mutational spectra in each environment, "mutator" *E. coli* strains will be genetically engineered through site-directed mutagenesis of genes involved in maintaining genome fidelity. Such genes may include those that encode products involved in DNA replication and repair, transcription and translations. This will facilitate the determination of the types of mutations (point mutations, insertions, deletions, chromosomal recombination, *etc*.) that predominate in aerobic and anaerobic environments.**

B. **The roles of cellular systems involved in maintaining genome stability under aerobic and anaerobic conditions will be determined from transcriptome analyses of cultures grown under aerobic and anaerobic conditions. Cellular systems which are differentially expressed in the environments will be disrupted using gene knockouts, to determine their effects on mutation accumulation in each culture condition. These genes are likely to be involved in DNA replication and repair; stress response pathways; the processing of reactive oxygen species and oxidatively damaged cellular components.**

C. **We will select for strains that have 'evolved' mechanisms that have enabled them to better survive under aerobiosis or anaerobiosis. The genomes of the selected strains will be sequenced and compared to the original ancestral strain in order to identify mutations that may play a role in providing the survival mechanism(s). The contributions of these genes to enhancing fitness in each environment will be determined through creating gene knockout strains (single and/or multiple gene knockouts, with deletion/disruption of the putative fitness enhancing genes) and competing these knockout strains against the ancestral strain to determine the degree to which the**

genes contribute to fitness.

D. In addition to above, *E. coli* strains containing gene expression constructs will also be developed to assess the complementation of mutations (naturally occurring, or genetically engineered) to determine gene function and the contribution of specific genes to fitness.

E. Standard genetic tools, cloning procedures and bacterial strains (including derivatives of *E. coli* K12 and B strains, which are non-pathogenic and non-toxic) will be used to develop the knockout, mutagenised and complementation strains. Standard published and commercially available vectors will be used as outlined in Section 3.3, and donor DNA will be sourced from *E. coli*, or will be chemically synthesised (based on *E. coli* sequences) to introduce specific mutations.

Short summary of purpose

Please provide a short summary of the purpose of the application. 255 characters or less, including spaces. Refer to page 11 of the user guide. This section will be transferred into the decision document.

To genetically modify *Escherichia coli* to understand the evolutionary processes involved in copying and repairing genetic material, and adapting to living, with or without oxygen.

Section Three –Description of the organism(s) to be developed

Refer to page 13 of the user guide.

3.1 Identification of the host organism to be modified

Complete this section separately for **each host organism** to be modified.

Latin binomial	***Escherichia coli* - non-pathogenic strains** Migula 1895; Castellani & Chalmers 1919
Common name(s)	***E. coli***
Type of organism	**Bacterium**
Taxonomy	Kingdom **Bacteria** Phylum **Proteobacteria** Class **Gamma Proteobacteria** Order **Enterobacteriales** Family **Enterobacteriaceae** Genus ***Escherichia*** Species ***coli***
Strain(s)	**K12 and B non-pathogenic laboratory strains and their derivatives**
Other information, including presence of any inseparable or associated organisms and any related animals present in New Zealand:	• **The micro-organisms described above are standard laboratory strains and to our knowledge contains no inseparable organisms.** • **There are no inseparable or associated organisms associated with the host species listed in this application.** • **There are no prohibited organisms involved in this application.**

3.2 Information on the host organism

Refer to pages 14-19 and pages 33-38 of the user guide for assistance in completing this section. Complete this section separately for **each host organism** to be modified.

	***Escherichia coli* (non pathogenic strains)**	**Yes**	**No**
1	Is the organism normally capable of causing disease in humans, animals, plants or fungi?		✘
2	Is the organism a human cell line?		✘
3	Is the organism native to New Zealand?		✘
4	Does the organism contain infectious agents normally able to cause disease in humans, animals, plants or fungi?		✘
5	Does the organism produce desiccation resistant structures (such as spores or cysts) that can normally be disseminated in the air?		✘
6	Is the organism characterised to the extent that its main biological characteristics are known?	✓	
7	Does the organism normally infect, colonise or establish in humans?		✘
8	If the organism is a whole plant or plant tissue, do you intend to: a) Allow it to develop reproductive structures. *If yes, please provide further information on containment in section 4* b) Keep it in a closed container?	**N/A**	
9	Is the host a Category 1 organism *(as defined in the HSNO (Low-Risk Genetic Modification) Regulations 2003)*?	✓	
10	Is the host a Category 2 organism *(as defined in the HSNO (Low-Risk Genetic Modification) Regulations 2003)*?		✘

3.3 Nature and range of the proposed genetic modification(s)

Refer to pages 15-19 and pages 33-38 of the user guide for assistance in completing this section. Complete this section separately for **each host organism** to be modified only if there are significant differences in the modifications for each of the host organisms listed above.

Information on how the new organism(s) will be developed

Vector system used *e.g. cloning or expression, plasmid, or viral*	**Commercially available and published *E. coli* cloning, mobilisable and expression plasmid vectors will be used. Such as:** – **Novagen's pET vectors for expression in *E. coli*, pKS Bluescript, pGEM, pLITMUS, pDONR, pTOPO, pACYC, pCR2.1, pCC and pUT vector series, GATEWAY vector systems, pIVETD, pUIC3.**
Range of elements that the vectors may contain	**These cloning and expression vectors generally containing the following elements:** – **Regulatory elements such as origins of replication, plasmid mobilisation regions, inducible promoters, selectable markers, transcription terminators.** – **The gene(s), or fragments of interest may include fusions or genetic elements encoding protein variants with deletions or additional amino acid residues designed to enhance expression, alter functional activity or correct protein folding.** – **May include DNA sequences encoding:** – **Protein tags (*e.g.* His, TAP and GST tags to aid protein purification).** – **reporter genes for fusion constructs (*e.g.* GFP, Luc or GUS reporter proteins to determine transgene localisation).** – **Epitopes to enhance detected and/or aid protein purification (*e.g.* ha, myc, AcV5 and FLAG).** – ***ccdB* gene and *att* recombination recognition sequences to allow positive selection of GATEWAY constructs containing insert fragments.** – ***sacB* gene to facilitate screening for chromosomal allelic exchange.** – **Multiple cloning cassette.** – ***lacZα* for beta galactosidase complementation (blue-white colony screening).** – **Cre recombinase gene for the recombination of *loxP* sites.** – **Standard and R6K origin of replication.** **Antibiotic selection genes such as those providing resistance to:** – **Spectinomycin/streptomycin, tetracycline, gentamycin, kanamycin, chloramphenicol.**
Type, source and function of any donor genetic material	**GMOs will use donor DNA from *E. coli*. The precise identities and functions of the genes and loci to be cloned, disrupted or mutagenised are not yet known, but will likely include those involved in DNA replication and repair, and systems that enhance adaptation to aerobic and anaerobic environments. These genes will be determined from transcriptome analyses and experimental**

	evolutionary experiments.
Exclusions	**<u>The modifications will exclude:</u>** – **Genetic material from New Zealand indigenous fauna and flora.** – **Genes encoding known or predicted vertebrate toxins.** – **Uncharacterised sequences from pathogenic microorganisms.** – **Genetic material that increases the pathogenicity, virulence, or infectivity of the host organism.** – **Genetic material that results in the modified organism having a greater ability to escape from containment than the unmodified host.**

Use of special genetic material

	Yes	No
Does the proposed modification use genetic material derived from organisms capable of causing disease in humans, animals, plants or fungi?		✘
Does the proposed modification use genetic material from native biota?		✘
Does the proposed modification involve human genetic material?		✘

Other details of the modification

Including any unusual manipulations. If the foreign genetic material is to be expressed, where it is expected to be expressed and what techniques will be used in the modification.

No unusual manipulations will be used. The expression of foreign genetic material (for gene complementation studies) will take place within bacterial cells. However, gene products may then be secreted or targeted to the cell membrane or cell wall as per their natural functions.

Constructs will be developed using standard recombinant DNA technologies and transformed into *E. coli* via electroporation, conjugation, or the use of chemically competent cells.

In conjugation experiments, mobilisable plasmids (which contain *mob* (mobilisation) but not *tra* (transfer) genes, and are not self-transmissible *per se*), will be mobilised to the recipient via *tra* gene functions conferred by the *E. coli* host (such as S17-1), or an helper *E. coli* strain (such as RK2013) in tri-parental conjugations. The conjugation proficient (either with or without helper strain) intermediates will not increase pathogenicity, infectivity or virulence of the non-pathogenic host, nor result in an organism with a greater ability to escape from containment. Thus this modification is considered Category A in nature.

3.4 Identify the category of experiments as described in the HSNO (Low-Risk Genetic Modification) Regulations, 2003.

Refer to pages 17-19 and pages 33-38 of the user guide for assistance in completing this section.

		Yes	No
1	Is the proposed modification to a Category 1 host organism?	✓	
2	Is the proposed modification to a Category 2 host organism?		✘
3	Will the proposed modification increase the pathogenicity, virulence, or infectivity of the host organism to laboratory personnel, the community, or the environment?		✘
4	Will the proposed modification result in a genetically modified organism with a greater ability to escape from containment than the unmodified host?		✘
5	Is the proposed modification to be carried out under a minimum of PC1 containment?	✓	
6	Is the proposed modification to be carried out under a minimum of PC2 containment?		✘
7	Does the proposed modification conform to the requirements of a Category A genetic modification?	✓	
8	Does the proposed modification conform to the requirements of a Category B genetic modification?		✘

Explanation of categorisation, if necessary. This is particularly important for work involving pathogenic microorganisms and viral vectors

Non-pathogenic laboratory strains of *E. coli* will be used as both host organisms and source of donor DNA. These strains are Category 1 host organisms and the modifications will not increase the pathogenicity, virulence, or infectivity of the host organism nor result in a GMO having a greater ability to escape from containment than the unmodified host organism. Therefore we consider this application to conform to the requirements of Category A genetic modification, requiring PC1 containment.

Section Four – The proposed containment system

Refer to page 20 of the user guide for assistance in completing this section.

Describe the containment facility and the proposed containment system (physical and operational)

Question	Answer
Which MAF/ERMA Standard is this containment facility approved under?	**Genetically modified organisms (GMOs) will be maintained under a minimum of PC1 in accordance with MAF Biosecurity / ERMA New Zealand Standard - Facilities for Microorganisms and Cell Cultures: 2007 (for bacteria and plant cell/tissue cultures).**
What physical containment level (AS/NZS 2243.3:2002) is this containment facility approved to operate at (where relevant)?	**The Australian/New Zealand Standard (AS/NZS) 2243.3:2002 Safety in Laboratories: Part 3: Microbiological aspects and containment facilities, at Physical Containment Level 1 (PC1) and Physical Containment Level 2 (PC2).**
What other physical measures do you propose to use to contain this organism?	**All experiments will be conducted in AgResearch containment facilities registered to the MAF Microorganism standard at PC1 or higher physical containment levels. No additional safety precautions beyond this are required.**
What procedural or operational measures do you propose to use to contain this organism?	**All experiments will be conducted using PC1 or higher conditions. All staff that will work on the project will be qualified to work under PC1 or higher conditions. All GMOs will be contained and stored according to PC1 or higher conditions. All cultures and consumables will be autoclaved prior to disposal and any clones relevant to the project stored at -20°C or -80°C.**
Any other information relevant to the containment of the organism.	**The *E. coli* strains are weakened compared to native strains and have a long historical record of safe usage in the world-wide molecular biology community. The described experiments are deemed to be of a low-risk nature.**

Section Five – Identification and assessment of adverse effects

Refer to page 21 of the user guide for assistance in completing this section.

This section should only be completed in detail if pathogenic microorganisms, human cells, native or valued flora and fauna were identified as host or sources of donor genetic material in section 3. It is expected that organisms meeting the low-risk regulations will not normally have any significant biological risks associated with them. However, there may still be some adverse effects that need to be identified and assessed. This might include economic, social and cultural adverse effects and other risks not addressed by the HSNO (Low-Risk Genetic Modification Regulations) 2003.

What adverse effects could this organism have on the environment - for all stages of the life cycle?

None identified. No pathogenic microorganisms, human cells, native or valued flora and fauna were identified as host or sources of donor genetic material in this application. Furthermore, the modified *E. coli* strains are unlikely to establish in the environment in the unlikely event of escape as these are derived from non pathogenic laboratory strains that have been adapted to grow in laboratory conditions and have specific nutritional requirements. These strains survive poorly in the environment, and are not known to have adverse effects on microorganisms or plants.

What adverse effects could this organism have on human health and safety?

None identified. The *E. coli* strains that will be used are non pathogenic laboratory strains. The *E. coli* K-12 strain is a debilitated strain and does not normally colonise the human intestine due to changes in its cell wall components that have affected its ability to recognise and adhere to the mucosal surface of cells lining the intestinal tract. The proposed genetic modifications are highly unlikely to increase its pathogenicity and therefore there are no increased risks to laboratory personnel.

What adverse economic effects could this organism have?

None identified. However, the significant knowledge advancements that will be made in understanding fundamental evolutionary processes from this project will improve New Zealand's international reputation in this area, and furthermore, may have implications in the understanding of the origins of cancer and the development of antibiotic resistances through mutagenic processes.

What adverse effects could this organism have on the relationship of Māori and their culture and traditions with their ancestral lands, water, sites, waahi tapu, valued flora and fauna and other taonga (taking into account the principles of the Treaty of Waitangi)? Include details of any consultation that you have undertaken.

None identified. These organisms will not contain any donor DNA from native flora or fauna or derived directly from humans. These organisms also have no increased pathogenicity and a low probability of surviving outside of laboratory artificial culture. As such, if released from Containment are unlikely to have any direct influence on Māori culture and traditions.

Are there any other potential adverse effects?

No.

The unmodified host organisms are commonly used laboratory strains and require no specialised handling requirements. They are not expected to stay alive when out of laboratory culture conditions, as they require a specialised environment to survive. Also, the genetic material placed within these host organisms is not expected to encode:

- **known or predicted vertebrate toxins,**
- **attributes that increases the pathogenicity, virulence, or infectivity of the host organism,**
- **attributes that results in the modified organism having a greater ability to affect humans, when compared to the unmodified host,**
- **attributes that results in the modified organism having a greater ability to escape from containment than the unmodified host.**

Therefore, in the unlikely event that these modified organisms escape from Containment, they are not expected to harm human health or harm the environment.

Section Six – Additional information

Refer to page 31 of the user guide for assistance in completing this section.

Additional Information	Y/N	If yes, explain
Do any of the organism(s) need approvals under any other New Zealand legislation?	**N**	
Does New Zealand have any international obligations relating to (any of) the organism(s)?	**N**	
Have any of the new organism(s) in this application previously been considered in New Zealand or elsewhere?	**N**	
Is there any additional information that you consider relevant to this application that has not already been included?	**N**	
Following the development of this organism what will the genetically modified organism be used for? *eg will experimental animals or plants be exposed to this organism?*	**N**	

Provide a glossary of scientific and technical terms used in the application:

Adaptation: The process whereby a population becomes better suited to its habitat.

Aerobic: Presence of oxygen.

Anaerobic: Absence of oxygen.

Antibiotic: A chemical that kills or inhibits growth.

Bacteria (eubacteria): One of the three taxonomic domains of life.

***ccdB* gene**: When expressed in the appropriate E. coli strain, is lethal. Provides positive selection for vector/cloning systems.

Chemically competent cells: Cells whose membranes have been treated to become more permeable to the uptake of DNA (usually by chilling in the presence of divalent cations such as Ca^{2+}).

Complementation: The rescue of function of a mutated gene by provision of the wild-type gene.

Conjugation: The natural transfer of DNA from one bacterial cell to another through cell-cell contact, or the formation of a bridge-like structure).

Construct: A vector that has been constructed to contain DNA segments of particular interest or function.

DNA: Molecules (nucleic acids) in which all hereditary information is contained. Nucleic acids are strung together in the form of genes that normally give rise to protein products.

Escherichia coli: A bacterium that lives in the lower intestine of mammals and common workhorse for molecular biology techniques.

Electroporation: A genetic transformation method using high voltage discharges to mediate entry of foreign DNA into a cell.

Experimental evolution: Testing hypotheses and theories of evolution using populations of organisms under controlled experimental conditions.

Expression: The physical manifestation of a gene, the process of being transcribed, translated and performance a function.

Fitness: The capacity of an individual to reproduce and pass on its genes to the next generation.

Gene deletion: The loss of genetic material resulting in the deletion of a gene, and loss of gene function.

Gene disruption: The disruption of a gene by deletion of a portion of it, or insertion of a sequence within it, that results in a loss of the gene's function.

Genome: The complete DNA sequence of an organism.

Knockout (gene): An organism containing an inactivated gene, usually through genetic modification resulting in gene deletion or gene disruption.

lacZα: Gene that encodes the alpha subunit of beta-galactosidase, a common reporter gene for molecular cloning that confers a screening system for clones.

***loxP*, *att* sites**: Specific DNA sequences that are the substrates of recombinase enzymes (Cre, for *loxP*, and BP and LR clonase enzymes for *att* sites). Facilitates cloning procedures.

***mob*:** Plasmid-borne gene that enables mobilisation of the plasmid from host to recipient cells *via* conjugation in the presence of transfer proteins.

Mutation: A change in the DNA sequence of a cell's genome.

Plasmid: Any DNA molecule in cells that replicates independently of the chromosome and regulates its own replication so that the number of copies of the DNA molecule remains relatively constant.

Reactive oxygen species: Highly chemically reactive oxygen containing molecules that contain unpaired electrons in an open (valence) shell format.

RNA: Ribonucleic acid is a nucleic acid polymer consisting of nucleotide monomers that plays several important roles in the processes that translate genetic information from DNA into protein products.

***sacB* gene:** Encodes levan saccharase which, in the presence of sucrose, facilitates the counter-selection of cells that do not contain *sacB* during allelic exchange modifications.

Selection (natural selection): The process by which heritable fitness-enhancing traits become more common in a population over successive generations.

Sequence: Sequence of nucleotides, usually of DNA.

Transcriptome: The set of all RNA molecules, including mRNA, tRNA rRNA, and non-coding RNA that are produced by an individual or population of cells.

Transformation: Process of introducing foreign DNA into a cell.

Vector: Plasmid that can be readily used as a vehicle for the cloning of foreign DNA fragments.

List of appendices attached:

None.

List of references attached:

None.