Lactococcus lactis-based vaccines
Current status and future perspectives

Mohammed Bahey-El-Din1,4 and Cormac G.M. Gahan1,3,*
1Department of Microbiology; 2School of Pharmacy; 3Alimentary Pharmabiotic Centre; University College Cork; Cork, Ireland; 4Department of Pharmaceutical Microbiology; Faculty of Pharmacy; Alexandria University; Egypt

Lactococcus lactis offers significant potential as a platform for the delivery of vaccines especially via mucosal routes of administration. The organism has an established history of safe use in the food industry and is highly amenable to genetic manipulation, with many systems available for efficient production of secreted and surface-expressed proteins. Here we describe the benefits of using this organism as a vaccine delivery platform and outline how L. lactis based antigen delivery may be improved. Finally we discuss the safe use of L. lactis vectors and outline the potential for use of biological containment systems and killed lactococcal preparations.

Benefits of Lactococcus lactis-based Vaccines

Lactococcus lactis is a GRAS (generally regarded as safe) bacterium that is widely used in the food industry. However intensive genetic and molecular research carried out on this organism over the past two decades has led to an extension of the use of L. lactis into potential new biomedical applications. These include vaccine delivery, gene delivery, heterologous protein expression and therapeutic drug delivery.1,5

L. lactis has a number of advantages over other bacterial species for use as a vaccine delivery platform. The bacterium has a well established safety profile through its long use in dairy food products. It is a Gram-positive bacterium and therefore does not possess endotoxic lipopolysaccharides (LPS) which are associated with commonly used Gram-negative delivery vehicles such as Escherichia coli or Salmonella typhimurium. Moreover, L. lactis has a smaller genome size and fewer native exoproteins than E. coli and therefore fewer native proteins are present to contaminate samples when the organism is used as an expression host for heterologous proteins.3,6-8

As mucosal surfaces represent an entry route for many pathogens, establishing specific mucosal immunity (including induction of secretory IgA antibodies) by mucosal (oral, intranasal (IN), vaginal or rectal) vaccination can aid the early and efficient elimination of infection. Moreover, mucosal vaccination has the ability to elicit both mucosal and systemic immune responses9 and the relative ease of mucosal vaccine administration ensures better patient compliance. Since the safety of L. lactis is well established, this organism has significant appeal as a mucosal vaccine delivery vector. L. lactis is non-invasive and non-commensal and thus has less potential to trigger immunotolerance or side effects upon prolonged use.2

In addition, L. lactis bacteria are similar in dimensions to microparticle vaccine delivery systems which are known to be taken up by the microfold cells (M cells) of the intestinal mucosa.10,11 The presentation of antigen to the immune system in the context of particulate Lactococcus cells avoids the development of host immunotolerance which is commonly induced by oral administration of soluble antigens.12 Furthermore, only a weak immune response is elicited against the Lactococcus vector despite its ability to
Numerous studies have utilised engineered *L. lactis* strains as delivery platforms for bacterial, viral and protozoal antigens and these have been reviewed in detail elsewhere in reference 4 and 14. In the majority of these studies vaccination resulted in a positive immunological outcome in the particular animal model used. However, the vaccination efficacy varies significantly depending on a variety of factors including the route of administration and the amount and nature of the antigen expressed. A number of studies have demonstrated the generation of optimal immune responses through systemic or IN administration of recombinant *L. lactis* strains but failed to demonstrate immunity when recombinant strains were administered orally. Upon oral administration *L. lactis* and accompanying antigens are subjected to a relatively harsh physico-chemical environment which is likely to affect bacterial survival and antigen production. Indeed, we have observed that *L. lactis* survives relatively poorly in this environment indicating that oral vaccination by *L. lactis* strains may be a challenge.

However, despite these observations some other studies have been able to elicit significant immune responses using *L. lactis* strains administered orally to mice. For example, oral immunization with *L. lactis* secreting the SARS-coronavirus nucleocapsid N protein induced significant N-specific IgG in the sera of vaccinated mice. In another study orally administered recombinant *L. lactis* expressing the pneumococcal protective protein A (PppA) generated appropriate mucosal immunity and provided protection against respiratory pneumococcal infection. It is possible that high level expression of the particular antigens by *L. lactis* in these studies compensated for the poor gastrointestinal survival of the vector.

Possible approaches towards improving vaccine efficiency include co-expression of immunostimulatory cytokines or co-delivery of an adjuvant. Indeed, Xin et al. developed an *L. lactis* strain expressing surface bound HIV envelope (Env) protein. They demonstrated that oral immunization with this strain was most efficient when delivered with inactivated cholera toxin as an adjuvant. Steidler and coworkers engineered *L. lactis* to secrete biologically active murine IL-2 and IL-6. Intra-nasal administration of *L. lactis* coexpressing tetanus toxin fragment C (TTFC) and IL-2 or IL-6 induced significantly higher serum anti-TTFC IgG compared to *L. lactis* expressing TTFC alone. Similarly when *L. lactis* expressing IL-12 was intranasally coadministered to mice with another *L. lactis* strain expressing cell wall-anchored E7 antigen of HPV-16 (Human Papilloma virus type-16), a significant potentiation of the E7-specific cellular immune response was observed. These results highlight the possibility of potentiating the vaccine response using adjuvants coexpressed or co-delivered with other antigens in *L. lactis*.

Finally, improved delivery of live strains may potentially be achieved through selection of novel *L. lactis* strains with enhanced stress resistance properties, genetic manipulation of strains to improve in vivo delivery or encapsulation of live organisms to enhance protection against stomach acids. Indeed Steidler and coworkers have utilised encapsulation of freeze-dried *L. lactis* vector for oral administration in humans. We have shown that bile tolerance of *L. lactis* can be improved by heterologous expression of a bile tolerance protein derived from *L. monocytogenes*. This engineered strain was shown to survive significantly better in the gastrointestinal tract of mice when compared to wild-type *L. lactis*. The approach is a good example of the Pathobiotecology concept for genetically improving the efficacy of live strains destined for biotechnological and biomedical uses. Potential innovations to improve oral delivery of *L. lactis*-based vaccines are outlined in the accompanying figure.
Overcoming Barriers to the Use of Genetically Modified *L. lactis* Vaccine Strains for Humans or Animals

Although wild-type *L. lactis* bacteria have GRAS (generally regarded as safe) status, engineered vaccine strains of *L. lactis* are classified as GMMs (genetically modified microorganisms). This naturally evokes concerns regarding the safety of GMMs for human or animal applications. These concerns include the risk of possible horizontal transfer of transgenes to other environmental microorganisms or the unexpected behaviour of the GMM in the environment. Clearly the use of antibiotic resistance markers in such strains is not feasible due to the risks of transfer of such genes to environmental microorganisms. The concerns outlined above have led to efforts to limit the spread of engineered strains in the environment (biological containment) or to use killed lactococcal particles as vaccine vectors.

**Biological Containment through Pyrimidine Synthesis Knock-Out Systems**

Steidler and colleagues employed a pyrimidine synthesis knock-out system to ensure strict thymidine auxotrophy in a genetically modified *L. lactis* MG1363 strain designed to secrete hIL-10 (human interleukin 10). In that study the essential *thyA* gene of *L. lactis* MG1363, encoding the thymidylate synthase enzyme, was replaced by the hIL-10 gene through homologous recombination. The resulting auxotrophic strain has no antibiotic resistance marker and the hIL-10 gene was stably integrated in the *L. lactis* chromosome. This hIL-10-secreting strain requires the continuous availability of thymidine for growth and survival and removing thymidine from the growth medium has a dramatic bactericidal effect on the *thyA* mutant strain. Thus the mutant GMM was described to be "biologically contained" since it cannot survive in the natural environment where thymine and thymidine are limiting. This strain, designated Thy12, is currently under clinical investigation to treat inflammatory bowel disease (IBD).

Although the concept of thymine-less death of bacteria has long been reported in the literature its use in *L. lactis* vectors for human applications has only been developed relatively recently. The acceptance of *thyA*-deficient *L. lactis* as a standard for biological containment is growing worldwide where it has been approved by the Belgian Biosafety Advisory Council, the Swedish Medical Products Agency, Health Canada and the Canadian Environmental Protection Agency.

We recently explored the feasibility of biological containment of an *L. lactis* vaccine strain using a similar pyrimidine synthesis knock-out approach but involving the essential cytidine triphosphate (CTP) synthase gene, *pyrG*, rather than *thyA*. The *pyrG* gene of *L. lactis* MG1363 strain was replaced by the gene encoding the listeriolysin antigen from *L. monocytogenes* through a double cross-over. The resulting auxotroph was effective as a vaccine inducing protective immunity against *L. monocytogenes* in mice. However, in-vitro removal of cytidine from the growth medium had a bacteriostatic rather than a bactericidal effect against the *pyrG* vaccine strain. These findings suggest that whilst limiting thymidine is bactericidal, the effect of limiting cytidine is bacteriostatic even though both involve knock-out of an essential pyrimidine synthesis pathway.

**Gram-Positive Enhancer Matrix (GEM) as a Novel Delivery and Adjuvant System**

Gram-positive enhancer matrix (GEM) is a novel *L. lactis*-based delivery and adjuvant system which potentiates the immune response against the GEM component itself when compared to administration of live *L. lactis* vectors. Overall, the GEM display system offers a novel approach towards the use of self-adjuvanted non-living and non-genetically modified vaccines which is worthy of further investigation for potential use in humans.

**Future Perspectives and Conclusions**

*L. lactis* provides a promising platform for the delivery of vaccine antigens via the mucosal route. Although intensive work has been done during the last 15 years on *L. lactis* as a vaccine vector, to our knowledge no *lactococcal* vaccine candidates are currently under clinical investigation. In contrast genetically manipulated live *Mycobacterium bovis* BCG vaccine candidates are being tested in clinical trials suggesting that live modified bacterial vectors may be acceptable to regulatory authorities for clinical evaluation in human subjects. The use of biological containment strategies may be necessary to prevent survival of genetically modified vaccine strains in the environment. However, although an efficient strategy,
the thyA knock-out approach, has been used in a hiL-10-secretting L. lactis which currently under clinical trials,25 this strategy has not been used yet for L. lactis vaccine vectors. In addition, further investigation of other highly stringent containment approaches may offer practical and diverse alternatives. This should be accompanied by environmental impact studies to analyse the survival of biologically contained strains in soil and other natural systems. The ultimate aim will be to develop environmentally safe, multivalent L. lactis vaccine vectors against a variety of different pathogens that can be delivered through mucosal routes of administration.

Acknowledgements

The authors wish to acknowledge funding received from the Health Research Board, Ireland and from the Science Foundation Ireland Centre for Science Engineering and Technology (CSET) programme through the Alimentary Pharmabiotic Centre.

References