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Immunomodulation by genetically engineered lactic acid bacteria

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1. ABSTRACT

The taxonomically diverse lactic acid bacteria (LAB) are unified by their capability to produce lactic acid from carbohydrates by fermentation. The LAB *Lactococcus (L.) lactis* has been characterized into great detail and is increasingly used as a production host for heterologous proteins. *L. lactis* is a non-pathogenic and non-colonizing LAB species and can be efficiently engineered to produce proteins of viral, bacterial or eukaryotic origin, both intra- or extracellularly. Importantly, orally formulated *L. lactis* strains (ActoBiotics™), engineered to synthesize and secrete therapeutic peptides and proteins in the gastrointestinal tract, are already in advanced stages of preclinical and clinical development. This review focuses on the genetic engineering of LAB in general and *L. lactis* in specific to secrete high-quality, correctly processed, bioactive molecules derived from a eukaryotic background. The therapeutic applications of these genetically modified strains are discussed, as well as the need for a sound environmental containment strategy, and a detailed review is presented on *Lactococcus* strains engineered to produce specific antigens, antibodies, cytokines and trefoil factors, with special regards to immunomodulation.

2. INTRODUCTION

Even though they are taxonomically diverse, lactic acid bacteria (LAB) are Gram positive and share a fermentative metabolism, converting carbohydrates into lactic acid. LAB either produce lactic acid as their main metabolite (homofermentative LAB) or several other compounds as well (heterofermentative LAB). The original niche of many LAB species is unidentified but several of them are commonly found among the gastrointestinal (GI) microflora of vertebrates. In general, LAB are best known for their use in the preparation of fermented foods such as dairy products and a wealth of studies has focused on the molecular basis of the productive assets of LAB, aiming to obtain better control of the industrial processes they are involved in. An important advantage of working with food-grade LAB lies in the fact that they are generally not associated with pathogenic effects. As such, several LAB species are "Generally Recognized As Safe" in the United States.

One of the main LAB used in genetic engineering of heterologous protein expression is *Lactococcus (L.) lactis,* a non-colonizing and non-pathogenic LAB species.

L. lactis has been used in the food industry throughout history, particularly in the production of cheese, and has been elaborately characterized, molecularly as well as genetically. *L. lactis* was also included in the evaluation list of Gram-positive, non-sporulating bacteria when the European Food Safety Authority (EFSA) introduced the concept of "Qualified Presumption of Safety" (QPS) for the safety assessment of microorganisms used in food production (1, 2).

3. ENGINEERING OF *L. LACTIS* FOR THE EFFICIENT SECRETION OF HETEROLOGOUS PROTEINS

L. lactis is considered a model organism and with the rise of highly proficient genetic tools (3-5), the genomes of several *L. lactis* subspecies have been entirely sequenced. Nowadays, many established plasmids are available (6, 7) and numerous expression systems have been successfully designed (8, 9), allowing for intra- or extracellular production of a great variety of 9.8 to 165 kDa proteins of viral, bacterial or eukaryotic origin (10).

In the engineering of heterologous protein expression, secretion is generally preferred over cytoplasmic production because it not only allows for continuous culturing but also simplifies purification. *L. lactis* is an efficient production host for several reasons. Not only does *L. lactis* secrete relatively few homologous proteins -an unknown secreted protein of 45 kDa (Usp45) being the most prominent one (11)-, selected laboratory strains also do not produce any extracellular protease, leaving secreted proteins less prone to extracellular degradation (12, 13). Furthermore, *L. lactis* possesses only one intracellular housekeeping protease, *HtrA*, which is involved in cell survival under stress conditions by degrading improperly folded proteins. *HtrA*-deficient *L. lactis* strains have been developed and these mutant strains allow for higher heterologous protein secretion because their degradation is impaired (14). Alternatively, *HtrA* proteolytic activity can be inhibited by adding salt to the culture medium. These salt concentrations do not affect the growth rate of *L. lactis* (9).

The lactose-inducible *lac* operon constituted the first controllable expression system for *L. lactis*, albeit at a low level of induction (15), and an expression system based on the *L. lactis nisA* promoter was developed soon thereafter (16, 17). Another important step in engineering efficient heterologous protein secretion was the design of a series of constitutive expression vectors (the pTREX series of Theta Replicating Expression plasmids) which replicate in a wide range of Gram-positive bacteria. Plasmid pTREX1 contains an expression cassette which incorporates the strong lactococcal promoter *p1* and both the translation initiation region and transcription terminator of the *Escherichia coli* bacteriophage T7 *gene10*. The translation initiator region has been modified at one nucleotide position to increase complementarity of the Shine Dalgarno sequence to the ribosomal 16S RNA of *L. lactis* (18).

Although one report indicated that secretion of *Escherichia coli* fimbrial adhesin was more effective in *L. lactis* when linked to a *Lactobacillus brevis* signal peptide (19), the Usp45 secretion signal is still the most frequently and most successfully used signal peptide for high-level secretion of biologically active heterologous proteins: no other homologous secretion signals *L. lactis* have been demonstrated to be superior to Usp45 in heterologous secretion (20, 21). Several other factors have a direct influence on secretion capacity as well and important considerations include (i) affinity between the mature protein and the signal peptide directing its secretion, (ii) capability for efficient cleaving of the precursor polypeptide by *lactococcal* signal peptidase, and (iii) proper escorting of the mature protein by the secretion machinery. Not surprisingly, some heterologous proteins are poorly, if at all, secreted (21, 22) and charges at the N-terminal part of the mature moiety may greatly affect the translocation efficiency across the cytoplasmic membrane (23).

Interestingly, the introduction of negative charges, through the insertion of a synthetic peptide between the final amino acid residue of the signal peptide and the first residue of the mature heterologous polypeptide, can improve the secretion efficiency and production yield of several heterologous proteins in *L. lactis* (24, 25). Furthermore, a labile protein can be stabilized by fusion to a stable protein-like *Nuc*, which enhances both cytoplasmic accumulation and secretion without affecting its enzymatic activity (26).

4. ENGINEERED LAB FOR THE DELIVERY OF THERAPEUTIC PROTEINS AND PEPTIDES

Over recent years, it has become clear that several bacterial species can be genetically engineered to secrete high-quality, correctly processed, bioactive molecules derived from a eukaryotic background. This review focuses on immunomodulatory applications of engineered LAB.

4.1. Therapeutic *in situ* production of antigens

4.1.1. Bacterial vaccines

Several studies have focused on the development of engineered LAB for induction of an antigen-specific immune response (for reviews see (27, 28)). In a pioneering study, oral as well as intranasal inoculation with an engineered *L. lactis* strain, designed to accumulate tetanus toxin fragment C (TTFC) intracellularly, increased Immunoglobulin G (IgG) serum levels and protected mice against a lethal challenge with tetanus toxin (29-31). Importantly, a similar antibody titer was induced using dead *Lactococci*, suggesting that *in situ* antigen synthesis was not essential (30). Two other groups have reported that mucosal immunization with a lactococcal vaccine effectively reduced infection. Immunization with *L. lactis* expressing the envelope protein of HIV on its cell surface protected mice when challenged with an HIV Env-expressing vaccinia virus (32). Similarly, mice vaccinated with *L. lactis* expressing the conserved C-repeat region of *Streptococcus pyogenes* M protein were protected against pharyngeal infection following a nasal challenge with *S. pyogenes* (33).

Over 2000 published papers have looked into using attenuated strains of pathogenic species (mainly belonging to the genera *Salmonella, Shigella, Yersinia* and *Listeria*) as tools for non-parenteral vaccination (for review, see (34)). Development of bacterial vaccine vehicles however can be problematic and because LAB are non-invasive, vaccine delivery to antigen-presenting cells may be less effective. Additionally, development of bacterial vaccine vehicles that carry a heterologous gene can be tedious and none have reached the market so far.

4.1.2. Engineered LAB can bias the immune system towards a T_h1-specific response

Since Type 1 helper T cells (T_h1) and T_h2 can antagonize each other, this capacity is of interest in Type 1 allergies, which are characterized by a pathological T_h2 response. A number of studies have demonstrated T_h1-polarizing effects of specific LAB strains *in vitro* (35-37). As such, the induction of a counter-regulatory, allergen-specific T_h1 response seems a promising therapeutic approach in the treatment of Type 1 allergies (38). Experimental evidence for this hypothesis came from two animal studies, in which mucosal administration of *L. lactis* and/or *Lactobacillus plantarum*, together with either a birch pollen allergen or with the major house dust mite allergen Der p 1, prior to or after sensitization, indeed resulted in a clear shift towards the T_h1 allergen-specific immune response in mice (39, 40).

The findings above have sparked the development of genetically modified (GM) LAB strains aimed at inducing a protective immune response at the site of allergen encounter. In mice, a shift towards the T_h1-specific immune response has been reported respectively following intranasal administration of live, recombinant LAB producing the major birch pollen allergen Bet v 1, following oral administration of GM *L. lactis* bacteria expressing the cow milk allergen bovine beta-lactoglobulin, as well as following oral administration of recombinant LAB, expressing the Der p 5 mite allergen (41-43).

In the first study, a clear shift towards the T_h1-specific Immunoglobulin G2a (IgG2a) antibody response was noted and allergen-specific mucosal Immunoglobulin A (IgA) levels were increased (41). The second study demonstrated that oral administration of recombinant *L. lactis* resulted in a higher protective effect than co-administration of the antigen with unmodified *L. lactis*. Furthermore, not only was a direct correlation reported between the amounts of allergen secreted and the recombinant strain's capability to induce a T_h1 response, fusion of the allergen to a carrier protein was clearly shown to further enhance inhibition of the pathological IgE response (42). A significant decrease in the synthesis of allergen-specific IgE was also reported in the third study and, contrasting with the effect of the engineered strain, neither recombinant allergen nor LAB alone were able to suppress allergen-induced airway inflammation and hyperreactivity (43).

4.1.3. Engineered LAB can also balance the immune response without direct T_h1-adjuvant effect

The T_h1-adjuvant effect described above apparently contrasts with the beneficial effect of specific LAB strains on the development of T_h1-mediated (autoimmune) diseases such as Type 1 diabetes and arthritis, which clearly suggests additional therapeutic pathways. Results from several studies indicate that engineered LAB could be an effective tool for the induction of antigen-specific tolerance, with possible application in the treatment of antigen-induced autoimmune diseases.

This was hinted upon by Kruisselbrink and colleagues, who did not find any increased T_h1 response or altered levels of IgE or IgG2a when Der p 1-immunized mice were treated with mucosally-delivered recombinant *Lactobacillus plantarum* expressing Der p 1 intracellularly (44). They did however report inhibition of both Interferon (IFN)-gamma and Interleukin-5 (IL-5) production, and while the reduction of IFN-gamma was shown to be an antigen-non-specific effect of *Lactobacillus plantarum,* the effect on the T_h2 cytokine IL-5 was only observed with the Der p 1-expressing strain. This study demonstrated that the *lactobacilli* ensured a well-balanced immune response to harmless antigen, rather than promoting the T_h1-response.

Additional indications for this rebalancing effect came from a recent study evaluating the efficacy of engineered *L. lactis* in the induction of antigen-specific peripheral tolerance (45). Ovalbumin (OVA)-immunized DO11.10 mice, bearing transgenic OVA-specific $CD4^+$ T cell receptors, were fed with OVA-secreting *L. lactis*. The development of OVA-specific systemic tolerance was demonstrated and this effect was mediated through the induction of $CD4^+CD25^-$ regulatory T cells, exerting their function in a Transforming Growth Factor (TGF)-beta-specific way. These results again indicated the therapeutic potential of engineered *L. lactis* in balancing aberrant immune responses.

4.2. Therapeutic *in situ* production of antibodies

Another therapeutic application of GM bacteria is the local delivery of therapeutic antibodies. The current therapeutic application of antibodies, aimed at alleviating mucosal infections, is limited due to short half-life of the polypeptides, issues with the mode of administration (often systemic injections, resulting in various unwanted side-effects) and high production costs.

In an explorative experiment, two strains of the Gram-positive commensal bacterium *Streptococcus gordonii* were engineered to either express an anti-idiotypic single chain antibody on its surface, mimicking a yeast killer toxin, or to secrete that same antibody in the extracellular environment. The *in vivo* activities of both strains were assessed in a well-established rat model of vaginal candidiasis. At day 21 after the start of treatment, full clearance of the *Candida albicans* infection was observed in 75% of the animals treated with the secreting strain, compared to only 37.5% of the animals treated with the surface-expressing strain. Control animals were still infected and the results clearly demonstrated an increase in

efficiency for engineered strains secreting antibodies over strains expressing the same antibodies at the cell surface (46).

4.3. 'Smart' modulation of the immune system: engineered *L. lactis* strains for the targeted treatment of inflammatory bowel disease

Inflammatory bowel disease (IBD) is a chronic, relapsing inflammatory GI disorder, likely resulting from reduced tolerance of the immune system towards the normal intestinal microflora. Crohn's disease (CD) and ulcerative colitis (UC) are the two major forms of IBD and have a yearly incidence of up to 10 and 15 per 100,000 respectively. While CD can affect any region of the GI tract from mouth to anus, chronic inflammation in UC specifically affects the colon, resulting in characteristic ulcers. In general, IBD patients experience (bloody) diarrhea or constipation, abdominal pain, fever and, often, weight loss. Several frequently occurring complications have been described in IBD patients, ranging from blockage of the intestine, sores affecting surrounding tissue (bladder, vagina or skin), fistulas and fissures in CD patients to ulcers in the mouth, inflammation of the iris, arthritis, inflammation of subcutaneous tissue, thrombosis, pulmonary embolism, anemia and even an increased risk for colon cancer in UC patients. To date, therapeutic options are limited and apart from surgical resection of the colon in UC patients, there is no known cure for IBD. Instead, therapy focuses on inducing and maintaining remission.

A significant step towards new treatment options for IBD is the development of biological drugs with high specificity and low inherent toxicity. However, while administration of potent immunomodulatory biologics can result in profound therapeutic effects, their use is hampered by difficulties encountered in the (costly) production steps and by the fact that they have to be systemically injected over regular intervals, which not only implies considerable patient discomfort, but also inherently ties administration to various systemic side effects.

Steidler and colleagues were the first to engineer *L. lactis* for efficient synthesis and secretion of eukaryotic proteins, e.g. murine (m) and human (h) Interleukin-2 (IL-2), Interleukin-6 (IL-6) and Interleukin-10 (IL-10), in quantities ranging from 50 µg to as much as 1 mg per liter of culture (mIL-2 and hIL-10, respectively), without affecting growth rate (47-50). These cytokines are small secreted proteins which mediate and regulate several key factors in vital processes such as immunity, inflammation and hematopoiesis. They generally act at very low concentrations and do not depend on complex glycosylation or secondary modifications to be biologically active. Importantly, even though cytokines display fairly simple structures, many of them critically depend on the correct formation of disulfide bridges in order to be biologically active. *Lactococci* are well capable of correct disulfide bridge formation in the processing of heterologous proteins, as shown by *in vitro* and *in vivo* bioactivity of recombinant mIL-2, mIL-6, hIL-10 and trefoil factor (TFF) peptides (48, 49, 51).

A first, eye-opening study demonstrated that intranasal administration of *L. lactis,* engineered to simultaneously express TTFC and either mIL-2 or mIL-6, produced significantly higher antibody titers than the parental strain expressing TTFC alone (49). Bacteria expressing TTFC in combination with mIL-6 were also capable of eliciting a serum anti-TTFC IgA response, indicating the possibility of 'smart' modulation of the immune system, hinting at the tremendous potential of active, *in situ* delivery of functional immunomodulatory peptides. Importantly, *in situ* secretion of the cytokines by viable bacteria was shown to be an essential part of the mechanism.

The first step towards the clinical development of GM bacteria for the production and delivery of immunomodulatory biological drugs, was the development of an orally formulated *L. lactis* strain, engineered to express and secrete IL-10 in the GI tract. IL-10 is a powerful anti-inflammatory cytokine with high therapeutic potential in IBD. This cytokine plays a central factor in the induction and maintenance of immune tolerance, as demonstrated by the chronic ileocolitis that develops in IL-10 knock-out mice (IL-$10^{-/-}$) (52) and by its therapeutic efficacy in various animal models of colitis (53-55). The clinical results after systemic administration of recombinant hIL-10 to IBD patients however, have been mostly disappointing, with limited clinical efficacy, various side-effects (56, 57) and injection of high systemic doses even leading to the induction of the pro-inflammatory IFN-gamma (58). Nevertheless, direct targeting of hIL-10 to the site of inflammation, e.g. the intestine, could solve many of these problems. Groundbreaking results for this approach have been obtained in animal models and even in IBD patients.

Daily intragastric administration of engineered *L. lactis*, secreting murine (m)IL-10, efficiently cured chronic dextran sulfate sodium (DSS)-induced colitis in mice, with an efficiency comparable to that of systemic treatment with prominent and well-established anti-inflammatory drugs (e.g. dexamethasone, anti-IL-12 antibodies), but at dose that was 10,000-fold lower than the dose used for systemically administered recombinant IL-10 (52). Daily treatment with mIL-10-secreting *L. lactis* also prevented the establishment of spontaneous enterocolitis in IL-$10^{-/-}$ mice (52). Killing the bacteria by UV irradiation prior to inoculation abrogated their curative effects, confirming *in situ* production of IL-10 as an essential feature of the mechanism of action. Importantly, these orally-formulated, live *L. lactis* strains not only limited systemic exposure and thus many of the aforementioned drawbacks associated with systemic IL-10 therapy, but also introduced the concept of an oral formulation not hampered by the extreme acid sensitivity of IL-10 (which explains why to date, no oral formulation of IL-10 has been marketed) (48, 59). More recently, *L. lactis*-mediated IL-10 delivery has also been validated in trinitrobenzene sulfonate (TNBS)-induced colitis (60) and remarkably, recombinant human (h)IL-10, secreted by GM *L. lactis*, has also been shown to be bioactive in mice and to reduce inflammation in murine DSS-induced colitis (61).

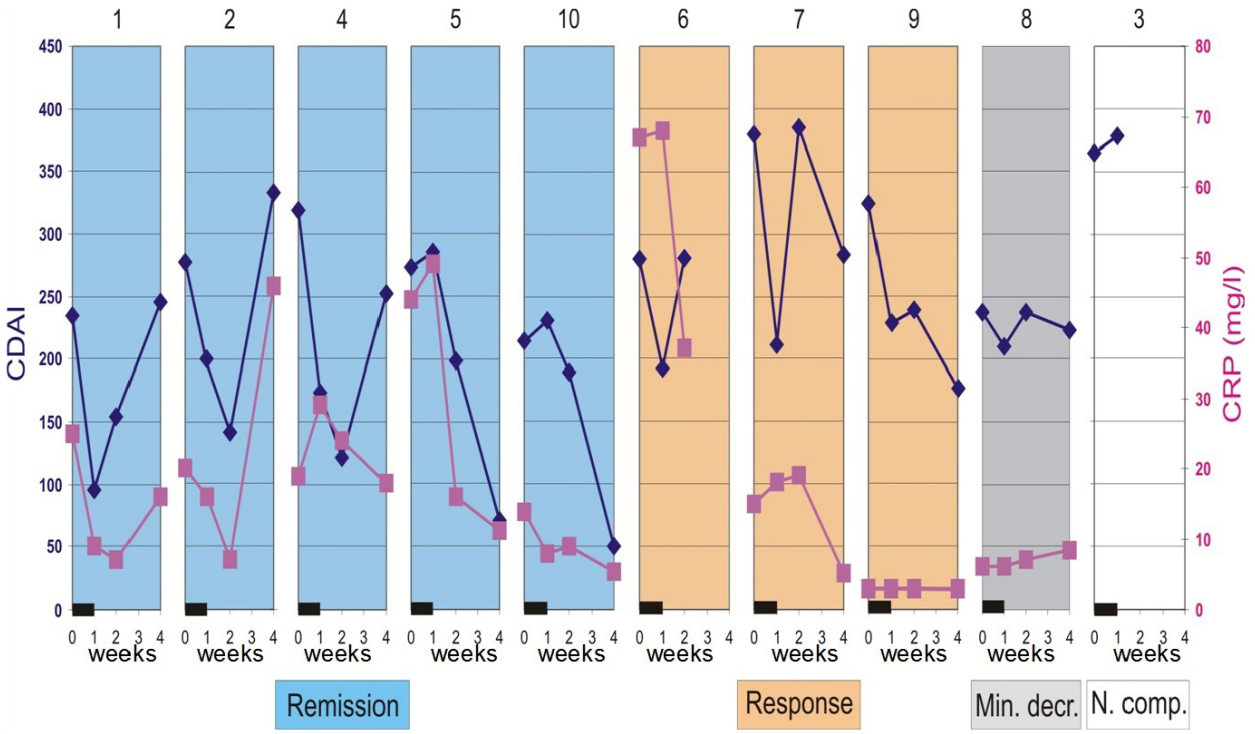

Figure 1. Clinical scores of patients before and after administration of IL-10-secreting *L. lactis*.Graphical representation of the data as presented in full in (76).CD Activity Index (◆) and C-reactive protein (■) levels in patients at day -1, 8, 14, and 28. The black bar indicates the treatment period of 7 days.

Expanding on the therapeutic efficacy of hIL-10 secreting *L. lactis* strains, a paper by Vandenbroucke *et al.* detailed the development of an orally administered *L. lactis* strain, engineered for the local synthesis and secretion of TFFs at the colonic mucosa (51). TFFs are considered epithelial healing factors and as such promising tools for the treatment of acute UC. Whether delivered orally, subcutaneously or rectally, there is strong *in vivo* evidence for a protective and healing influence on various forms of mucosal injury (62-73). The orally formulated, TFF-secreting *L. lactis* strain not only abrogated the need for systemic delivery but also addressed key issues associated with oral TFF treatment; orally administered, purified TFFs tend to stick to the mucus, becoming metabolically inert, and are removed from the lumen at the caecum or small bowel (67). Indeed, despite their extreme stability and resilience to acid denaturation and proteolytic degradation, most studies indicate that TFFs are more efficacious when they are subcutaneously administered rather than orally applied.

Daily intragastric administration of a TFF-secreting *L. lactis* strain, prior to or during induction of colitis in mice, resulted in a lower mortality rate, a reduced loss of body weight, a substantial improvement in colon histology and a significant reduction in inflammatory infiltrates. Importantly, oral administration of high amounts of purified TFF alone did not ameliorate acute colitis, whereas rectal administration only had some effect at doses 2,500-fold higher than in *L. lactis*-mediated delivery. Complementing these findings, the protective effect was shown to be dependant on *de novo* TFF synthesis by live *L. lactis* and bioactivity of the secreted molecules was demonstrated *in situ (*51). Cyclooxygenase (COX)-2, a known target for TFF signaling (74, 75), was strongly induced in the intestines of mice treated with TFF-secreting *L. lactis* and in addition, inhibition of COX-2 by meloxicam substantially abrogated the prophylactic effect on acute DSS-induced colitis. This indicated that, although probably not the only factor involved, up-regulation of COX-2 through *L. lactis*-secreted TFFs is vital in the protection from and restoration of acute colitis (51). Furthermore, the authors substantiated the hypothesis that GM *L. lactis* can engage in intimate contact with basolateral colon cells, either following transport by M-cells or through ruptures in the epithelium, enabling the TFF peptides to accumulate out of reach of complexing mucins and allowing them to interact with the putative basolateral TFF receptors on enterocytes.

Finally, a so-called ActoBiotic or orally formulated *L. lactis* strain, engineered to secrete therapeutic peptides and proteins in the GI tract, has also been evaluated in a pioneering open-label clinical trial. An environmentally contained *L. lactis* strain, engineered to secrete hIL-10, was administered to ten patients with moderate to severe CD (59, 76). Considerable clinical improvement was observed in eight of them: five patients went into complete clinical remission and three patients showed a significant clinical response (Figure 1). This trial

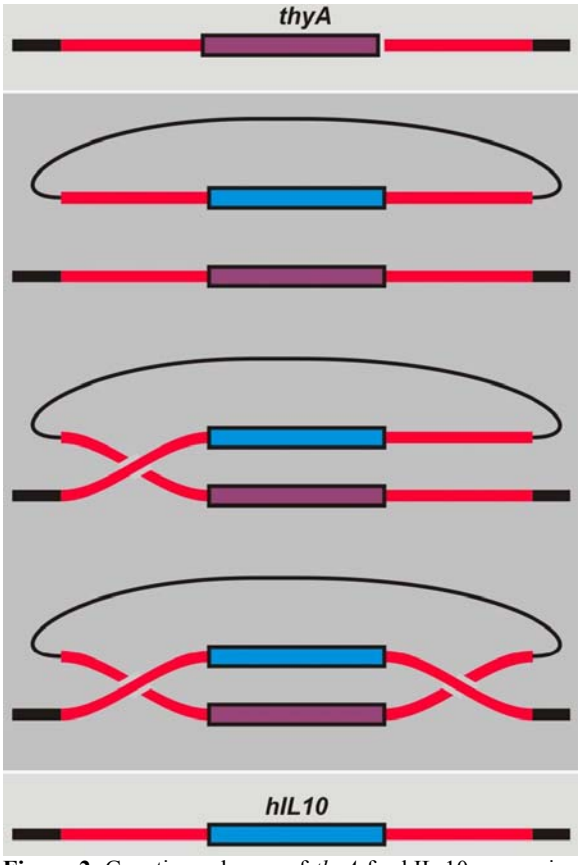

Figure 2. Genetic exchange of *thyA* for hIL-10 expression cassette. Genetic exchange of *thyA* for *hIL-10* involved the introduction of a non-replicative integration plasmid followed by upstream and downstream cross-over. Cross-over events occurred at target sites (red) which were present in both the integration plasmid and the *L. lactis* chromosome.

-the first one *ever* in its kind- not only demonstrated the safety and tolerability of the viable, orally formulated GM bacteria in patients, but also verified that the bacteria were environmentally contained and in addition, provided promising indications for therapeutic efficacy (76). Large-scale, double-blind, placebo-controlled trials, set to fully evaluate the therapeutic potential of hIL-10-secreting *L. lactis,* are currently in preparation.

5. ENVIRONMENTAL CONTAINMENT

Both "active" and "passive" environmental containment systems exist. Active systems usually depend on the production of a toxic compound, either under tightly controlled expression by environmental factors or temporarily suppressed by an immunity factor. While these systems are able to conditionally eliminate their host, they are plasmid borne and therefore prone to horizontal transfer to other microorganisms and in addition often depend on the introduction of a large amount of foreign DNA.

Passive containment strategies overcome these shortcomings. Here, growth is dependent on complementation of an engineered auxotrophy or other induced gene defect, necessitating the supplementation of either the essential metabolite or the intact gene. Passive systems, however, have the drawback that they often affect only bacterial growth and not survival.

Steidler and colleagues have developed an ingenious passive containment system with both bacteriostatic and bactericidal properties, centered on removal of the thymidylate synthase (*thyA*) gene. The choice of *thyA* as target gene combined the advantages of both passive and active containment systems. *ThyA* codes for an essential enzyme in the synthesis of the DNA constituents thymine and thymidine (59). In absence of thymine/thymidine, *thymineless death* is triggered. Described as early as 1954 (77), *thymineless death* involves activation of the SOS repair system and rapid DNA fragmentation, essentially constituting an indigenous suicide system. Therefore, thymine- and thymidine-dependence is intrinsically different from other auxotrophies, since lack of the essential component not only affects growth but also survival of the host (59). Genetic exchange of the chromosomal *thyA* gene for a specific gene of interest thus provided a sound strategy for inheritable growth and survival control of engineered *L. lactis* for several reasons: (i) an absolutely minimal amount of foreign DNA is present in the GMO, (ii) no resistance markers are required to guarantee stable inheritance of the transgene, (iii) upon thymine/thymidine starvation, death is rapidly induced, (iv) in the unlikely event that lateral transfer of intact *thyA* would occur, this would reciprocally remove the *thyA* gene from the recipient, (v) the risk of disseminating the GM trait through lateral gene transfer is minimized because the gene of interest is integrated into the *L. lactis* chromosome, and (vi) the chances for uptake of naked DNA following release by *L. lactis* are minimal, since *thymineless death* results in DNA fragmentation before lysis. Additionally, phage replication is severely impaired in *thyA*-deficient *Lactococci*, disabling phage-mediated transduction of host genetic material (78, 79).

This innovative approach resulted in the creation of a GM *L. lactis* strain, Thy12, in which *thyA* was replaced by an hIL-10 expression cassette, essentially inducing strict thymine- and thymidine-dependence AND incorporating therapeutic secretion of hIL-10 (Figure 2) (59). Thy12 provided a satisfactory solution to concerns about biosafety because of the *thymineless death* containment system described above, strengthened by the fact that acquisition of *thyA* from other microorganisms could not be experimentally demonstrated (59), and by the careful selection of a parental strain (*L. lactis* subsp. *cremoris* MG1363) which was already disabled in a number of mechanisms for lateral gene transfer. Keeping into account that *L. lactis* is naturally confined to select niches, MG1363 is even more contained because it was cured of all naturally resident plasmids (80), lacking not only factors essential for its metabolism but also a host factor required for conjugative transposition. Taken together, this approach provided an elegant complementary and innovative strategy for environmental containment of engineered bacteria,

essentially relying on multiple mechanisms to constitute a fail-safe system with the advantages of both passive and active containment strategies, without the associated drawbacks.

6. PERSPECTIVE

It should be clear that the number of possible applications of engineered bacteria in modern medicine is enormous. With the rise of engineering proficiency, new strategies are being developed at an ever increasing pace. While cutting-edge approaches such as *L. lactis*-mediated topical delivery of therapeutic proteins may open up a vast new spectrum of medical applications, the use of a genetically modified organism (GMO), especially in healthcare, raises legitimate concerns on safety and on deliberate release, survival and propagation of a GMO in the environment and potential transfer of its genetic modifications to other microorganisms.

With the design of an adequate environmental containment strategy, engineering of well characterized LAB is increasingly opening new therapeutic avenues. Synoptically, engineered LAB strains provide not only one of the most elegant ways of therapeutic drug delivery, but also a highly cost-effective secretion vehicle for a vast array of bioactive molecules. Multiple new applications are currently being addressed and orally formulated, environmentally contained GM *L. lactis* strains (ActoBiotics™), engineered to synthesize and secrete therapeutic peptides and proteins in the GI tract, are already in advanced stages of preclinical and clinical development.

Potential applications of engineered bacteria in humans immediately enclose inflammatory as well as allergic diseases, but are likely not limited thereto. Giomarelli and co-workers already documented the engineering of *Streptococcus gordonii* to provide the host with sustained delivery of recombinant antibodies, effectively inhibiting dental caries. Another recent study also showed the effectiveness of *L. lactis,* engineered to express *Yersinia pseudotuberculosis* V-antigen, in the treatment of experimental enterocolitis, broadening the battery for therapeutic intervention to pathogen-derived factors (81).

In a time where compelling and adept biological drugs are becoming increasingly available, safety and patient compliance are major considerations. GM LAB strains for targeted synthesis and secretion of therapeutic proteins provide a new concept in sustained delivery of bioactive molecules and offer the means to treat diseases with high unmet medical needs. Groundbreaking research has shown that therapeutic strains can be engineered to produce and release highly potent bioactive molecules at their desired site of action. This not only abrogates systemic side-effects, constituting a safer drug, but also implies increased patient compliance and a more cost-effective approach. With potential indications ranging over a wide variety of diseases, engineered LAB might one day even represent a new generation of pharmaceuticals.

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Abbreviations: COX-2: cyclooxygenase 2, DSS: dextran sulfate sodium, Env: envelope protein of HIV, GI: gastrointestinal, GM: genetically modified, GMO: genetically modified organism, h: human, IBD: inflammatory bowel disease, IFN-gamma: Interferon gamma, IgA: Immunoglobulin A, IgE: Immunoglobulin E, IgG: Immunoglobulin G, IgG2a: Immunoglobulin G2a, IL-2: Interleukin-2, IL-5: Interleukin-5, IL-10: Interleukin-10, IL-$10^{-/-}$: IL-10 knockout (mice), IL-12: Interleukin-12, kDa: kiloDalton, LAB: lactic acid bacteria, *L. lactis*: *Lactococcus lactis,* m: murine, OVA: Ovalbumin, pTREX1: series of Theta Replicating Expression plasmids, QPS: Qualified Presumption of Safety, *S.*: *Streptococcus,* TGF-beta: transforming growth factor beta, T_h1: Type 1 helper T cell, T_h2: Type 2 helper T cell, TFF: trefoil factor, TNBS: trinitrobenzene sulfonate, TTFC: tetanus toxin fragment C, Usp45: Unknown secreted protein.

Key Words: Lactic Acid Bacteria, *Lactococcus Lactis*, Interleukin-10, Trefoil Factors, Antigen, Inflammatory Bowel Disease, Antibodies, Immunomodulation, Genetic Engineering, Environmental Containment, Review

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