The Journal of Microbiology, June 2003, p.63-72 Vol. 41, No. 2

MINIREVIEW

In situ Delivery of Therapeutic Proteins by Recombinant *Lactococcus lactis*

Lothar Steidler* and Sabine Neirynck

University College Cork, Western Road, Cork, Ireland

(Received May 2, 2003)

Chronic inflammatory bowel disease (IBD) such as Crohn's disease or ulcerative colitis, affects around 2 in every 1000 individuals in western countries and its incidence, particularly amongst children, is increasing. IBD shows extreme morbidity with impact on all aspects of quality of life. If left untreated, IBD can lead to death. Conventional treatment of IBD involves powerful immunosuppressive chemotherapies and surgical intervention. Long-term anti-inflammatory medication is required and so patients are often subject to a spectrum of unpleasant side effects. Interleukin-10 (IL-10) is a cytokine that acts to suppress inflammation. When however administered by injection, the high levels of IL-10 that are distributed throughout the body also lead to side effects. *Lactococcus lactis* can be genetically engineered to secrete biologically active cytokines. When applied to the mucosa, these *L. lactis* can actively deliver such cytokines. By use of this principle we developed a new therapeutic approach for IBD. Administration of *L. lactis* that secretes murine IL-10 cures and prevents IBD in mice. The use of the engineered *L. lactis* gets around the problem of delivering IL-10, allowing dramatic reduction of the effective dose. A sincere concern exists about the possible dangers of uncontrolled, deliberate release of genetically modified microorganisms, such as could occur following application in healthcare. We engaged in the establishment of adequate means for biological growth control of engineered *L. lactis* by targeted gene exchange between *thyA* and *hIL-10*.

Key words: *Lactococcus lactis*, inflammatory bowel disease, *thyA*, interleukin-10

IBD: a consequence of disrupted intestinal tolerance

Inflammatory bowel disease (IBD) comprises intestinal inflammatory pathology that can be subdivided in ulcerative colitis (UC) and Crohn's disease (CD) based on typical clinical manifestations. The symptoms of both are extremely unpleasant and have a major impact on all aspects of quality of life. They include diarrhea, abdominal pain, rectal bleeding, fever, nausea, weight loss, lethargy and loss of appetite. If left untreated, malnutrition, dehydration and anemia follow, which, in extreme cases, can even lead to death. Although UC and CD show a considerable degree of similarity in etiology and epidemiology, they are quite different in pathology. UC is restricted to the colon. CD, however, has been observed throughout the whole intestinal tract, from the mouth to the rectum. Whereas in CD the inflammation can be transmural i.e. penetrating the bowel wall, inflammation is restricted to the mucosa in UC. This often leads to the development of perianal fistulae. An imbalance in T-helper (Th) subsets of T-cells, so called Th1 and Th2 (Mosmann and Coffman, 1989) differentiates CD-showing predominant Th1 cytokines (IL-12, IFN-γ) from UC-where Th-2 type cytokines (IL-4, IL-5) prevail (Niessner and Volk, 1995; Fuss *et al.*, 1996; Kakazu *et al.*, 1999).

The cause of IBD is unknown. The pathogenesis of CD and UC probably involves interaction between genetic and environmental factors, such as bacterial agents (reviewed by Sartor (1997)). Today, the leading theory, supported by findings in experimental models (Cohen and Barner, 1954; Czerucka, 1999), is that misinterpretation inducing immunity rather than tolerance of commensal antigen causes an abnormal immune response that reacts towards the cognate intestinal microflora. Due to the massive presence of bacteria in the gut, tissue damage arising from the ongoing inflammation obviously leads to an increased influx of antigen, thereby establishing a vicious circle of repeated priming and response. All steps in the process leading to an appropriate decision from the immune system to either induce tolerance or immunity against luminal antigen can be disregulated in IBD patients. Such steps include epithelial barrier function, antigen presentation, lymphocyte medi-

* To whom correspondence should be addressed.
(Tel) 353-21-490-1383; (Fax) 353-21-490-3877
(E-mail) l.steidler@ucc.ie

ated regulatory loops and apoptosis of activated T-cells.

Breach of tolerance towards normal intestinal microflora, as also appears in TNBS induced colitis, may thus be the driving force behind IBD. The administration of IL-10, a central mediator in downregulation of immune reactions, can restore the healthy status by re-establishing tolerance (Duchmann *et al.*, 1996). Also, IL-10 deficient mice develop intestinal inflammation (Kuhn *et al.*, 1993). IL-10 producing T-cells (Tr1) further perform a key role in the development of tolerance (Asseman and Powrie, 1998; Asseman *et al.*, 1999). Because of this central role of IL-10 in the onset and maintenance of immune tolerance, researchers have been tempted to administer recombinant IL-10 to IBD patients to investigate its potential in counteracting the disease. The outcome of these studies (van Deventer *et al.*, 1997) has led to a moderate optimism. However, when applied through the systemic route this protein suffers from a number of drawbacks. Mild to moderate side effects are induced (Fedorak *et al.*, 2000; Schreiber *et al.*, 2000; Tilg, 2002), thereby encumbering its long term use at elevated concentrations. Moreover, IL-10 is extremely acid sensitive, impeding circumvention of the above side effects by targeted delivery through the digestive tract.

There appears to be a distinct role for certain pathogens in the onset of IBD. The development of IBD has often been proposed to be a consequence of viral or bacterial infection (*Mycobacteria, Shigella, Salmonella, Yersinia, Clostridium difficile, Bacteroides vulgatus*) but up to date no etiological agent has been identified for IBD. Recently, however, a DNA sequence has been identified in lamina propria mononuclear cells of which the presence and serum reactivity towards the according peptide highly correlate with CD. This presently unknown sequence is not of human origin and shows homology with bacterial tetR/acrR transcription regulators (Sutton *et al.*, 2000). It has now been demonstrated that people deficient in molecules such as CARD15 (Hugot *et al.*, 2001; Ogura *et al.*, 2001) and Tlr4 (H. Braat, personal communication) that recognize bacterial pathogen associated patterns and switch on innate immunity, are at stake of developing CD. One of the hypotheses behind this remarkable finding - a deficiency in a proinflammatory loop leading to pathological inflammation - is that uncontrolled early acute inflammation, as is the case in bacterial infection, could induce larger and longer than desirable tissue disturbance and could lead to active priming of acquired immunity. For this reason we argue that acute colitis may be a valuable target for preventing IBD onset. For this reason we are currently developing *L. lactis* strains that can produce trefoil factors, peptides that assist in tissue repair.

Probiotic bacteria and functional microflora

Probiotic microorganisms - predominantly lactic acid bacteria (LAB) but also *E. coli* and yeast - have been used for extended times. Originating as food supplements, now, still they are most often administered through the oral route and offer an attractive alternative for treatment of intestinal disorders. Until recently very little was known on the mechanisms that underlie these health beneficial effects. Now, however, we can discriminate a number of mutually not exclusive probiotic mechanistic pathways. They involve most of the interactions of the host with its commensal microflora.

Alteration of the microbial content and displacement of nocuous bacteria may be achieved by modifying pH or production of antibacterial compounds such as antibacterial peptides (Jack *et al.*, 1995; Lievin *et al.*, 2000), low molecular weight antimicrobial compounds (Niku-Paavola *et al.*, 1999) or non protein, lipophilic antibacterial molecules (Lievin *et al.*, 2000). Also the effects of toxins produced by these pathogenic bacteria can be inhibited, as for the yeast *S. boulardii* that produces a 120 kDa protein, protective against cholera toxin (Czerucka *et al.*, 1994).

Physical competition for binding to host tissues may displace pathogens. *B. casei* GG impedes the invasion of *Salmonella typhimurium* (Hudault *et al.*, 1997). A selection of probiotic strains can interfere with adherence of pathogenic *E. coli* and *Salmonella* to human intestinal glycoproteins (Tuomola *et al.*, 1999). Adherent human bifidobacterial strains inhibit colonization by a number of diarrheagenic bacteria (*E. coli* 0157 and *S. typhimurium*) and viruses (murine and rhesus rotavirus) (Duffy, 2000).

Some bacteria show a direct influence on the immune system. Strain specific induction of cytokines after oral *Lactobacillus* administration (Hessle *et al.*, 1999; Maassen *et al.*, 2000) may indeed provide a way to modulate local immune circuits in any desired (e.g. Th1, Th2, Th3 or Tr1) direction. The probiotic preparation VSL#3 induces IL-10 production (Helwig *et al.*, 1999). Some bacteria can actively interfere with the proinflammatory IκB/NFκB signaling pathway in intestinal epithelial cells by blockade of IκB-α degradation, thus adding to tolerance in the intestinal tract (Neish *et al.*, 2000). Feeding *Lactobacillus* GG prenatal to mothers and postnatal to infants was effective in prevention of allergy in children that, due to genetic predisposition, are at high risk (Kalliomaki *et al.*, 2001). IgA promotes the intestinal immunological barrier and is of major importance in the establishment of T-cell independent tolerance towards the indigenous microflora (Macpherson *et al.*, 2000). *Lactobacillus* GG promotes the number of IgA secreting plasma cells in CD patients (Malin *et al.*, 1996). Fecal levels of total IgA were significantly higher in healthy children upon intake of viable bifidobacteria (Fukushima *et al.*, 1998).

Some strains improve the gut wall barrier function. *Lb. plantarum* - a species that exerts beneficial effects on intestinal disorders (Mao *et al.*, 1996; Schultz, 1998;

Nobaek *et al.*, 2000) - is capable of generating nitric oxide through arginine catabolysis (Jonsson, 1983). Low levels of NO, as obtained from constitutive synthesis, promote organ integrity (Geroulanos *et al.*, 1992; Wright *et al.*, 1992). *Lb. plantarum* also induced MUC2 and MUC3 mRNA expression levels in HT-29 cells (Mack *et al.*, 1999), thus possibly enhancing the secretion of protective mucus. *Lb. casei* or *Clostridium butyricum* markedly enhanced gut epithelial cell proliferation - up to 200% in the colon - in rats and can thus enhance tissue repair (Ichikawa *et al.*, 1999). *Bifidobacteria*, *Staphylococcus aureus*, and *E. coli* were found to bind significant quantities of aflatoxin B1 (Datley *et al.*, 2000), a food and feed contaminant of fungal origin that damages the epithelial barrier.

Ammonia is a putative tumour promoter produced by bacterial degradation of protein and urea. *Bifidobacterium longum* administration reduces the number of aberrant crypts in the colon induced by the carcinogen azoxy-methane, possibly by reducing beta-glucuronidase activity and ammonia concentration (Rowland *et al.*, 1998). Detoxification of the intestinal content is clearly an option that could be addressed through GM probiotics. *Bifidobacterium*, *Enterococcus*, or *Lactobacillus* increased total short chain fatty acid concentration - a positive factor in counteracting colitis (Roediger, 1980) - in the colon (Sakata *et al.*, 1999).

A better mechanistic understanding of such functional microorganisms now opens the possibilities of designing novel probiotic strains. Through genetic engineering, not only will we be able to strengthen the effects of existing strains but we will also be able to create completely novel probiotics. The identification of adhesions, functional metabolites, scavenging factors and other components provide tools that can now be combined in a rational manner. These tools need however not necessarily be composed of bacterial products only but can also comprise elements of regulatory systems or enzymes derived from foreign - human - source. Modulating the immune system by engineering bacteria for the production of immunological messengers such as cytokines (Steidler *et al.*, 1995, Steidler *et al.*, 2000) may thus give rise to new probiotic strains. Strains that were engineered to secrete antibodies or antibody fragments may assist in immune surveilance (Beninati *et al.*, 2000; Kruger *et al.*, 2002). If designed carefully and with absolute attention for biological safety in its broadest sense, the development of genetically modified probiotics has the potential to revolutionize alimentary health.

Lactococcus lactis is a non-pathogenic and non-colonizing bacterium used mainly in dairy industries. As viable cells are regular constituents of the human diet, their harmless nature is generally accepted. They have accordingly been attributed the generally regarded as safe (GRAS) status by the US FDA. For this reason, the organism has been considered a good candidate to serve as a live vaccine delivery vehicle for mucosal immunization. Along the line of this rationale it has been shown that intranasal and oral immunization with recombinant *L. lactis* constitutively expressing tetanus toxin fragment C (TTFC) elicits protective high level immune responses against a challenge with the complete toxin (Wells *et al.*, 1993). We have focused on this organism as a carrier host for genetic modifications and have thus established a number of conceptual proofs.

Cytokine delivery at mucosal surfaces

Cytokines - such as interleukins and interferons - are small soluble proteins that, together with numerous growth factors and chemokines, provide the means by which cells of the immune system communicate with each other and with most other tissues in the body. As such, these molecules are able to regulate many aspects of the immune response in which numerous cells and tissues may be involved at any one time. Because of their activity as regulators of the immune system, cytokines and growth factors provide interesting tools for redirecting immunity. However, the production of these proteins is often complex and inappropriate targeting of these potent molecules may have devastating effects on the organism.

During engineering and manipulating recombinant *L. lactis* strains we were struck by the apparent ease with which this organism can secrete the above-mentioned factors without any apparent counterselection on its viability. Therefore we have taken up the project of developing genetically engineered *L. lactis* strains for the delivery of immunomodulatory cytokines at mucosal surfaces. We have demonstrated the potential of *L. lactis* cells to secrete fully biologically active cytokines, such as murine interleukins 2, 6 and 10, human interleukins 2, 6 and 10 and murine soluble type 1 and type 2 TNF receptors (Steidler *et al.*, 1995; Steidler *et al.*, 1998; Steidler *et al.*, 2000; Steidler *et al.*, 2003). In general, both IL-2 and IL-6 act as potent stimulators in the onset and maintenance of immune reactions. To investigate whether the mucosal and systemic responses to TTFC, responsible for the above-mentioned protection, can be enhanced or modulated we have constructed strains of *L. lactis* that produce TTFC intracellularly and secrete functional murine IL-2 or IL-6 (Steidler *et al.*, 1998). This was done by engineering recombinant operons which drive both the expression of TTFC and of the desired cytokine. Groups of six female Balb/c mice were immunized with these recombinant strains as well as with the relevant control strains. Immunizations were performed for three times, with intermediate 2 weeks spacing. Anti-TTFC serum antibody responses were significantly higher, up to 15 folds, in mice immunized intranasally with those strains of *L. lactis* secreting IL-2 or IL-6. Also the concentration of serum IgA reactive with TTFC was considerably higher after immunization with the IL-6 secret-

ing strain. This is in good agreement with the biological properties of IL-6 as a B-cell growth and IgA secretion-stimulating factor.

Delivery of IL-10 for IBD therapy

Above we discussed the central role of IL-10 in the establishment and maintenance of tolerance. It is however clear that IL-10 suffers from an important number of drawbacks. We wanted to investigate whether *in situ* synthesis of this promising therapeutic protein could remedy these shortcomings. We investigated this in two different mouse models for IBD (Steidler *et al.*, 2000). The repeated addition of DSS to the drinking water of Balb/c mice leads to the induction of chronic colitis (Kojouharoff *et al.*, 1997) reminiscent of IBD. The histology is characterized by a gradual decrease of goblet cell presence in the epithelial layer, disappearance of crypt architecture, infiltration of lymphocytes in the lamina propria and submucosa, thickening of the mucosa, appearance of crypt abscesses, swollen lymph follicles and ulceration (Fig. 1). Occasionally we have observed the formation of polyps, adenoma and squamous metaplasia. The colonic inflammation can be rated by blindly interpreting the appearance of the above-mentioned phenomena in histology. Typically, a mean score of 5 points was recorded. The inflammation is further associated with the up-regulation of several pro-inflammatory cytokines such as IFN-γ, IL-12 and TNF and can be cured by the systemic administration of neutralizing antibodies towards these proteins. Interleukin-10 is a very potent anti-inflammatory mediator, both at the level of antigen presentation and T-cell activity. Recombinant IL-10 is very acid sensitive and is therefore rapidly degraded when given orally. However, when given systemically, 5 consecutive doses of 5 μg daily will substantially decrease the inflammation. The daily ingestion, for 2 weeks, of *L. lactis* cells engineered to produce mIL-10 results in the acquisition of a score of approximately 1 in 40% of the treated mice, which is a status equal to that of healthy control mice. Most other animals only showed minor patchy remnants of the inflammation (Fig. 1). As killing of the IL-10 producing bacteria prior to inoculation can abrogate the effects reported here, these can be attributed strictly to the active *in vivo* delivery of IL-10. The observed healing is comparable to systemic treatment with prominent anti-inflammatory drugs but the amount of IL-10 required to score this effect is 10,000-fold lower, pointing at a dramatically improved delivery. Daily treatment with IL-10 producing *L. lactis* leads to the prevention of colitis, normally associated with the IL-10 -/- genotype in 129 Sv/Ev mice. When left untreated, 129 Sv/Ev IL-10-/- mice developed colitis from week 3 on, resulting in a mean histological score of 6 points at week 7. Littermates treated from week 3 on, stalled at a histological score of approximately 1.5 points.

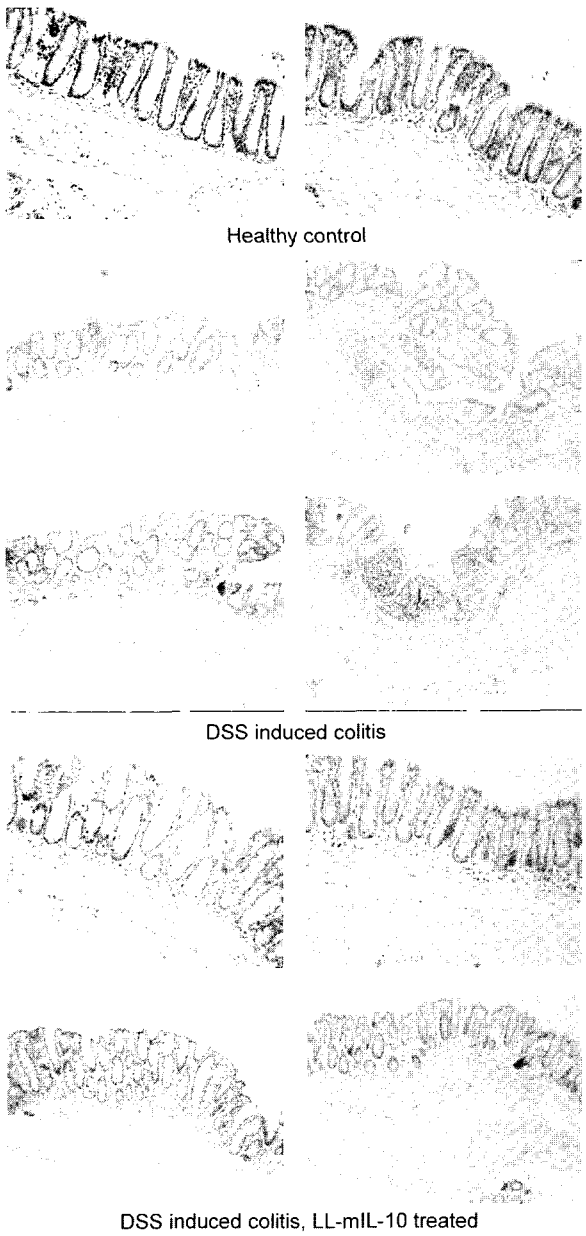

Fig. 1. Haematoxylin and eosin stained histological slides from colon tissues. The pictures show representative images of healthy control, mice in which chronic colitis has been induced by the repeated addition of DSS to the drinking water (DSS induced colitis) and mice which have undergone the same induction of chronic colitis followed by a treatment with mIL-10 producing *L. lactis* (LL-mIL-10 treated). The degree of inflammation can be expressed on a histological scale and interpreted statistically. This shows that LL-mIL-10 treatment significantly ($p=0.015$) reduces inflammation by 50%.

Tools for the design of biologically contained GM probiotics

The final goal of any designer probiotic strain is obviously its use in humans or animals to counteract or prevent disease. A point of major attention in the discussion of genet-

ically engineered probiotics is therefore that such medical use of GMO, in fact represents a deliberate releases of a GMO in the environment. The design of recombinant functional microflora should therefore be such that subsequent to its use, safety is guaranteed. This essentially relates to the prevention of lateral dissemination of the genetic modification and antibiotic selection markers to other bacteria and the prevention of accumulation of the GMO in the environment. It is optimal and most elegant if these concerns can be addressed through a biological system that is propagated along with the host. Such systems are then termed biological containment systems. Any biological containment strategy should meet all of the above-mentioned concerns. As currently no reports have been made on the use of GMO probiotics in human or veterinary medicine, the literature on the specific subject of biological containment of probiotics is very scarce. The strategies that are likely to be followed will however be quite similar to those used in other disciplines in which proficient use is made of GMO. Biological containment systems can be subdivided in active and passive. The former essentially provides control through the conditional production of a bacterial toxin via tightly regulated gene expression which is controlled by an environmentally responsive element or suppressed by an immunity factor. Passive systems render growth dependent on complementation of an auxotrophy or other gene defect, by supplementing either the intact gene or the essential metabolite.

Active biological containment systems that control GM dissemination.

Molina and coworkers (Molina *et al.*, 1998) designed contained GM bacteria that degrade a model pollutant, 3-methylbenzoate, on the basis of the *Pseudomonas putida* TOL plasmid for aromatic hydrocarbon metabolism. In *P. putida* Δ*asd*, a background necessary to enforce the system (Ronchel and Ramos, 2001), a fusion with the Pm promoter controls *lacI*. *E. coli* Gef, a porin-inducing protein, is produced under the control of the P*lac* promoter. The positive regulator of the Pm promoter, XylS, is active when 3-methylbenzoate is present. Degradation of the pollutant from the environment leads to decrease of LacI, Gef production and killing of the host. Though an elegant system, its applications are very restricted due to the high integration of the various components. In a streptavidin-based suicide system (Kaplan *et al.*, 1999), streptavidin expression was induced in a similar way by the absence of 3-methyl benzoate, resulting in 1000-fold reduction of *P. putida* viability within eight hours.

E. coli K12 *relA* mutants die faster than wild type. When supplemented with a system in which the alkaline phosphatase gene promoter drives the phage T7 lysozyme gene the killing rate further increases following phosphate depletion (Schweder *et al.*, 1995). Positioning of the *E. coli* alkaline phosphatase gene promoter so that it controls the *parB* locus of plasmid R1 makes that the resulting plasmids are stably inherited and that the carrier strains die as a consequence of phosphate starvation (Schweder *et al.*, 1992). The activity of the *E. coli rrnB* P1 promoter is completely turned off in the presence of the "alarmone" guanosine tetraphosphate, a compound that is produced following starvation. It can thus be used as a biosensor for poor growth conditions such as encountered upon release in the environment (Tedin *et al.*, 1995). The toxin in an active biological containment system can also be an endonuclease, countered by a methylase, as in the type II *Eco*RI restriction-modification system. The *eco*RIR lethal restriction gene therein is present on a plasmid. The *eco*RIM gene encoding the cognate *Eco*RI neutralising methylase is placed on the chromosome (Torres *et al.*, 2000). Lateral dissemination of the plasmid to recipient bacteria causes destruction of the DNA and evidently death. It can however be questioned whether such system is suitable for use in the intestine as a large part of the microbiota consists of *E. coli*. Other species could further acquire the methylase from this large common gene pool. The bacterial toxin colicin E3 displays an endonucleolytic activity towards the highly conserved 3' end of the 16S ribosomal RNA. Cleavage inhibits protein synthesis. Combination of the genes for both colicin production and colicin immunity renders a viable strain. When the E3 gene in such strain is closely linked to a genetic modification, lateral gene transfer of the later decreases by several orders of magnitude (Diaz *et al.*, 1994). A plasmid containment system using the lethal *E. coli relF* gene was shown effective *in vitro* but also in rat intestine (Knudsen *et al.*, 1995).

Passive systems for biological containment

A number of food grade strategies have been described that eliminate the need for antibiotic selection markers. Likewise, some of the gene deletion mutants utilized therein are essentially dependent for growth on the presence of a particular compound and may in that way also be used for the development of contained strains. D-alanine is an essential component for cell wall biosynthesis of many LAB. Alanine racemase (Alr) catalyses the conversion of L-alanine to D-alanine and therefore, *L. lactis* Δ*alr* and *Lb. plantarum* Δ*alr* strains showed auxotrophy for D-alanine (Hols *et al.*, 1997). The plasmid borne *alr* genes of both species have been used as food-grade selection markers to complement for Δ*alr*. Plasmids carrying a heterologous *alr* were stably inherited in a Δ*alr* background (Bron *et al.*, 2002). *alr* deletion has been used by Hillman and coworkers for containment of the GM *S. mutans* designed for anti-caries therapy, using *Streptococcus mutans* (Hillman, 2002) Δ*ldh adh*+ (J. Hillman, personal communication). Fu and Xu (2000) have described a similar containment system for *Lb. acidophilus* using

the thymidylate synthase gene (*thyA*) from *Lb. casei* as a selective marker for plasmid maintenance. In this case, a foreign gene is used to avoid the reversion of the mutation by backward recombination of the marker gene. Platteeuw and coworkers used the soluble carrier enzyme IIALac encoded by the 300 bp *lacF* as a selection marker in *L. lactis* Δ*lacF* (MacCormick *et al.*, 1995; Platteeuw *et al.*, 1996). This allows stable inheritance of the plasmid when lactose is used as a carbon source. An amber suppressor, *supD*, has been utilized as a selectable marker for plasmid maintenance to complement suppressible pyrimidine auxotrophs (Sorensen *et al.*, 2000). This host-plasmid combination is effective in any pyrimidine-free medium.

Nisin does not have the obvious drawbacks of a classical antibiotic. By placing the nisin immunity gene *nisI* on a plasmid, its maintenance through bacterial generations can be assured when nisin is used as a selection antibiotic (Takala and Saris, 2002). Integration of expression units in the chromosome of the carrier strain is very useful architecture because the GM character then is stably inherited and lateral gene transfer is reduced strongly in comparison with plasmid borne systems. Often however the level of expression of the transgene decreases significantly as a consequence of reduced copy number. Leenhouts and coworkers describe a method to obtain multiple integrations in a way that is compatible with the use in humans. The integrated plasmid is devoid for *repA*, an essential replication factor for derivatives of the lactococcal plasmid pWV01, and carries the sucrose utilization genes of the lactic acid bacterium *Pediococcus pentosaceus* (Leenhouts *et al.*, 1998a; Leenhouts *et al.*, 1998b). Intermediate engineering can be done in *L. lactis* strains which produce the pWV01 RepA protein. Single-crossover integration can be strongly enhanced on medium containing sucrose as the only carbon source.

Potential of thymidylate synthase gene exchange

When induced to execute their designed function, active containment systems provide effective killing of the host. They however have some important shortcomings. Firstly these systems often involve the introduction of a large amount of foreign, often pathogen derived DNA. From the regulatory perspective, this is highly undesirable for human applications. Secondly, a lot of these systems are plasmid borne. In any such system, function will depend on the relative expression of the different components. It remains to be demonstrated that, when integrated in the bacterial chromosome to reduce lateral dissemination, performance is maintained. Passive systems overcome these defects but are often bacteriostatic rather than bactericidal.

The choice of *thyA* as a target gene combines the advantages of both passive and active containment systems. "Thymine less death" (Ahmad *et al.*, 1998) was described as early as 1954 (Cohen and Barner, 1954) and involves activation of the SOS repair system and DNA fragmentation, thereby essentially using an indigenous suicide system. Thymine and thymidine growth dependence is intrinsically different from other auxotrophs (Ahmad *et al.*, 1998) because lack of the essential component is bactericidal rather than bacteriostatic. We recently reported targeted replacement of *L. lactis thyA* by *hIL-10* as a containment strategy (Steidler *et al.*, 2003). The *thyA* gene from *L. lactis* MG1363 had been cloned. However, the known flanking sequences are too short to allow for efficient crossover. For this reason we isolated the entire *thyA* locus of MG1363 (GenBank AF462070). Comparison with the *thyA* locus of *L. lactis* IL1403 showed that both *thyA* genes share 88% homology. The sequences flanking *thyA* are, however, completely unrelated. Conditionally replicative plasmids were used for targeted gene exchange by double homologous crossover (Fig. 2). Erythromycin (Em) selection forces integration by homologous recombination at either one of the target regions. This was confirmed by PCR. That subsequent random loss of the Em resistance marker had occurred by the alternative cross over was verified by PCR. Survival of the *thyA*-deficient strains is critically dependent on the presence of thymidine or thymine in the growth medium. These strains show a strong preference to utilize thymidine over thymine. Thymidine auxotrophs are self-limiting because

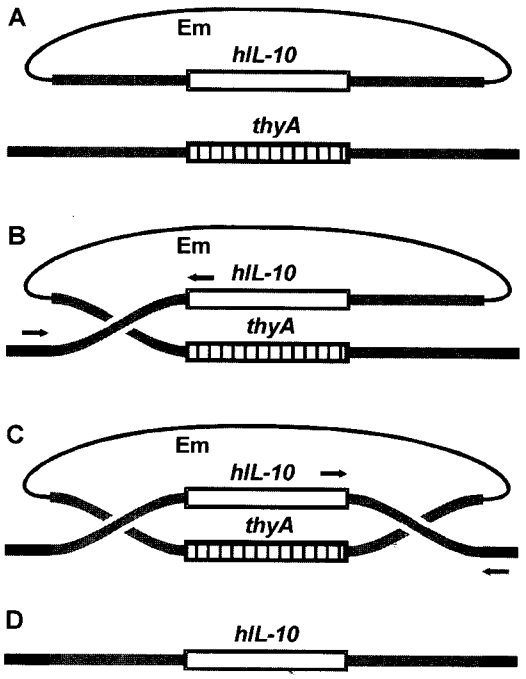

Fig. 2. Overview of the strategy used to exchange *thyA* and *hIL-10* genes. Gray lines represent target areas for recombination, thick black lines represent non-target MG1363 chromosome fragments and thin black lines represent plasmid vector. Different stages include: A) introduction of the nonreplicative vector; B) first crossover, forced by erythromycin selection and identified by PCR; C) second crossover in the absence of Em identified through PCR and D) acquisition of the desired transgenic chromosome organization.

depletion of the essential component immediately induces cell death. Although limited thymidine is present in the small intestine, *thyA* deficient strains show a substantial reduction in viability following passage through porcine intestine. We could never force the acquisition of foreign *thyA* genes to complement *thyA* deficiency. All of the strains constructed by exchanging *thyA* for *hIL-10* showed the production of *IL-10*, both *in vitro* and *in vivo*. It is remarkable that the strain that carried the least foreign DNA - Thy12 - showed the highest *hIL-10* synthesis.

Thy12 answers biosafety questions most adequately. No resistance marker is required to guarantee stable inheritance of the transgene. Accumulation of the GMO in the environment and the acquisition of *thyA* from other microorganisms are very unlikely. The most likely candidate donor for effective *thyA* reverse-in-recombination would be *L. lactis* subsp. *cremoris*. This event would however automatically remove *hIL-10*, generating the non modified state. The risk of disseminating the genetic modification through lateral gene transfer is reduced maximally by integrating *hIL-10* in the *L. lactis* chromosome. This system of biological containment was subject to the scrutiny of the Dutch authorities and was corollary allowed for use in humans. This will be the first time ever that a GMO bacterium will be used as a therapeutic.

Needs and necessities for future developments of functional microflora

Although in use for prolonged time and described for almost a century, we only now see the onset of medical use for probiotics. Especially in the area of intestinal disorders, where quite often life long treatment is required, they promise elegant alternatives to high impact chemical therapeutics. Gradually, reliable clinical studies have demonstrated efficacy. Probiotic microorganisms can be effective in IBD therapy through a number of mechanistic pathways. Current probiotic treatment of IBD however is still limited to remission maintenance. Genetic engineering to adapt current mechanisms or to establish novel mechanistic pathways may open new perspectives to obtain more powerful strains.

It is conceivable that the creation of novel probiotic organisms through the aid of genetic engineering will have a revolutionizing impact in this field. With the advent of GMO probiotic strategies, mechanistic tools can be taken from foreign sources and combined to make new organisms. Very much like traditional pharmacology will extract and copy specific compounds from nature for their interesting properties, active components, even when derived from pathogens, can now be placed in unsuspected carrier strains through genetic engineering and so provide a safe use for such elements. Molecular medical research has furnished a large collection of ingenious therapeutic compounds. Their application is however not rarely complicated by their *in vivo* sensitivity and the cost of their production. The use of GMO probiotics can circumvent short half-life and fragility of the therapeutics and yield very cost-effective access to such expensive therapeutics.

It is clear that GMO probiotics have a myriad of applications. It is however also clear that uninhibited spreading of GMO microorganisms in the environment is highly undesirable. Proactive engagement in the discussions on biosafety is therefore a necessity. When designing or applying novel functional microflora one should further realize that these are in many ways similar to novel chemical medicaments and require the same caution when testing them *in vivo*. Especially antigenic reactions are a key consideration. A strain that brings in a foreign protein may well provoke an immune reaction. This will not only jeopardize future use of this medication but could cause bystander recognition and allergy. It is appreciable that man, as a consequence of life long consumption, shows tolerance to lactic acid bacteria, *S. cerevisiae* and other carriers. But how will the organism react upon extensive exposure to a foreign (bacterial, animal, plant) enzyme? Will this provoke an immune reaction and if so, could it be cross-reactive to indigenous analogues? Will such hypothetical cross-reaction extend to the carrier? It is clear that these questions may limit the broadness of possible applications and call for caution when considering the clinical use of these systems. This however need not be an unsurpassable hurdle as similar problems in other areas have been overcome through "humanization" of the foreign proteins.

The functional microflora that have to date been designed are the first attempts of what has the potential to become a novel pharmacology. Although the goals are clearly ambitious, the problems encountered in real-life application are often immense. This is however not different in any novel, cutting edge development. Now the challenge is to creative molecular biologists and venturing physicians to conceive a new generation of powerful tools to combat what are amongst the most malicious diseases.

Acknowledgments

The authors thank Inge Bruggeman, Hilde Devlies and Koen Van Laer for expert technical assistance. Their studies were in part financed by VIB, Ghent University (GOA project nos. 12050700 and 12051501) and the commission of the European Communities, specific RTD programme "Quality of life and Management of Living Resources" QLK1-2000-00146 "Probiotic strains with designed health properties". LS and SN are subsidized through the Science Foundation of Ireland (SFI/01/F.1/B036).

References

Ahmad, S.I., S.H. Kirk, and A. Eisenstark. 1998. Thymine metab-

olism and thymineless death in prokaryotes and eukaryotes. *Annu. Rev. Microbiol.* 52, 591-625.

Asseman, C., S. Mauze, M.W. Leach, R.L. Coffman, and F. Powrie. 1999. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J. Exp. Med.* 190, 995-1004.

Asseman, C. and F. Powrie. 1998. Interleukin 10 is a growth factor for a population of regulatory T cells. *Gut* 42, 157-158.

Beninati, C., M.R. Oggioni, M. Boccanera, M.R. Spinosa, T. Maggi, S. Conti, W. Magliani, F.De Bernardis, G. Teti, A. Cassone, G. Pozzi, and L. Polonelli. 2000. Therapy of mucosal candidiasis by expression of an anti-idiotype in human commensal bacteria. *Nat. Biotechnol.* 18, 1060-1064.

Bron, P.A., M.G. Benchimol, J. Lambert, E. Palumbo, M. Deghorain, J. Delcour, W.M.De Vos, M. Kleerebezem, and P. Hols. 2002. Use of the alr gene as a food-grade selection marker in lactic acid bacteria. *Appl. Environ. Microbiol.* 68, 5663-5670.

Cohen, S.S. and H.D. Barner. 1954. Studies on unbalanced growth in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 40, 885-893.

Czerucka, D., I. Roux, and P. Rampal. 1994. *Saccharomyces boulardii* inhibits secretagogue-mediated adenosine 3',5'-cyclic monophosphate induction in intestinal cells. *Gastroenterology* 106, 65-72.

Diaz, E., M. Munthali, V. de Lorenzo, and K.N. Timmis. 1994. Universal barrier to lateral spread of specific genes among microorganisms. *Mol. Microbiol.* 13, 855-861.

Duchmann, R., E. Schmitt, P. Knolle, K.H. Meyer zum Buschenfelde, and M. Neurath. 1996. Tolerance towards resident intestinal flora in mice is abrogated in experimental colitis and restored by treatment with interleukin-10 or antibodies to interleukin-12. *Eur. J. Immunol.* 26, 934-938.

Duffy, L.C. 2000. Interactions mediating bacterial translocation in the immature intestine. *J. Nutr.* 130, 432S-436S.

Fedorak, R.N., A. Gangl, C.O. Elson, P. Rutgeerts, S. Schreiber, G. Wild, S.B. Hanauer, A. Kilian, M. Cohard, A. LeBeaut, and B. Feagan. 2000. Recombinant human interleukin 10 in the treatment of patients with mild to moderately active Crohn's disease. The Interleukin 10 Inflammatory Bowel Disease Cooperative Study Group. *Gastroenterology* 119, 1473-1482.

Fu, X., and J.G. Xu. 2000. Development of a chromosome-plasmid balanced lethal system for *Lactobacillus acidophilus* with *thyA* gene as selective marker. *Microbiol. Immunol.* 44, 551-556.

Fukushima, Y., Y. Kawata, H. Hara, A. Terada, and T. Mitsuoka. 1998. Effect of a probiotic formula on intestinal immunoglobulin A production in healthy children. *Int. J. Food Microbiol.* 42, 39-44.

Fuss, I.J., M. Neurath, M. Boirivant, J.S. Klein, C. de la Motte, S.A. Strong, C. Fiocchi, and W. Strober. 1996. Disparate CD4+ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. *J. Immunol.* 157, 1261-70.

Geroulanos, S., J. Schilling, M. Cakmakci, H.H. Jung, and F. Largiader. 1992. Inhibition of NO synthesis in septic shock. *Lancet.* 339, 435.

Helwig, U., F. Rizzello, and A. Venturi. 1999. Effect of probiotic therapy on pro- and anti-inflammatory cytokins in pouchitis. *Gut* 45, A104.

Hessle, C., L.A. Hanson, and A.E. Wold. 1999. Lactobacilli from human gastrointestinal mucosa are strong stimulators of IL-12 production. *Clin. Exp. Immunol.* 116, 276-282.

Hillman, J.D. 2002. Genetically modified Streptococcus mutans for the prevention of dental caries. *Antonie Van Leeuwenhoek* 82, 361-366.

Hols, P., C. Defrenne, T. Ferain, S. Derzelle, B. Delplace, and J. Delcour. 1997. The alanine racemase gene is essential for growth of *Lactobacillus plantarum*. *J. Bacteriol.* 179, 3804-3807.

Hudault, S., V. Lievin, M.F. Bernet-Camard, and A.L. Servin. 1997. Antagonistic activity exerted in vitro and in vivo by *Lactobacillus casei* (strain GG) against *Salmonella typhimurium* C5 infection. *Appl. Environ. Microbiol.* 63, 513-518.

Hugot, J.P., M. Chamaillard, H. Zouali, S. Lesage, J.P. Cezard, J. Belaiche, S. Almer, C. Tysk, C.A. O'Morain, M. Gassull, V. Binder, Y. Finkel, A. Cortot, R. Modigliani, P. Laurent-Puig, C. Gower-Rousseau, J. Macry, J.F. Colombel, M. Sahbatou, and G. Thomas. 2001. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* 411, 599-603.

Ichikawa, H., T. Kuroiwa, A. Inagaki, R. Shineha, T. Nishihira, S. Satomi, and T. Sakata. 1999. Probiotic bacteria stimulate gut epithelial cell proliferation in rat. *Dig. Dis. Sci.* 44, 2119-2123.

Jack, R.W., J.R. Tagg, and B. Ray. 1995. Bacteriocins of gram-positive bacteria. *Microbiol. Rev.* 59, 171-200.

Jonsson, S., E. Clausen, and J. Raa. 1983. Amino acid degradation by a *Lactobacillus plantarum* strain from fish. System *Appl. Microbiol.* 4, 148-154.

Kakazu, T., J. Hara, T. Matsumoto, S. Nakamura, N. Oshitani, T. Arakawa, A. Kitano, K. Nakatani, F. Kinjo, and T. Kuroki. 1999. Type 1 T-helper cell predominance in granulomas of Crohn's disease. *Am. J. Gastroenterol.* 94, 2149-2155.

Kalliomaki, M., S. Salminen, H. Arvilommi, P. Kero, P. Koskinen, and E. Isolauri. 2001. Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial. *Lancet.* 357, 1076-1079.

Kaplan, D.L., C. Mello, T. Sano, C. Cantor, and C. Smith. 1999. Streptavidin-based containment systems for genetically engineered microorganisms. *Biomol. Eng.* 16, 135-140.

Knudsen, S., P. Saadbye, L.H. Hansen, A. Collier, B.L. Jacobsen, J. Schlundt, and O.H. Karlstrom. 1995. Development and testing of improved suicide functions for biological containment of bacteria. *Appl. Environ. Microbiol.* 61, 985-991.

Kojouharoff, G., W. Hans, F. Obermeier, D.N. Mannel, T. Andus, J. Scholmerich, V. Gross, and W. Falk. 1997. Neutralization of tumour necrosis factor (TNF) but not of IL-1 reduces inflammation in chronic dextran sulphate sodium-induced colitis in mice. *Clin. Exp. Immunol.* 107, 353-358.

Kruger, C., Y. Hu, Q. Pan, H. Marcotte, A. Hultberg, D. Delwar, P.J. van Dalen, P.H. Pouwels, R.J. Leer, C.G. Kelly, C. van Dollenweerd, J.K. Ma, and L. Hammarstrom. 2002. In situ delivery of passive immunity by lactobacilli producing single-chain antibodies. *Nat. Biotechnol.* 20, 702-706.

Kuhn, R., J. Lohler, D. Rennick, K. Rajewsky, and W. Muller. 1993. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* 75, 263-274.

Leenhouts, K., A. Bolhuis, J. Boot, I. Deutz, M. Toonen, G. Venema, J. Kok, and A. Ledeboer. 1998. Cloning, expression, and chromosomal stabilization of the *Propionibacterium shermanii* proline iminopeptidase gene (pip) for food-grade application in *Lactococcus lactis*. *Appl. Environ. Microbiol.* 64, 4736-4742.

Leenhouts, K., A. Bolhuis, G. Venema, and J. Kok. 1998. Construction of a food-grade multiple-copy integration system for *Lactococcus lactis*. *Appl. Microbiol. Biotechnol.* 49, 417-423.

Lievin, V., I. Peiffer, S. Hudault, F. Rochat, D. Brassart, J.R. Neeser, and A.L. Servin. 2000. *Bifidobacterium* strains from resident infant human gastrointestinal microflora exert antimicrobial activity. *Gut* 47, 646-652.

Maassen, C.B., C. van Holten-Neelen, F. Balk, M.J. den Bak-Glashouwer, R.J. Leer, J.D. Laman, W.J. Boersma, and E. Claassen. 2000. Strain-dependent induction of cytokine profiles in the gut by orally administered *Lactobacillus* strains. *Vaccine* 18, 2613-2623.

MacCormick, C.A., H.G. Griffin, and M.J. Gasson. 1995. Construction of a food-grade host/vector system for *Lactococcus lactis* based on the lactose operon. *FEMS Microbiol. Lett.* 127, 105-109.

Mack, D. R., S. Michail, S. Wei, L. McDougall, and M. A. Hollingsworth. 1999. Probiotics inhibit enteropathogenic *E. coli* adherence *in vitro* by inducing intestinal mucin gene expression. *Am. J. Physiol.* 276, G941-950.

Macpherson, A.J., D. Gatto, E. Sainsbury, G.R. Harriman, H. Hengartner, and R.M. Zinkernagel. 2000. A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. *Science* 288, 2222-2226.

Malin, M., H. Suomalainen, M. Saxelin, and E. Isolauri. 1996. Promotion of IgA immune response in patients with Crohn's disease by oral bacteriotherapy with *Lactobacillus* GG. *Ann. Nutr. Metab.* 40, 137-145.

Mao, Y., S. Nobaek, B. Kasravi, D. Adawi, U. Stenram, G. Molin, and B. Jeppsson. 1996. The effects of *Lactobacillus* strains and oat fiber on methotrexate-induced enterocolitis in rats. *Gastroenterology* 111, 334-344.

Molina, L., C. Ramos, M.C. Ronchel, S. Molin, and J.L. Ramos. 1998. Construction of an efficient biologically contained *Pseudomonas putida* strain and its survival in outdoor assays. *Appl. Environ. Microbiol.* 64, 2072-2078.

Mosmann, T.R., and R.L. Coffman. 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.* 7, 145-173.

Neish, A.S., A.T. Gewirtz, H. Zeng, A.N. Young, M.E. Hobert, V. Karmali, A.S. Rao, and J.L. Madara. 2000. Prokaryotic regulation of epithelial responses by inhibition of IkappaB-alpha ubiquitination. *Science* 289, 1560-1563.

Niessner, M., and B.A. Volk. 1995. Altered Th1/Th2 cytokine profiles in the intestinal mucosa of patients with inflammatory bowel disease as assessed by quantitative reversed transcribed polymerase chain reaction (RT-PCR). *Clin. Exp. Immunol.* 101, 428-435.

Niku-Paavola, M.L., A. Laitila, T. Mattila-Sandholm, and A. Haikara. 1999. New types of antimicrobial compounds produced by *Lactobacillus plantarum*. *J. Appl. Microbiol.* 86, 29-35.

Nobaek, S., M.L. Johansson, G. Molin, S. Ahrne, and B. Jeppsson. 2000. Alteration of intestinal microflora is associated with reduction in abdominal bloating and pain in patients with irritable bowel syndrome. *Am. J. Gastroenterol.* 95, 1231-1238.

Oatley, J.T., M.D. Rarick, G.E. Ji, and J.E. Linz. 2000. Binding of aflatoxin B1 to *bifidobacteria* in vitro. *J. Food. Prot.* 63, 1133-1136.

Ogura, Y., D.K. Bonen, N. Inohara, D.L. Nicolae, F.F. Chen, R. Ramos, H. Britton, T. Moran, R. Karaliuskas, R.H. Duerr, J.P. Achkar, S.R. Brant, T.M. Bayless, B.S. Kirschner, S.B. Hanauer, G. Nunez, and J.H. Cho. 2001. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* 411, 603-606.

Platteeuw, C., I. van Alen-Boerrigter, S. van Schalkwijk, and W.M. de Vos. 1996. Food-grade cloning and expression system for *Lactococcus lactis*. *Appl. Environ. Microbiol.* 62, 1008-1013.

Roediger, W.E. 1980. The colonic epithelium in ulcerative colitis: an energy-deficiency disease? *Lancet.* 2, 712-715.

Ronchel, M.C., and J.L. Ramos. 2001. Dual system to reinforce biological containment of recombinant bacteria designed for rhizoremediation. *Appl. Environ. Microbiol.* 67, 2649-2656.

Rowland, I.R., C.J. Rumney, J.T. Coutts, and L.C. Lievense. 1998. Effect of *Bifidobacterium* longum and inulin on gut bacterial metabolism and carcinogen-induced aberrant crypt foci in rats. *Carcinogenesis* 19, 281-285.

Sakata, T., T. Kojima, M. Fujieda, M. Miyakozawa, M. Takahashi, and K. Ushida. 1999. Probiotic preparations dose-dependently increase net production rates of organic acids and decrease that of ammonia by pig cecal bacteria in batch culture. *Dig. Dis. Sci.* 44, 1485-1493.

Sartor, R.B. 1997. Pathogenesis and immune mechanisms of chronic inflammatory bowel diseases. *Am. J. Gastroenterol.* 92, 5S-11S.

Schreiber, S., R.N. Fedorak, O.H. Nielsen, G. Wild, C.N. Williams, S. Nikolaus, M. Jacyna, B.A. Lashner, A. Gangl, P. Rutgeerts, K. Isaacs, S.J. van Deventer, J.C. Koningsberger, M. Cohard, A. LeBeaut, and S.B. Hanauer. 2000. Safety and efficacy of recombinant human interleukin 10 in chronic active Crohn's disease. Crohn's Disease IL-10 Cooperative Study Group. *Gastroenterology* 119, 1461-1472.

Schultz, M. 1998. Continuous feeding of Lactobacillus plantarum attenuates established colitis in interleukin-10 deficient mice (abstr). *Gastroenterology* 114, A1081.

Schweder, T., K. Hofmann, and M. Hecker. 1995. *Escherichia coli* K12 relA strains as safe hosts for expression of recombinant DNA. *Appl. Microbiol. Biotechnol.* 42, 718-723.

Schweder, T., I. Schmidt, H. Herrmann, P. Neubauer, M. Hecker, and K. Hofmann. 1992. An expression vector system providing plasmid stability and conditional suicide of plasmid-containing cells. *Appl. Microbiol. Biotechnol.* 38, 91-93.

Sorensen, K.I., R. Larsen, A. Kibenich, M.P. Junge, and E. Johansen. 2000. A food-grade cloning system for industrial strains of *Lactococcus lactis*. *Appl. Environ. Microbiol.* 66, 1253-1258.

Steidler, L., W. Hans, L. Schotte, S. Neirynck, F. Obermeier, W. Falk, W. Fiers, and E. Remaut. 2000. Treatment of murine colitis by *Lactococcus lactis* secreting interleukin-10. *Science* 289, 1352-1355.

Steidler, L., S. Neirynck, N. Huyghebaert, V. Snoeck, A. Vermeire, B. Goddeeris, E. Cox, J.P. Remon, and E. Remaut. 2003. Biological containment of genetically modified *Lactococcus lactis* for intestinal delivery of human interleukin 10. *Nat. Biotechnol.* (in press).

Steidler, L., K. Robinson, L. Chamberlain, K.M. Schofield, E. Remaut, R.W. Le Page, and J.M. Wells. 1998. Mucosal delivery of murine interleukin-2 (IL-2) and IL-6 by recombinant strains of *Lactococcus lactis* coexpressing antigen and cytokine. *Infect. Immun.* 66, 3183-3189.

Steidler, L., J.M. Wells, A. Raeymaekers, J. Vandekerckhove, W. Fiers, and E. Remaut. 1995. Secretion of biologically active

murine interleukin-2 by *Lactococcus lactis* subsp. *lactis*. *Appl. Environ. Microbiol.* 61, 1627-1629.

Sutton, C.L., J. Kim, A. Yamane, H. Dalwadi, B. Wei, C. Landers, S.R. Targan, and J. Braun. 2000. Identification of a novel bacterial sequence associated with Crohn's disease. *Gastroenterology* 119, 23-31.

Takala, T.M. and P.E. Saris. 2002. A food-grade cloning vector for lactic acid bacteria based on the nisin immunity gene nisI. *Appl. Microbiol. Biotechnol.* 59, 467-471.

Tedin, K., A. Witte, G. Reisinger, W. Lubitz, and U. Blasi. 1995. Evaluation of the *E. coli* ribosomal rrnB P1 promoter and phage-derived lysis genes for the use in a biological containment system: a concept study. *J. Biotechnol.* 39, 137-148.

Tilg, H., H. Ulmer, A. Kaser, and G. Weiss. 2002. Role of IL-10 for induction of anemia during inflammation. *J. Immunol.* 169, 2204-2209.

Torres, B., S. Jaenecke, K.N. Timmis, J.L. Garcia, and E. Diaz. 2000. A gene containment strategy based on a restriction-modification system. *Environ. Microbiol.* 2, 555-563.

Tuomola, E.M., A.C. Ouwehand, and S.J. Salminen. 1999. The effect of probiotic bacteria on the adhesion of pathogens to human intestinal mucus. *FEMS Immunol. Med. Microbiol.* 26, 137-142.

van Deventer, S.J., C.O. Elson, and R.N. Fedorak. 1997. Multiple doses of intravenous interleukin 10 in steroid-refractory Crohn's disease. Crohn's Disease Study Group. *Gastroenterology* 113, 383-389.

Wells, J.M., P.W. Wilson, P.M. Norton, M.J. Gasson, and R.W. Le Page. 1993. *Lactococcus lactis*: high-level expression of tetanus toxin fragment C and protection against lethal challenge. *Mol. Microbiol.* 8, 1155-1162.

Wright, C.E., D.D. Rees, and S. Moncada. 1992. Protective and pathological roles of nitric oxide in endotoxin shock. *Cardiovasc. Res.* 26, 48-57.