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Biocontainment strategies for live lactic acid bacteria vaccine vectors

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Stability is an important issue when engineering bacteria for use as live vaccine vectors. For the majority of live bacterial vaccines, the antigen-encoding gene is either plasmid located or integrated into the chromosome. Regardless, several safety concerns can be raised for both instances. One concern when using plasmid-encoded antigens is the transfer of antibiotic resistance markers. Alternatively, for chromosomal integrated antigens however, the concern focuses on the spread and possible release of genetically-modified microorganisms (GMM) into the environment, which is problematic. Their recombinant nature calls for a proper bio-containment strategy to be implemented or in place before any realistic attempt at releasing a live bacterial vaccine. No examples of human bacterial vaccines causing problems among animals have been found in the literature but the possibility exists and has to be both tested and evaluated before release of a live bacterial vaccine. The ideal GMM for use in humans should therefore contain the minimal amount of foreign DNA and must not include an antibiotic resistance marker. Furthermore, the possibilities of transgene horizontal transfer must be minimized, and GMM lethality for biocontainment should be achieved in an unconfined environment.

Key words: biocontainment, lactic acid bacteria, *Lactococcus lactis*, vaccine vector

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Biosafety and Containment

Increased knowledge of mucosal immunity and the availability of genetic tools for heterologous gene expression has renewed interest in the concept of live vaccine vehicles. To circumvent some of the safety and environmental issues inherent to the wide-scale dissemination of engineered pathogens, the suggested use of lactic acid bacteria (LAB) as vaccine carriers has potential promise. Dietary LAB have a long history of beneficial use in the food industry and are mostly known for their widespread use in the production and preservation of fermented foods and as such have obtained the "generally regarded as safe" (GRAS) status.[1] Their safe status and immune modulating capacity have already been tested using a wide array of components like antigens from infectious diseases, allergy promoting proteins, and therapeutic antibodies.[2,3] Interestingly, LAB have demonstrated a strain dependent induction of cytokines, thus it can be concluded that immune polarization towards a Th_1 or Th_2 response can be obtained using different LAB strains. As such, the intrinsic immune modulatory capacity of the LAB must be evaluated and selected to fit the purpose of vaccination.[4,5] Two members of the LAB family that have been frequently and successfully used as live vectors under laboratory conditions are *Lactococcus lactis* and *Streptococcus gordonii*.

Since LAB are more suited to survive in nature than other types of bacteria, vaccines based on recombinant LAB may result in the release of these bacteria, especially non-colonizing strains (e.g., *L. lactis*), into nature. The use of genetically modified organisms raises legitimate concerns about their survival and propagation in the environment, and about the dissemination of antibiotic selection markers or other genetic modifications to other microbes. Microorganisms have evolved highly efficient systems for horizontal gene transfer such as transformation, conjugation, retromobilization and transduction to improve their adaptation to changes in their ecological niche. In a

recent study, Toomey et al.[6] highlighted the involvement of LAB, (*Enterococcus faecalis* and *Lactococcus lactis*) as a potential source of resistance determinants that may be disseminated between LAB and pathogenic strains such as *E. coli*, Listeria spp, Salmonella spp and *Staphylococcus aureus.* Surprisingly, no resistance transfer was observed with *E. coli*, Salmonella spp and *Staphylococcus aureus*; however, erythromycin resistance was transferred by both LAB to Listeria spp. Moreover, a high frequency transfer of both erythromycin and tetracycline-resistance was observed between LAB species.

Therefore, a bio-containment strategy for preventing the escape of genetically modified organisms (GMO) into the environment needs to be considered before they can be applied out in the "real world." Biological containment systems can be subdivided into two groups: active and passive systems.[3] Active containment is based on the conditional genetic control of either activation of a killing gene/compound or repression of an essential gene, whose expression is tightly controlled by an environmentally responsive element.[7] Some well-integrated active systems of this type have been developed.[7-13] Although active containment systems provide actual killing of the host, they have notable drawbacks. First, these systems introduce a large amount of foreign DNA, which inhibits their use in humans. The probability of a harmful effect due to integration of foreign DNA into a host genome has been calculated be $<10^{-16}$ to 10^{-19} per DNA molecule.[14] Second, many are plasmid borne, and it remains to be demonstrated that function is maintained when the plasmids become integrated in the bacterial chromosome to reduce lateral dissemination. Third, mutations can occur that either inactivate a killing gene/compound or result in constitutive expression of the essential gene. However, it may be possible to minimize these problems by combining more than one system in a defined recombinant strain for redundancy.

In contrast, passive containment systems are robust and very simple in design circumventing these limitations. They are based mainly on complementation of an auxotrophy or other gene defect by supplementation with either the intact gene or the essential metabolite. This metabolite is ideally not available or occurs in extremely low amounts in the environment. Passive systems have the drawback that they are often bacteriostatic rather than bactericidal.[7,15-17] However, two auxotrophies have high potential for human application since they are bactericidal. Alanine racemase mutants that demonstrate a requirement for D-Ala can be obtained in a large number of bacteria including LAB.[15,18,19] D-Ala starvation results in cell death via a lysis process. The second bactericidal auxotrophy is based on a thymidine synthase (*thy*A) mutant of *L. lactis*.[20] The choice of *thy*A as a target gene combines the advantage of passive and active containment systems. Thymine starvation results in activation of the SOS repair system and DNA fragmentation, thus constituting an intrinsic suicide system, and is referred to as "Thymine-less death",[21] which was described as early as 1954.[22] Thymine and thymidine growth dependence differs from most other auxotrophies in that absence of the essential component is bactericidal in the former and bacteriostatic in the latter.[7]

As such, one may exploit these genes by implementing an auxotroph transgene containment method (ATCM) that basically involves replacing an essential gene involved in the survival of the LAB with that of an antigen of interest. Having the antigen chromosomally integrated thereby avoids the use of antibiotic selection markers and effectively eliminating the requirement for a plasmid-based system adding more stability, which is an important issue when engineering bacteria for use as live vaccine vectors. In the unlikely event that the GM strain survives out of the host and acquires an intact gene from a donor, the transgene would be eliminated from the genome and the GM strain would revert to being normal wild-type. So, by analogy, using ATCM, one could create a GM strain with an effective "expiration date" once out of the host and into the environment. In fact, Steidler et al.'s[20] use of ATCM in *L. lactis* replacing *thy*A with a synthetic human IL-10 transgene led to the creation of strain Thy12, which has been validated in pigs, and has been approved by Dutch authorities as an experimental therapy for humans with IBD.

In the previous paper, presented by Bahey-El-Din and colleagues, the authors employ ATCM to engineer a cytidine transgene *L. lactis* auxotroph as a possible alternative biological containment strategy au lieu of the one demonstrated by Steidler et al.[20] Bahey-El-Din and colleagues targeted the *pyr*G gene, encoding for CTP synthase, which is responsible for converting UTP to CTP during de novo pyrimidine synthesis in *L. lactis*. Their results indicate that cytidine auxotrophy in *L. lactis* is bacteriostatic in contrast to thymidine auxotropy which is bactericidal[20] whilst in media depleted of pyrimidines. Although both auxotrophies are pyrimidine related, the mechanisms underpinning these different responses remain unknown. As a consequence, in future studies, it will be very interesting to determine whether the creation of a double mutant in both *thyA* and *pyrG* can offer additional benefits over a single *thyA* mutation alone; such as redundancy with respect to biological containment, increased antigen expression if two transgene copies are utilized, and the applicability of expressing two different antigens simultaneously using different transgenes.

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