University of Sydney Institutional Biosafety Committee

- This form is to be completed by the Principal Investigator and attached to the project proposal.

Name of Principal Investigator:
Dr Nicholas Coleman

Project title:
Construction of a new cloning system in E.coli based on integron recombination and natural transformation

Type of project proposal:		
X Exempt Dealing	☐ NLRD	☐ DNIR

RIMS Project ID Code: (for projects that are part of a funded research grant)

- For completion by IBC.

IBC Reference Number

Name of IBC Chair	Signature	Date of approval
Prof Anthony Weiss		

University of Sydney Institutional Biosafety Committee – Exempt Dealing application form

1. Exemption Category – Please place an X in the appropriate box/s

OGTR Item #	Mark with an X	Description of Dealing
2		A dealing with a genetically modified *Caenorhabditis elegans*, whereby:
		a) an advantage is not conferred on the animal by the genetic modification; and
		b) as a result of the genetic modification, the animal is not capable of secreting or producing an infectious agent.
3		A dealing with an animal into which genetically modified somatic cells have been introduced, if:
		a) the somatic cells are not capable of giving rise to infectious agents as a result of the genetic modification; and
		b) the animal is not infected with a virus that is capable of recombining with the genetically modified nucleic acid in the somatic cells.
3A		A dealing with an animal whose somatic cells have been genetically modified *in vivo* by a replication defective viral vector, if:
		a) the *in vivo* modification occurred as part of a previous dealing; and
		b) the replication defective viral vector is no longer in the animal; and
		c) no germ line cells have been genetically modified; and
		d) the somatic cells cannot give rise to infectious agents as a result of the genetic modification; and
		e) the animal is not infected with a virus that can recombine with the genetically modified nucleic acid in the somatic cells of the animal.

4	X	1) Subject to subitem 2) below, a dealing involving a host/vector system mentioned in Part 2 of Schedule 2 and producing no more than 25 litres of GMO culture in each vessel containing the resultant culture.
		2) The donor nucleic acid: a) must meet either of the following requirements: i) it must not be derived from organisms implicated in, or with a history of causing, disease in otherwise healthy human beings, animal, plants or fungi;
		ii) it must be characterised and the information derived from its characterisation show that it is unlikely to increase the capacity of the host or vector to cause harm; and
		b) must not code for a toxin with an LD_{50} of less than 100µg/kg; and
		c) must not code for a toxin with an LD_{50} of 100µg/kg or more, if the intention is to express the toxin at high levels; and
		d) must not be uncharacterised nucleic acid from a toxin-producing organism; and
		e) must not include a viral sequence, unless the donor nucleic acid: i) is missing at least 1 gene essential for viral multiplication that: A) is not available in the cell into which the nucleic acid is introduced; and B) will not become available during the dealing; and ii) cannot restore replication competence to the vector.
5		A dealing involving shot-gun cloning, or the preparation of a cDNA library, in a host/vector system mentioned in item 1 of Part 2 of Schedule 2, if the donor nucleic acid is not derived from either: a) a pathogen; or b) a toxin-producing organism.

2. Name and full professional address of Principal Investigator submitting proposal
Name: Dr Nicholas Coleman Department: School of Molecular and Microbial Bioscience Room No: 564 Building Name: G08 Tel: 9351 6047 Email: nicholas.coleman@sydney.edu.au

3. Name(s) of other Principal Investigators responsible for the project. Please provide their professional addresses if different from that above.
Dr. Hannah Nicholas. School of Molecular Bioscience. (project co-supervisor) hannah.nicholas@sydney.edu.au

4. Describe the aim of the work
The aim is to provide proof of principle for a new gene cloning and expression method, which is useful for assembly of multiple genes at a single locus. The method combines integron-mediated site-specific recombination, and natural transformation of DNA. This phenomenon has been seen before in Pseudomonas, our goal is to make this occur also in E.coli. We will remodel the class 1 integron recombination and expression system in E.coli, to separate the recombination and cassette-expression functions, and bring these under the control of arabinose and lactose, as inducers, respectively. We will then investigate the effect of co-expression of genes potentially involved in natural transformation alongside the integron system, to see if we can demonstrate uptake and recombination of gene cassettes, as occurs in e.g. Pseudomonas stutzeri. These experiments will be done in the framework of the iGEM competition, which is an international undergraduate science fair. iGEM hosts an open-source Parts registry of DNA elements which we will draw from and contribute to as part of this project. The iGEM foundation strongly promote the principles of ethical and safe use of genetic modification techniques, and this document will be used as part of our proof that we have complied with iGEM safety regulations.

5. Describe the main experimental procedures of the work
All work will be done in E.coli TOP10 or in similar E.coli K12-derived laboratory strains of E.coli. Synthetic pieces of DNA ("gBlocks") or PCR products will be joined to a cloning vector (pSB1C3 or pUS41 or pUS201) by the Gibson assembly method (exonuclease/ polymerase/ ligase mixture). The resultant DNAs will be transformed into E.coli TOP10. The phenotype of the resultant recombinants will be investigated via recombination assays to determine their ability to take up and integrate foreign DNA provided in the form of non-replicative gene cassetes. The genes to be constructed / amplified are described in detail on the next page. Different combinations of integron recombination genes, natural transformation-related genes, and regulatory systems will be cloned in the three different vectors pSB1C3, pUS41 and pUS201. The effect of these different constructs on DNA transformation and integron recombination will be tested by adding synthetic gene cassettes to E.coli cells carrying the plasmids. Successful recombination results in GmR blue colonies of E.coli. If successful, we will expand the work to include making gene cassettes from the other chromoprotein genes listed on the next page. The gene cassettes are non-replicative DNA circles containing aacC1 (GmR) and aeBlue (chromoprotein), linked to an integron recombination site (attC). The cassettes are made in-vitro by PCR and self-ligation.

6. Details of biological system Note: Any substantial change in the system will require submission of another proposal
(a) Describe the biological source of the donor DNA to be used – include the genus, species and strain or organ/tissue as applicable. Include the specific genes to be involved in the dealing.

E.coli genomic DNA or various plasmids will be used as the source of DNA for amplification of PCR products and for the design of gBlocks. The gBlock method will be used when restriction sites need to be removed from the sequences – in these cases, the sequence will be modified as minimally as possible, to preserve the sequence of any known protein coding regions.

The complete list of genes and sources is described below.

1. Resistance genes

- aacC1 Gentamicin resistance (GmR) from cloning vector pUCP24
- aph3A Kanamycin resistance (KmR) from cloning vector pK18

2. Regulatory genes and promoters

- lacI-lacO-pLAC from E.coli P801
- araC-araO-araI-pBAD from E.coli P801
- tetR-tetO-pTET from Tn10 (orig. in R100 plasmid, *Shigella flexneri*)

3. Chromogenic marker genes

- GFP*mut3* from pUS23 (orig. from jellyfish *Aequoria victoria*)
- amilGFP from iGEM parts kit (orig. from coral *Acropora millepora*)
- amilCP from iGEM parts kit (orig. from coral *Acropora millepora*)
- aeBlue from iGEM parts kit (orig. from anemone *Actinia equina*)
- eforRed from iGEM parts kit (orig. from coral *Echinopora forskaliana)*

4. Genetic components of the integron recombination system

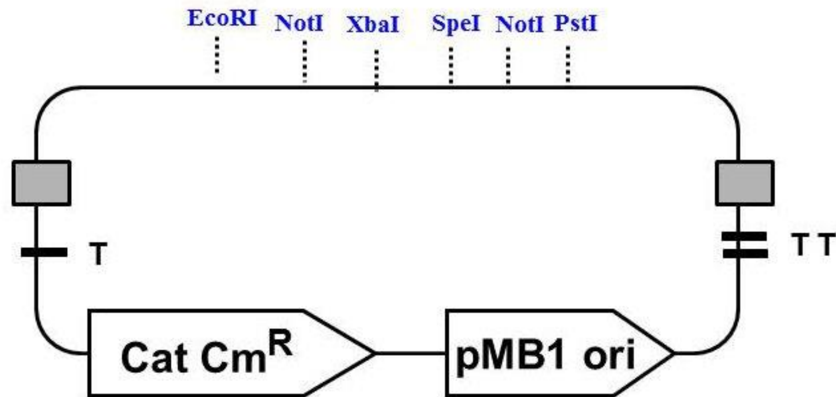
- intI1 site-specific recombinase gene from plasmid R388 (orig. from E.coli)
- attI1 recombination site from from plasmid R388 (orig. from E.coli)
- $attC_{aadB}$ recombination site from plasmid pUS23 (synthetic site)

5. Genes proposed to be involved in natural transformation

- sxy from E.coli P801
- comE from E.coli P801
- comA from E.coli P801
- pilQ from E.coli P801
- pilE (fimH) from E.coli P801
- pilG from E.coli P801
- pilF from E.coli P801

(b) Describe the host organism or tissue to be used – include the genus, species and strain where applicable. If not a commonly used laboratory strain, include the name of the strain from which it is derived. Escherichia coli TOP10 – a standard and very widely used cloning host found in Schedule 2, Part 2 as an exempt host system. This is a K12 derivative, and like most E.coli lab strains, it is considered non-pathogenic. No reports of human infection arising from this strain could be found.
(c) Describe the vectors or methods to be used to transfer donor DNA to the host. Include information regarding the origin and properties of the vector and confirm that all bacterial plasmid vectors are non-conjugative. If your project involves the use of a replication defective viral vector (unable to transduce human cells), please provide an explicit description of the assay you intend to perform to exclude the presence of replication competent virus. Three plasmid vectors will be used, described below. These (and their derivatives) will be introduced into E.coli TOP10 competent cells by heat shock, and potentially also by natural transformation. pSB1C3 (2.0 kb) Pre-existing – ref. (1). A narrow host range E.coli plasmid derived from the pUC plasmids. Selection is via the chloramphenicol resistance gene catA1. Non conjugative, and non-mobilisable. Contains transcription terminators flanking a multiple cloning site. pUS41 (8.9 kb) Pre-existing – ref. (2). A broad host range mobilisable but non-conjugative plasmid, with the rep, oriV, oriT, mob, StrAB, Sul genes from the resistance plasmid RSF1010, and a multiple cloning site derived from the plasmid pZERO-2. pUS201 (2.8 kb). Propose to construct this here, by joining the KmR gene from plasmid pK18 to the minimal F plasmid replicon (oriV, repE, IncC) from the plasmid pCC1FOS. This low-copy KmR plasmid pUS201 will then be used for further cloning steps.

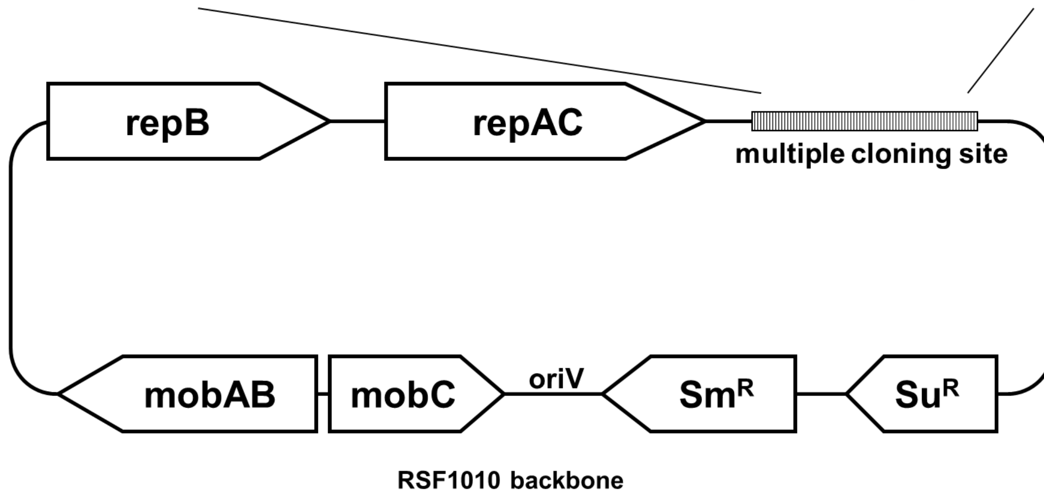
pSB1C3 2.0 kb, pMB1 oriV (E.coli only). Chlorampenicol resistance due to catA1 gene. Multiple cloning site (EcoRI, NotI, XbaI, SpeI, NotI, PstI) is flanked by transcription terminators

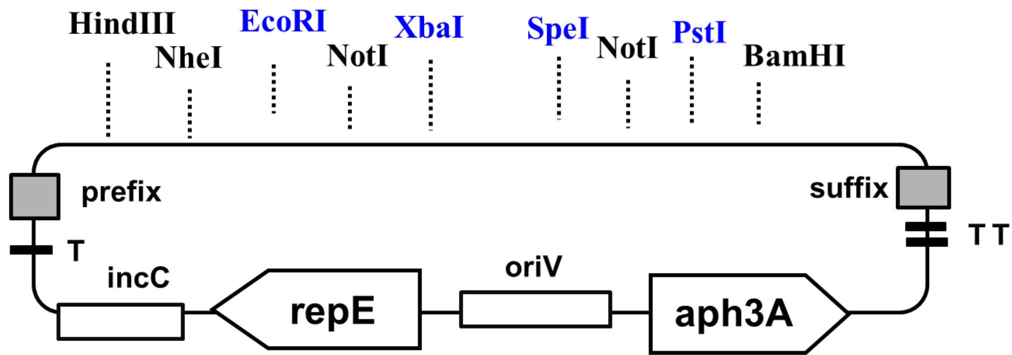


pUS41 8.9 kb **SmR, SuR, BHR**

RSF1010 derivative containing 9 unique cloning sites.
MCS derived by PCR from plasmid pZERO-2.

BstEII, SalI, ApaI, XbaI, XhoI, SpeI, BamHI, KpnI, HindIII, BglII, BstEII




pUS201
2.8 kb, low copy, E.coli only,
Mini-F replicon, KmR,
HindIII
NheI
EcoRI
NotI
XbaI
SpeI
NotI
PstI
BamHI
prefix
suffix
T
TT
incC
repE
oriV
aph3A

7. If you believe the protein/gene is characterised, and unlikely to increase the capacity of the host or vector to cause harm, briefly explain why, referring to what is known about its structure, function and/or genetics.

<u>1. Resistance genes</u>

The KmR gene aph3A is widely used in cloning vectors, and gives resistance to kanamycin and neomycin. This gene and similar aminoglycoside phospotransferases (e.g. *aphA1/neo*) are widely dispersed in Nature, and are very well-studied genes/enzymes; they exist naturally in environmental and clinical bacteria, and have been released to the environment in GMO plants such as the RoundUp ready soybean. Kanamycin and neomycin are not major clinically-important antibiotics. Due to the containment protocols in place, and the fact that KmR genes are already ubiquitous in the environment, the risk of harm from the KmR bacteria or genes here is exceedingly low.

The GmR gene *aacC1* gives gentamicin resistance. This gene has found use in a few cloning vectors but is not as widely used as the KmR gene. The aacC1 gene is found in some Gram-negative pathogenic bacteria, which contain these genes as integron gene cassettes. Gentamicin is an important clinical antibiotic, so the use of this antibiotic and the corresponding resistance genes here does pose a hazard, but due to the containment protocols in place (e.g. autoclaving all GMOs after use in experiments), the risk of harm due to GmR bacteria or genes escaping is judged to be very low.

<u>2. Regulatory genes and promoters</u>
The lactose, arabinose, and tetracycline control systems to be used here are some of the best studied genes and DNA sequences known. These are found naturally in E.coli and many other bacteria, and all are used very widely in the construction of expression vectors. No substantial risks are posed by these regulatory genes and sequences.

<u>3. Chromogenic marker genes</u>
There are five proposed chromogenic marker genes, these include the very widely used green fluorescent protein GFP, and some newer variants of GFP, which are sourced from a variety of marine organisms, especially corals. The GFP and related chromoproteins pose no conceivable risks.

<u>4. Genetic components of the integron recombination system</u>
The integron recombination and expression system is responsible for assembly of arrays of antibiotic resistance genes. The class 1 integron in particular is notorious for this, and class 1 integrons can be readily detected in a large proportion of Gram-negative clinical isolates. Here we will deconstruct the integron system, and change the way that the recombination and cassette expression work in the integron.

There is an element of risk in this work, for example, if our lab integron works better than wild-type integrons at capturing resistance genes, and this system got loose into Nature, this might accelerate the spread of resistance genes. This is very unlikely, firstly because the triggers for recombination and

expression in the recombinant system are millimolar levels of the sugars lactose and arabinose; these conditions would very rarely occur in Nature. Secondly, the control mechanisms in the natural system are ‘better’ in terms of being of adaptive value to the bacteria. Thirdly, the lab containment protocols will prevent any GMO integrons from leaving the lab.

5. Genes proposed to be involved in natural transformation

We are going to test the effect of overexpression of one regulatory gene (sxy) and six structural genes (com/pil) that have been proposed to be involved in natural transformation of DNA in bacteria. Our aim is to make an E.coli strain that is (more) naturally transformable. There are two risks here that must be addressed, firstly, that pilus production might be a virulence factor in itself, and secondly, that the naturally-transformable phenotype could make a pathogen more dangerous because it is better at acquiring DNA.

It must be noted that pilus genes can potentially be pathogenicity determinants, since these may influence binding to surfaces, and play a role in export of toxins or other virulence factors. The type 1 pilus structure that will be studied here (notably pilE) is found in approx. 70% of all faecal E.coli isolates (Ornroff and Bloch, 1990), most of which are commensals, so it is certainly not a dedicated virulence factor. However, the type 1 pilus helps the E.coli cells bind to mannose receptors on human cells, so it is a potential virulence factor under certain circumstances.

We believe that the issue of potential pathogenicity arising from pilus production is not significant, based on consultation of the literature on pathogenic Ecoli strains, and consideration of the other conditions of our experiments, particularly the use of a tame lab strain such as TOP10 as a host. Multiple virulence factors are necessary to create a dangerous E.coli; these include especially the presence of toxins, invasins, fimbriae, and capsule. Overexpressing the genes for a pilus which is already found in most E.coli strains will not change the non-pathogenic status of E.coli TOP10.

In a clinical context, a naturally transformable strain could conceivably be more dangerous than a non-transformable strain, since it would be more able to acquire antibiotic resistance genes or virulence genes from its environment. This is a subtle and indirect risk, but it is a real one. This risk will be controlled in several ways; first, the strain used for the cloning experiments (TOP10) is an attenuated lab strain, not a pathogenic E.coli; second, biological containment protocols will prevent any GMOs leaving the lab, and third, none of the plasmids used are conjugative – this will stop the spread of the genes even if the GMOs escaped the lab.

8. Please attach printed evidence (such as the results of a Medline or PubMed search) that the donor DNA does not code for a toxin.

Search ‘toxin’ and ‘integron’

- 28 results total. 25 of these relate to analysis of plasmids or bacteria which contain integrons and also contain toxins of various types. 3 of these describe toxin/antitoxin gene cassettes found in some integrons (note these are antibacterial toxins, not human toxins). none suggest the integron itself is toxic

Search ‘toxin’ and ‘integrase’
- 49 articles retrieved. all of these describe various kinds of mobile genetic elements, especially bacteriophage, which use an integrase enzyme to move around, and which also contain toxin genes of one sort or another. none of thee articles suggest that the integrase protein is itself a toxin.

Search ‘toxin’ and ‘sxy’
- no results

Search ‘toxin’ and ComE or ‘toxin’ and ComA
- too many non-specific results (hundreds), due to gene names also being words ‘come’ and ‘coma’.

Search ‘toxin’ and ‘pilus’ and ‘ComE’
- no results

Search ‘toxin’ and ‘pilus’ and ‘ComA’
- no results

Search ‘toxin’ and ‘pilQ’
- no results

Search ‘toxin’ and ‘pilus’ and ‘pilE’
- three results, all relating to the toxin co-regulated pilus protein TcpA in Vibrio cholera – this is a PilE homolog, and is an important virulence factor, although not directly a toxin.

Search ‘toxin’ and ‘pilus’ and ‘pilG’
- no results

Search ‘toxin’ and ‘pilF’
- one result, a paper that studied cholera toxin and pilFin Vibrio bacteria. this paper did indicate that certain pilF types of V.cholerae were more virulent than others, so in this sense pilF is a virulence factor, at least in Vibrio spp.

I have not done this search for the antibiotic resistance genes or the fluorescent marker genes, since these are gene types that are very widely used, commercially available, and very low risk. The focus above is on the integron system, and the genes related to natural transformation; the latter do seem to present some level of hazard, if not risk.

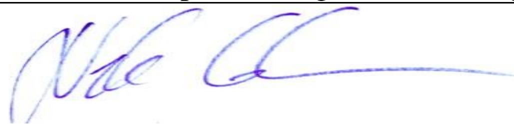
Any genes relating to synthesis of pili are possible virulence factors due to the importance of pili in transfer of macromolecules (e.g. toxins) into the host. Some of our target genes such as pilF and pilE are known to be virulence factors in other genera of bacteria, such as Vibrio (see literature search below). However, in the case of E.coli TOP10, I strongly believe that the proposed over-expression of these pilus-formation genes will not alter the pathogenicity of this organism, which will remain non-pathogenic. As discussed above, it takes many virulence factors together to give a pathogenic E.coli, notably toxins, which are absent here. Providing just the pilus machinery will not cause any problems in the case of TOP10 since there are no toxins or other 'nasties' for this pilus to transport.

9. Where will this work be conducted? Please provide Room Number, Building Name and Building Code.
Building G08, Room 564.

10. Do you have approval to use this facility?	
Yes [X]	No [ ]

11. Proposed date of commencement of work.
As soon as permission is granted by the IBC.

12. Likely duration of work.
2 years

13. Signature of Principal Investigator submitting this proposal.
 *Date* 28/5/2014

1. **Shetty R, Lizarazo M, Rettberg R, Knight TF.** 2011. ASSEMBLY OF BIOBRICK STANDARD BIOLOGICAL PARTS USING THREE ANTIBIOTIC ASSEMBLY, p. 311-326. *In* Voigt C (ed.), Synthetic Biology, Pt B: Computer Aided Design and DNA Assembly, vol. 498.
2. **Gestal AM, Liew EF, Coleman NV.** 2011. Natural transformation with synthetic gene cassettes: new tools for integron research and biotechnology. Microbiology **157:**3349-3360.