WETENSCHAPPELIJK INSTITUUT
VOLKSGEZONDHEID
INSTITUT SCIENTIFIQUE
DE SANTÉ PUBLIQUE

Division of Biosafety and Biotechnology

Heterologous expression of Mycolactone in *Mycobacterium smegmatis*

1. Aim of the project

Mycobacterium ulcerans is an emerging human pathogen of class of risk 3 harboured by aquatic insects, and is the causative agent of Buruli ulcer, a skin disease prevalent in tropical and subtropical regions, characterised by development of chronic necrotic but otherwise painless ulcers. The mode of transmission is unknown, although there is some evidence that it is transmitted through bites of aquatic insects. There is no vaccine (apart from a short lived protection with BCG) and so far no effective drug treatment. This pathology is attributed to mycolactone, a cytotoxic lipid consisting of a polyketide side chain attached to a lactone core that apparently has cytotoxic, analgesic and immunosuppressive activities. In studies using a guinea pig model of the disease, purified mycolactone injected subcutaneously induces extensive tissue damage and immunosuppression implying a key role for the toxin in pathogenesis. Three genes, lying on the circular virulence plasmid pMUM001 of *M. ulcerans* and encoding giant polyketide synthases are responsible for the production of mycolactone.
The aim of the study is the heterologous expression of mycolactone or isolated polypeptides in *Mycobacterium smegmatis*, a fast growing non pathogenic mycobacterium, for the use in diagnostic methods, kits, vaccines, therapy and for the production of mycolactone derivatives or novel polyketides by combinatorial synthesis.

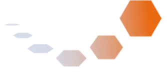
The method involves the following 2 steps

- Step 1: Transfer of the genes encoding the enzymes responsible for the synthesis of the 80 kbp mycolactone core structure (called hereafter core fragment) into *M. smegmatis*:
  1) a bacterial artificial chromosome clone (BAC) Mu0022B04, containing the core fragment is subcloned in *E.coli* by means of the shuttle vector pBEL, harbouring a gene conferring apramycine resistance;
  2) *M. smegmatis* is electroporated with DNA prepared from a apramycine resistant *E. coli* clone to produce a GM *M. smegmatis* called *M. smegmatis*::core.

- Step 2: Transfer of the genes encoding the enzymes responsible for the synthesis and the attachment of the mycolactone side chain structure (called hereafter side chain fragment) into *M. smegmatis:: core:*
  1) a bacterial artificial chromosome clone (BAC) Mu0022D03, encoding the side chain fragment and containing genes allowing autonomous replication of pMUM001, is submitted to transposon mutagenesis using the EZ:TN system which randomly inserts a kanamycine resistance gene (selection of a mutant with the kanamycine resistance in a gene not essential for mycolactone biosynthesis);
  2) *M. smegmatis*::core is electroporated with DNA prepared from a kanamycin resistant clone to produce a GM *M. smegmatis, called M. smegmatis::mycolactone* resistant to both apramycine and kanamycine and producing mycolactone and its co-metabolites.

2. Vector/plasmid

1) The shuttle vector used in step 1 is a well-characterized vector type pBEL, containing an L integrase gene, a mycobacterial L5 attachment side and an apramycine resistance gene. It is used to clone the above mentioned core fragment in *E.coli*. The L intergrase will facilitate the stable



integration in the mycobacterial host cell. Apramycine is an antibiotic for veterinary use and is employed to treat intestinal infections in pigs. It is not used for treatment of human disease.

2) The BAC clone Mu022D03 used as shuttle vector in step 2 contains a kanamycine resistance gene, widely used as a marker gene. Kanamycine is also an antibiotic for veterinary use and is employed to treat pulmonary infections in cats and dogs.

3. Inserts

The inserts harbour genes encoding the enzymes responsible for the synthesis of the mycolactone core structure, and to genes encoding the enzymes responsible for the synthesis and the attachment of the mycolactone side chain structure. As selectable marker antibiotic resistance genes against apramycine and kanamycine.are used.
Mycolactone is a toxin with a predominant role in the pathogenesis of *M. ulcerans*. Quite surprisingly, it was demonstrated that purified core lactone alone has also a cythopatic effect comparable to mycolactone, although 10.000 fold less active.

4. Localization of inserted genetic material

1) The insert containing the genes encoding the enzymes responsible for the synthesis of the mycolactone core structure is stably integrated (action of Phage integrase L5) in the genome of the first recipient organism *M. smegmatis*.

2) The insert containing the genes encoding the enzymes responsible for the synthesis and the attachment of the mycolactone side chain structure is harboured by the shuttle plasmid Mu0022D03 with a kanamycine resistent marker.

5. Receptor organisms

E.coli and M. smegmatis mc2 155 used in the first step are a non-pathogenic strains belonging to the **class of risk 1**. *M. smegmatis mc2 155* is often used as model organism in research on mycobacteria. The receptor organism involved in the second step is the resulting GMM of the first step (see below).

6. Biological hazards and/or considerations related to the manipulation of the resulting genetically modified micro-organism (GMM)

The two steps involved in the construction of the final GM *M. smegmatis* mycolactone production strain give each rise to a GM *E. coli* strain and two different GM *M. smegmatis* strains:

1) In the first step, transformation of *E.coli* and the *M. smegmatis* strain *mc2 155* leads to the construction of a GM *E. coli* and a GM *M. smegmatis* called *M. smegmatis:: core.* These GMMs harbours the genes encoding the mycolactone core. Since the core lactone in itself can have a low cytopathic effect (see above), the resulting GMMs should be assigned to **class of risk 2**.

2) The final mycolactone producing GM *M. smegmatis* obtained in step two harbours both the genes encoding the mycolactone core and side chain, leading to the production of the toxin mycolactone. This GM *M. smegmatis* mycolactone production strain should be assigned to the **class of risk 3.** Indeed, the genetic modification confers the recipient organism with the capacity to produce a toxin that is the major pathogenicity determinant in *M. ulcerans*. Moreover, the toxin alone is sufficient to induce the pathogenic phenotype (as confirmed in *in vivo* studies), and it will be produced in a fast growing mycobacterium.
The main risk related to the manipulation of these toxin producing strains is exposure of damaged skin.

7. Class of risk of the resulting GMMs

The GM *E.coli* strain is assigned to **class of risk 2.**
The GM *M. smegmatis:: core* used in this project is assigned to **class of risk 2**
The GM *M. smegmatis* mycolactone production strain is assigned to **class of risk 3**

8. Class of risk of the activity

Cloning of BAC fragments in *E.coli* laboratory strains is of class of risk 1. Transformation of *M. smegmatis* leading to the production of the toxin mycolactone is of class of risk 2 since, unlike mycobacteria of the *Mycobacterium tuberculosis* complex, the GMM as well as the recipient and donor organisms are not transmitted by air.

9. Recommended containment measures

All bacterial genetic manipulations involving transformation of the *M. smegmatis* strain should be performed in a standard biosafety level 2 facility **(BSL2)**. Gloves should be worn to prevent the toxin to penetrate damaged skin.

10. Reference and further reading

- Timothy P. Stinear, Melinda J. Pryor, Jessica L. Porter and Stewart T. Cole (2005): Functional analysis and annotation of the virulence plasmid pMUM001 from Mycobactarium ulcerans. Microbiology, 151, 683-692.

- Armand Mve-Obiang, Richard E. Lee, Françoise Portaels, and P.L.C. Small (2003): Heterogeneity of mycolactones produced by clinical isolates of *Mycobacterium ulcerans*: implications for virulence. Infection and Immunity, vol 71, No 2, p 774-783.

- Mycolactone locus: an assembly line for producing novel polyketides, therapeutic and prophylactic uses.
  United States Patent 20060024806
  Publication date: 02/02/2006
  http://www.freepatentsonline.com/y2006/0024806.html