Clinical Potential of Lactococcus lactis Mediated Delivery of Human Interleukin-10 and Trefoil Factors

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Systemic treatment with infliximab, a monoclonal antibody that binds TNFα is among the most potent therapies for Crohn’s disease. Despite initial successes with intravenous addition of IL-10, this treatment was abandoned in later trials. Both treatments suffered a serious drawback from the fact that they had to be administered systemically and thus caused potentially serious side effects. We used two mouse models for IBD to evaluate the efficacy of L. lactis mediated topical delivery of IL-10. In the Dextran Sulphate Sodium (DSS)-induced chronic colitis intragastric administration of mIL-10-secreting L. lactis led to a 50% reduction in inflammation. Also, daily administration of the engineered strain prevented the onset of colitis, normally associated with the IL-10 knockout genotype. In both models, the effect was strictly dependent on delivery of live bacteria. We engineered a strain exhibiting biological containment. To this end, the thymidilate synthase (thyA) gene of L. lactis was replaced with a synthetic human IL-10 gene. ThyA-deficient bacteria are suicidal in the absence of thymine or thymidine and therefore cannot accumulate in the environment. A limited clinical trial in Crohn’s patients under physical containment proved that the treatment was safe, the biological containment strategy was effective and the results obtained suggested a clinical effect. In contrast to oral administration of purified protein, intragastric administration of Trefoil factor-secreting L. lactis was very effective in prevention and healing of acute DSS colitis. In addition this approach was successful in improving established chronic colitis in IL-10 knockout mice.

Key words: Lactococcus lactis; GMO containment; interleukin-10; trefoil factors; inflammatory bowel disease

INTRODUCTION

The lactic acid bacteria (LAB) represent a taxonomically diverse group of gram-positive bacteria that share the property of converting fermentable carbohydrates primarily to lactic acid and thus acidify the medium in which they grow. The LAB family naturally occupies quite different ecological niches. They are aerotolerant anaerobes. They are found on plant surfaces, as members of the resident microflora of the gastrointestinal tract of vertebrates, as well as in sewage. The members of the LAB group are best known for their extensive use in the manufacture of a variety of fermented food and feed products, such as preparation of fermented dairy products (cheeses, sour milks, yogurts), meats and sausages.

Over the past decades interest in the study of LAB has dramatically increased. This reflects not only a growing industrial importance of these bacteria for a wide variety of fermentation processes, but also evidences the emerging—or perhaps more correctly put, the revival (28)—of their application as so called ‘probiotics’, i.e. strains with attributed nutritional and human/animal health beneficial properties (55). The fact that selected probiotic strains, which may or may not be natural residents of the human GI tract, may influence the intestinal physiology through modulation of the endogenous flora or the immune system is presently well recognized. In particular, members of the genera Lactobacillus and Bifidobacterium have received much attention and their beneficial effect in reducing tissue damage as seen in patients with Inflammatory Bowel Disease (IBD) or in animal models for this disease is well-documented (46).

Inflammatory bowel disease

IBD is a group of chronic intestinal inflammatory diseases, of which the most common forms are ulcerative colitis (UC), an inflammation of the large intestine, and Crohn’s disease (CD), which can affect any part of the gastrointestinal tract. This subdivision was originally based on typical clinical manifestations, endoscopic
pathogens while at the same time maintaining tolerance between developing an immune response to invading system of the GI tract maintains a delicate balance. In the healthy body the mucosa-associated immune α Necrosis factor (TNF- gut. Both pro-inflammatory cytokines, like Tumor β growth Factor- (TGF- β), are known to play a key role in the process. In many cases, IBD is provoked by acute bacterial food poisoning. There is, however, increasing evidence that T-cells, which recognize antigen from common commensal bacteria, play an important role in the establishment of pathogenesis. These T-cells are activated upon encounter with the abundantly and continuously present antigen, as presented on APC, and drive the inflammation by the concurrent production of pro-inflammatory cytokines. Tolerance may on the other hand be re-established by defined subsets of T-cells. With this knowledge in hand, IBD can be counteracted in a rational manner. Novel anti-inflammatory therapies, which make use of neutralizing monoclonal antibodies or anti-inflammatory cytokines, show the possibility to modulate the immune deregulations causative to IBD.

A highly prominent and effective new therapy is systemic treatment with anti-TNF monoclonal antibodies (13, 40, 67). Single intravenous doses of the cA2 infliximab antibody resulted in a drastic clinical improvement in active Crohn’s disease. A drawback of this approach became apparent with increasing numbers of patients suffering potentially serious side effect such as the reactivation of tuberculosis.

While infliximab and other anti-TNF-α agents correct the immune imbalance in IBD by interfering with the action of pro-inflammatory cytokines, IL-10 treatment intends to exploit the anti-inflammatory properties of the product itself. Intravenous administration of recombinant IL-10 resulted in reduced Crohn’s disease activity scores and increased remission (66). However, larger trials could not corroborate these positive effects. Besides, many patients developed systemic side effects.

A common aspect of the above named treatments is that they require systemic administration, whereby only a fraction of the drugs reaches the intended target, i.e. the gastrointestinal tract. This is not only an inefficient way to deliver drugs, but, more importantly, many patients suffer unpleasant side effects that result from the high levels of the drugs in other otherwise healthy tissues and organs of the body.

We have investigated the feasibility of topical delivery of therapeutic proteins trough oral administration of recombinant Lactococcus lactis, engineered to secrete the protein in situ, i.e. in the gastrointestinal tract.

Engineering Lactococcus lactis for cytokine secretion

Whilst rapid progress is being made in the molecular analysis of all species of lactic acid bacteria, the mesophilic lactococci remain by far the best characterized.

In the present communication we shall concentrate on work performed with Lactococcus lactis. This species is a mesophilic and microaerophilic LAB, commonly occurring in nature, especially on plant material. It is not a permanent resident of the gastrointestinal tract, nor is it usually found in fecal material. Raw cow’s milk,
however, consistently contains \textit{L. lactis}, presumably entered from the exterior of the udder during milking and also from the feed. Although \textit{L. lactis} has never been shown to have probiotic properties, it has in recent years enjoyed an increasing interest for its use in nutraceuticals (15) and as carrier for the delivery of biologically active molecules.

We were first to report on the use of recombinant \textit{L. lactis} strains for the production of cytokines of both murine and human origin (Table 1) (47, 51). The rationale for choosing \textit{L. lactis} as an expression host derived from the availability of well-characterized plasmids as well as an efficient transformation procedure (69).

Surprisingly, and in contrast to the situation in \textit{Escherichia coli}, the synthesis of cytokines did not influence the growth rate of the organism. This may very well be a consequence of the secretion of the possibly detrimental proteins. At that time the use of \textit{L. lactis} as a carrier for a foreign antigen, tetanus toxin fragment C (TTFC), had been demonstrated. Intranasal or oral inoculation of such engineered bacteria resulted in protective immune responses (71). We modified \textit{L. lactis} strains to allow accumulation of TTFC intracellular and co-secretion of either murine interleukin-2 (mIL-2) or mIL-6. Upon inoculation in the nostrils, mice showed a significant and substantial increase in the immune reaction against the foreign antigen. The mIL-2 or mIL-6 mediated increase in TTFC specific IgG titer was strictly dependent on the viability of the bacterial strains. This shows that recombinant \textit{L. lactis} can actively deliver these cytokines \textit{in situ} (50).

Table 1.  

<table>
<thead>
<tr>
<th>Protein</th>
<th>Yield secreted protein</th>
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<tbody>
<tr>
<td>murine interleukin 2</td>
<td>100 ng/ml</td>
</tr>
<tr>
<td>murine interleukin 4</td>
<td>3 ng/ml</td>
</tr>
<tr>
<td>murine interleukin 6</td>
<td>100 ng/ml</td>
</tr>
<tr>
<td>murine interleukin 10</td>
<td>100 ng/ml</td>
</tr>
<tr>
<td>murine interferon-β</td>
<td>not detectable</td>
</tr>
<tr>
<td>murine 55 kDa soluble TNF receptor</td>
<td>50 ng/ml</td>
</tr>
<tr>
<td>murine 75 kDa soluble TNF receptor</td>
<td>10 ng/ml</td>
</tr>
<tr>
<td>human interleukin 2</td>
<td>50 ng/ml</td>
</tr>
<tr>
<td>human interleukin 6</td>
<td>100 ng/ml</td>
</tr>
<tr>
<td>human interleukin 10</td>
<td>20 ng/ml</td>
</tr>
<tr>
<td>\textit{Staphylococcus aureus} protein A</td>
<td>1500 ng/ml</td>
</tr>
<tr>
<td>\textit{Streptomyces avidinii} streptavidin</td>
<td>3000 ng/ml</td>
</tr>
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To further investigate this potential we addressed the question whether a cytokine-secreting strain, administered by the oral route, could reverse the inflammation associated with experimental colitis in mice, as a model for the study of human inflammatory bowel disease. As a therapeutic tool we chose murine interleukin-10 (mIL-10).

**MATERIALS AND METHODS**

**Bacterial strains, media and plasmids**

\textit{Lactococcus lactis} subsp.\textit{cremoris} MG1363 is a derivative of NCD0712 (National Collection of Dairy Organisms, Reading, England), which was cured of all resident plasmids (11). \textit{L. lactis} strain LL108 has the \textit{repA} gene from plasmid pORI19 integrated into its chromosome (21). Bacteria were routinely grown as standing cultures at 30°C in M17 broth (Difco Laboratories, Detroit MI) supplemented with 0.5% glucose (GM17). Thymidine free medium (TFM) was obtained by growing a \textit{thyA} \textit{L. lactis} strain to saturation in M17 broth supplemented with 0.5% glucose. All viable bacteria were removed from the growth medium by centrifugation, subsequent filtration over a 0.22 μm pore size membrane and sterilization. GM9 is minimal salts M9 supplemented with 0.5% casitone and 0.5% glucose. BM9 means GM9 buffered with 50 mM carbonate (44). Stock suspensions of all strains were stored at ~20°C in 50% glycerol in GM17. For intragastric inoculations, stock suspensions were diluted 200-fold in fresh GM17 and incubated at 30°C. They reached a saturation density of $2 \times 10^9$ colony-forming units (cfu) per ml within 16 hours. Bacteria were harvested by centrifugation and concentrated 10-fold in BM9 medium. For treatment, each mouse received 100 μL of this suspension daily by intragastric catheter. Plasmid pORI19 specifies resistance to erythromycin (Em) and is \textit{repA} (20). Plasmid pVE6007 encodes a temperature sensitive RepA protein and carries chloramphenicol (Cm) resistance (2). pTI1mIL10 contains the coding region of mature murine interleukin-
10 (mIL-10) fused to the lactococcal usp45 secretion leader (64), preceded by the coliphage T7 gene-10 ribosome binding site and the lactococcal P1 promoter (68).

Recombinant DNA technology

Standard recombinant DNA technology was used. Restriction enzymes were from New England Biolabs, MA, USA. PCR amplification for plasmid construction was performed with Vent® thermostable DNA polymerase (New England Biolabs, MA, USA). Transformation of L. lactis was as described (69).

Specific variants of genes of interest were assembled in vitro trough PCR amplification and ligation of a series of overlapping oligonucleotides (52).

Animals

Eleven-week old female BALB/c mice were obtained from Charles River Laboratories (Sulzfeld, Germany). They were housed in a conventional animal facility. IL-10 knockout mice (129Sv/Ev IL-10–/–) (19) were housed and bred under SPF conditions. All mice were fed standard laboratory feed and tap water ad libitum. The animal studies were approved by the Ethics Committee of the Department for Molecular Biomedical Research, Ghent University.

Histological analysis

The colon was prepared out, cleaned and opened longitudinally. A segment of 1 cm was taken from the middle part of the colon, embedded in paraffin and sectioned longitudinally. Three sections of 4 μm were cut at 200 μm intervals and stained with hematoxylin/eosin. Colon sections were numbered randomly and interpreted semi-quantitatively in a blinded manner by a pathologist. The histological score is the sum of the epithelial damage and lymphoid infiltration, each ranging from 0 to 4 as described (18).

Quantification of L. lactis-secreted interleukins and trefoil factors

The biological activity of murine IL-10 was determined in a proliferation assay with the MC/9 mouse mast cell line by measuring the stimulation of [3H] thymidine incorporation in the proliferating mast cells (58). A standard of known specific activity (BioSource International, Camarillo, CA) was used as an internal control.

Quantification of hIL-10 in L. lactis supernatants was performed in a sandwich ELISA with rat anti-human IL-10 antibody (BD, Pharmingen, San Diego, CA) as the primary antibody followed by revelation of the bound antibody with biotinylated rat anti-human IL-10 antibody conjugated to horseradish peroxidase-coupled streptavidin and TMB as the chromogenic substrate.

Myc-tagged TFF secreted in vitro were quantified by direct adsorption of crude L. lactis supernatants to ELISA plates and subsequent detection with a specific mouse mAb against the Myc epitope (Sigma, St. Louis, MO).

For quantification of Myc-tagged TFF secreted in vivo in colon tissue, the entire colon was homogenized in PBS containing 1% BSA and sonicated. Myc-tagged TFF were captured from the suspension by immobilized polyclonal rabbit anti-Myc Ab (MBL, Naka-ku Naoya, Japan), and quantified by anti-Myc biotin-conjugated mouse mAb and revealed with horseradish peroxidase-conjugated streptavidin and reaction with TMB substrate.

Wound healing assay

An epithelial monolayer wound repair assay was performed essentially as described (40) but instead of the Caco-2 cell line, we used the mouse rectal carcinoma derived CMT-93 epithelial cell line (No. CCL-223, American Type Culture Collection, Manassas, VA).

RESULTS

Topical L. lactis-mediated delivery of IL-10 cures and prevents intestinal inflammation in murine models for IBD

We constructed a gene fusion between the secretion leader sequence of the lactococcal usp45 gene and the sequence encoding mature murine interleukin-10 (mIL-10). The carrier plasmid drives the expression of the usp45-mil-10 gene from the constitutive lactococcal P1 promoter. Strain L. lactis MG1363 transformed with this plasmid was designated LL-mIL10. During growth in GM17 or GM9, L. lactis produces lactate, so that the acidity of the culture drops to pH 5.5. Presumably because of its known acid sensitivity, no mIL-10 protein was detected in the culture supernatant. Therefore we tested a series of carbonate buffered media. Optimal and stable mIL-10 protein secretion was obtained in BM9 medium buffered to pH 8.5 (Fig.1). The profile of proteins secreted from the novel strain showed a very prominent supplementary band with mobility as expected for IL-10. The protein was identified as recombinant mIL-10 in Western blot, in ELISA and following N-terminal protein sequencing. The recombinant protein had the same specific biological activity as the protein isolated from the natural source (44).
We evaluated the effect of topical *L. lactis*-mediated delivery of mIL-10 on inflammation of the colon in two murine models for IBD, (i) a model in which colitis is induced by chemical treatment and (ii) a model in which colitis develops spontaneously in IL-10 knockout mice (48).

Treatment of chemically induced colitis: The addition of 5% dextran sodium sulfate (DSS) to the drinking water of Balb/c mice provokes acute colitis within a period of 7 days. Fourfold repetitive administration of this eroding compound during 7 days, alternated with intermediate 10-day periods of recovery with normal drinking water results in the establishment of long lasting chronic colitis (18, 31). Mice in which colitis had been induced in this way showed a thickened mucosa. The predominant epithelial damage was loss of goblet cells and of crypts. The infiltrate was restricted to the mucosa and consisted mainly of lymphocytes and macrophages and to a lesser degree (<15%) of granulocytes. In this experimental model for IBD a more severe tissue disturbance is observed in the distal part of the colon. The inflammation would typically last for at least 3 months after the induction.

Daily intra-gastric inoculations with *LL-mIL10* cultures were initiated at day 21 following the last DSS cycle. Untreated, healthy mice had a histological score of 1. Mice with chronic colitis had a score slightly over 5. All control groups, i.e. the strains carrying no plasmid or the empty vector, fluctuated around this number. However, in mice treated for 14 days with *LL-mIL10* followed by 14 days of recovery, a clear reduction in the histological score to an average of 3 points was observed, representing a decrease in pathology of nearly 50% as compared to untreated or mock treated control groups.

Due to the culture conditions used, a minor amount of mIL-10 is present in the supernatant of the *LL-mIL10* inoculation suspension. The fate of this residual mIL-10 is most likely acid denaturation and subsequent breakdown in the stomach and duodenum. Treatment of diseased mice with UV-killed *LL-mIL10* completely abrogated curing. This shows that the residual amount of mIL-10 was not responsible for the observed effect and that physiologically active bacteria are required. We demonstrated that recombinant *L. lactis* can actively produce mIL-10 in the colon by using IL-10 knockout mice and measuring the amount of mIL-10, which was delivered *in situ*.

When comparing the *in situ* delivery of mIL-10 with systemic recombinant IL-10, anti IL-12 and dexamethasone treatment - powerful conventional anti-inflammatory methods - all showed to be equally efficient. *LL-mIL10* treatment however required 10,000 fold lower amounts of mIL-10.

Prevention of colitis in IL-10 knockout mice. IL-10 knockout mice spontaneously develop a generalized enterocolitis between three and eight weeks of age (19). Inflammatory changes first appear in the cecum, ascending and transverse colon. When three weeks old mice were treated for four weeks by daily intra-gastric inoculation with *LL-mIL10* the development of colitis could be prevented. The non-treated mice showed a mean histological score of approximately 4.5. Mice treated with *LL-mIL10* showed a mean histological score of 1.5, which is only slightly over values reported for 3 week old mice.

This novel strategy reduces the inflammation in chronic colitis induced with DSS and prevents the onset of colitis in IL-10 knockout mice by localized delivery of IL-10 through in situ secretion by recombinant *L. lactis*. The efficacy of the method is comparable to well-established and accepted therapies relying on the systemic administration of anti-inflammatory proteins. The amount of the therapeutic compound administered could, however, be decreased by approximately four orders of magnitude, which points to a highly efficient mechanism of action. We shall address the discussion on the mechanism(s) of action further in the present contribution.

![Fig. 1. Western blot detection of secreted murine IL-10. One ml of culture supernatant was analyzed. Lane C, *L. lactis* MG1363, containing the empty vector pTREX 1, grown in GM9 medium; lanes 1 to 7, *LL-mIL10* grown in BM9 medium buffered at the indicated pH values; lane 8, *LL-mIL10* grown in GM9.](image-url)

A biologically contained system for *L. lactis*-mediated delivery technology

When applicable in man, this approach may open a vast spectrum of new medical applications. One could speculate that a multitude of protein therapeutics - not necessarily restricted to cytokines - could be delivered...
through *L. lactis*. The spectrum of activities would thus not be limited to intestinal inflammatory disorders but could be as broad as all diseases treatable through mucosal application of protein drugs.

There remains, however, a legitimate concern with regard to the deliberate release of genetically modified (GM) bacteria in the environment. This concern relates to the dissemination of antibiotic selection markers that are present on the expression plasmids, the survival and propagation of the GM organism in the environment and the dissemination of the genetic modification to other microorganisms.

As early as 1954 it was observed that thymine auxotrophs of *Escherichia coli* very rapidly lost colony forming ability upon starvation for thymine (8). The phenomenon was called ‘Thymineless Death’. Thymine dependence differs from most other auxotrophies in that the absence of the essential component is bactericidal rather than bacteriostatic (1). This is caused by the fact that thymine exhaustion triggers activation of the SOS repair system and DNA fragmentation, which is responsible for the rapid and efficient cell death. Thymineless death can thus be considered an indigenous suicide system. We decided to make use of this simple system to develop a biologically contained, IL-10-secreting *L. lactis*. The thymidylate synthase gene, *thyA*, which is essential for the growth of *L. lactis*, was replaced through double crossover with the expression cassette for human interleukin-10 (hIL-10) (49).

Cloning of the *thyA* locus of *L. lactis* MG1363: The nucleotide sequence of the *thyA* gene from *L. lactis* MG1363 has been determined (41). Because the published sequences flanking the *thyA* gene are too short to allow for efficient crossover but also to avoid problems arising from minor sequence differences between different sources of *L. lactis* MG1363 we cloned the entire *thyA* locus of the strain that is routinely used in our laboratory. Comparison of the MG1363 *thyA* locus to the *thyA* locus of *L. lactis* IL1403 showed that both *thyA* genes share 88% homology (4). The sequences upstream of *thyA*, however, are completely unrelated and a 1276-bp sequence downstream from MG1363 *thyA* is absent in IL1403. The sequence further downstream, which is part of the putative *rma1* gene, showed 86% homology. It should be recalled in this respect that strain MG1363 belongs to the subspecies *cremoris* whereas IL1403 is an *L. lactis* subsp. *lactis* strain.

Chromosomal replacement of *thyA* with the hIL-10 coding region: Plasmids with conditional replication were used for targeted gene exchange by double homologous crossover (Fig. 2). Starting from pORI19 we built plasmids in which 1 kb upstream and 1 kb downstream of *thyA* flank different types of hIL-10 expression cassettes. As their replication is dependent on *in trans* supply of RepA protein, the plasmid constructions were transformed into *L. lactis* LL108 (repA<sup>+</sup>) for structural analysis. In a next step *L. lactis* MG1363 containing the helper plasmid pVE6007 (temperature sensitive RepA protein, Cm resistance) was transformed with these constructs. The helper plasmid was subsequently diluted out by a temperature shift from 30°C to 37°C, while continuous selection for erythromycin resistance detected transformants in which chromosomal integration of the plasmid had occurred by homologous recombination at either one of the *thyA* flanking regions. Potentially interesting Cm<sup>+</sup>, Em<sup>+</sup> colonies were detected by PCR screening. Colonies that gave an amplified product of the appropriate size were selected and grown for many generations in GM17 broth supplemented with 50 μg thymidine/ml. When at least 90% of all bacteria had lost the Em resistance marker, candidate colonies for double homologous recombination were identified by PCR analysis. Finally, further PCR screening and Southern blotting identified those colonies that had lost *thyA* but contained the hIL-10 sequence inserted between the *thyA* flanking regions. Following DNA sequencing and assessing production capacity for hIL-10 of several candidates, we focused on a strain designated LL-Thy12.

Strain LL-Thy12 has the hIL-10 coding sequence fused to the usp45 secretion leader, preceded by the ribosome-binding site of *thyA* under transcriptional control of the *thyA* promoter. Also, the construction was such that the downstream fusion was established exactly at the respective stop codons of hIL-10 and *thyA*. In LL-Thy12 the sequence of the hIL-10 coding region is a synthetic one, assembled from oligonucleotides (52) and adapted to the preferred codon usage in *L. lactis* (10). In addition the proline residue - the first amino acid of the mature protein in native human IL-10 - was replaced by an alanine residue. This resulted in a marked increase in the amount of secreted hIL-10. It seems probable that a proline residue at the -1 position of the signal peptidase cleavage site interferes with efficient processing of the precursor polypeptide. Not unexpectedly, however, the amount of hIL-10 secreted by LL-Thy12 was about 10-fold lower than that of a plasmid borne hIL-10 producer and most likely merely reflects a ‘gene dosage’ effect. N-terminal protein sequencing of the secreted protein showed correct processing. The recombinant protein displayed full biological activity in a hIL-10 bioassay (58).
In vitro and in vivo viability of thyA deficient strains:

In order to ascertain the dependence of LL-Thy12 on the presence of exogenous thymidine or thymine, single colonies were inoculated in thymidine-depleted M17 medium (TFM) containing 0.5% glucose. In the absence of thymidine the number of colony forming units (cfu) of LL-Thy12 decreased over six orders of magnitude after approximately 60 hours of incubation; beyond 72 hours no viable cells could be detected. In low thymidine concentration (0.4 μM), an initial slight increase in cfu was immediately followed by a similarly rapid decrease as in the absence of thymidine. A high concentration of thymidine (10 μM) supported growth of the culture till saturation, immediately followed by a steep decrease in viability. No viability could be detected in cultures initiated with low or high concentrations of thymidine after 175 and 250 hours respectively.

We also determined the viability of LL-Thy12 in vivo. We chose pigs as experimental animal because the porcine digestive system is very similar, both in physiology and in size, to that of man. HPLC analysis on total extracts of porcine ileum indicated a thymidine concentration of approximately 1 μM. We introduced lyophilized cells of either LL-Thy12 or MG1363, incorporated in gelatin capsules coated with an enteric coating designed for release of their bacterial content in the ileum, trough a fistula accessing the proximal duodenum. A second fistula was created to sample the ileal content. Samples were taken from both the ileal content and from the feces. Comparison of the bacterial cfu counts showed that, despite the presence of low concentrations of thymidine, the viability of LL-Thy12 decreased approximately 20 fold more rapidly after intestinal passage than that of the parental MG1363 strain.

We believe that the properties of LL-Thy12 satisfy biosafety concerns in a most adequate way at several levels. The strain is devoid of plasmids and does not depend on antibiotic selection markers for stable propagation. The transgene is stably incorporated in the bacterial chromosome, as a consequence of which lateral dissemination is reduced to a very low probability. In this context it is noteworthy that a number of mechanisms for lateral gene transfer are disabled in LL-Thy12. Indeed, the parental strain L. lactis subsp. cremoris MG1363 lacks a host factor required for conjugative transposition (7). Also, phage replication is severely impaired in thyA-deficient L. lactis, thereby disabling phage mediated transduction of host genetic material (34).

In summary, this method provides a biologically contained bacterium that is strictly thymidine or thymine dependent. As thymine is scarce in the environment, these organisms will not survive long after passage through the GI tract. The method should be widely applicable not only to chromosomal insertion of genes other than IL-10 in L. lactis but in a broader sense most likely also be functional in other bacterial species that exhibit the ‘thymineless death’ suicidal phenotype.

Interleukin-10 secreting L. lactis for the treatment of Crohn’s disease

Based on the above-described characteristics of LL-
minor adverse events were being reported with flatulence. Treatment with the bacterium was safe as only determined the number of extracted bacterial DNA from stool samples and being the most prominent temporary side effect. We therapy no such DNA sequences were detected. Taking synthetic equivalents by quantitative PCR (Q-PCR) of the lyophilised immunosuppressive drugs disease chemical contamination or during relapses of Crohn’s epithelial barrier as a result of bacterial infection, Beneficial effects of Trefoil factors in acute colitis therapy no such DNA sequences were detected. Taking into account the detection limit of the method, this means that less then 10^4 cells per gram stool were present. We confirmed that growth of these LL-Thy12 isolates was strictly dependent on the addition of thymidine and that IL-10 production was exclusively associated with the LL-Thy12 genotype. Finally, after one week of treatment patients showed a decrease in CDAI of an average of 71.7 and a prominent reduction in serum levels of C-reactive protein. Clinical benefit was observed in 8 of 10 patients: five patients went into complete clinical remission and three patients showed a clinical response.

We conclude from these limited data that the use of genetically modified bacteria for mucosal delivery of proteins would emerge as a viable strategy in humans. The biological containment strategy was effective and the results obtained suggest a clinical effectiveness of LL-Thy12 (6).

Beneficial effects of Trefoil factors in acute colitis

Acute colitis is caused by a breakdown of the intestinal epithelial barrier as a result of bacterial infection, chemical contamination or during relapses of Crohn’s disease (5).

Therapy in acute colitis is restricted to antibiotics and immunosuppressive drugs (3).

Trefoil factors (TFF) are promising tools for treatment of acute colitis because of their important roles in the protection and repair of the intestinal epithelium (32, 33). The mammalian TFF family consists of three small peptides that have either one copy (TFF1 and TFF3) or two copies (TFF2) of the so-called P-domain, a triple loop (trefoil) structure created by three intrachain cysteine bonds (43). TFF have dense structures that make them extremely protease-resistant. In normal conditions, TFF are expressed in the gastrointestinal tract in a tissue-specific manner. In humans, TFF1 and TFF2 are expressed in mucus-producing cells in the stomach and duodenum, whereas TFF3 is expressed primarily in goblet cells in the small and large intestines. In the case of gastric ulceration (38, 72) or inflammatory bowel disease (37) there is coordinated, highly up-regulated expression of all three TFF. This suggests that trefoil peptides may have a damage repair function in the gastrointestinal tract, acting as naturally occurring curative factors (35). The importance of TFF for normal mucosal function has also been investigated by disruption of TFF1 and TFF3 genes (22, 26). TFF3 knockout mice have impaired mucosal healing and die from extensive colitis after oral administration of DSS, a situation that could be circumvented by luminal administration of recombinant TFF3. Although several studies have documented a protective or healing effect of trefoil peptides in models of gastric ulceration, colitis (63) and necrotizing enterocolitis (73), to our knowledge there are no reports on development of a trefoil factor-based, orally administered drug for colitis. The main reason for this is that orally administered TFF stick to the mucus of the small bowel and are removed from the lumen at the caecum (36).

We evaluated the protective and curative effects of orally administered L. lactis-mediated secretion of murine TFF1, TFF2 or TFF3 in the DSS model for acute colitis, a model that reproduces many of the histopathological and clinical features of acute colitis in humans (65).

In vitro and in vivo secretion of TFF by L. lactis: The DNA sequences encoding mature mTFF2 and mTFF3 were retrieved from GenBank (Acc. Nos. X51697 and D38410) (60, 61). We designed tff-DNA sequences with optimal L. lactis codon usage, and synthesized them by PCR assembly of 40mer oligonucleotides (52). The cDNA of the mtff1 gene and the synthetic genes mtff2 and mtff3, extended at their 5’ ends with a sequence encoding the Myc-tag, were fused to the usp45 secretion signal, downstream of the lactococcal P1 promoter. MG1363 strains transformed with plasmids carrying mtff1, mtff2 or mtff3 were designated LL-mTFF1, LL-mTFF2 and LL-mTFF3, respectively. LL-pTREX1, which is MG1363 containing the empty vector pTREX1 (70), served as control.

Synthesis of mTFF was evaluated by Western blot and
ELISA (Fig. 3). After 24 hours of growth, mTFF was found in the culture supernatant at concentrations ranging form 531 ± 92 ng/mL (LL-mTFF2) to 312 ± 68 ng/mL (LL-mTFF3). The constitutive mTFF secretion did not alter L. lactis growth rate. All three mTFF proteins had correct amino-termini as detected by automated Edman degradation.

L. lactis-derived mTFF showed full bioactivity in an in vitro wound-healing assay in that they enhanced CMT-93 cell monolayer reconstitution to the same extent as did purified mTFF1 produced by recombinant Pichia pastoris.

In order to measure the in vivo secretion of TFF ten inocula of 2 × 10⁹ cfu of LL-mTFF1, LL-mTFF2 or LL-mTFF3 were administered intragastric with 30 minutes interval to female BALB/c mice. We detected 8.7 ng (mTFF1), 10.5 ng (mTFF2) and 7.5 ng (mTFF3) L. lactis derived, Myc tagged TFF in the colon homogenates obtained one hour after the last inoculation. The average cfu value in the colon was 5 × 10⁸ (Table 2).

L. lactis mediated TFF-secretion protects against DSS-induced acute colitis: Acute colitis was induced in mice by administration of 5% DSS in the drinking water. This caused loose bloody stools, progressive weight loss, a significantly shortened colon, and prominent epithelial damage characterized by goblet cell and crypt depletion. Thickening of the mucosa was caused by a severe inflammatory infiltrate, consisting mainly of granulocytes, which led to increased myeloperoxidase (MPO) activity in colon biopsies.

During DSS administration, groups of mice received daily inocula of 2 × 10⁹ cfu of LL-mTFF1, LL-mTFF2, LL-mTFF3 or LL-pTREX1 (empty vector control). In two additional groups we administered UV-killed LL-mTFF1 or L. lactis, secreting interleukin-10 (LL-mIL10).

Continuous DSS administration caused high mortality in all groups except for the ones treated with the various live LL-mTFF cultures, in which mortality was impressively reduced. A similar positive effect, attributable to live LL-mTFF cultures, was observed for the others parameters of acute colitis, i.e. reduction of weight loss and a significant lowering of the levels of 

Table 2. In vivo detection of administered L. lactis cells and their secreted products

<table>
<thead>
<tr>
<th></th>
<th>PBS</th>
<th>LL-pTREX1</th>
<th>LL-mTFF1</th>
<th>LL-mTFF2</th>
<th>LL-mTFF3</th>
</tr>
</thead>
<tbody>
<tr>
<td>cfu</td>
<td>0.20 × 10¹</td>
<td>5.24 × 10⁸</td>
<td>5.72 × 10⁸</td>
<td>4.04 × 10⁸</td>
<td>5.28 × 10⁸</td>
</tr>
<tr>
<td>ng MYC-tagged protein</td>
<td>0.00 ± 0.00</td>
<td>0.02 ± 0.00</td>
<td>8.77 ± 0.32</td>
<td>10.56 ± 0.26</td>
<td>7.51 ± 0.34</td>
</tr>
</tbody>
</table>

ng MYC-tagged protein represents the amount protein per colon ± SEM. PBS, phosphate buffered saline.
MPO, a well-known marker for neutrophil infiltration and acute inflammation in the colon (27).

Histological analysis of the middle colon showed that LL-mTFF treatment reduced epithelial damage and inflammatory infiltrate in acute colitis by 50%. We conclude that daily intragastric inoculation of mice with $2 \times 10^9$ cfu of LL-mTFF1, LL-mTFF2 or LL-mTFF3 had a substantial protective effect against all aspects of acute DSS-induced colitis (Fig. 4).

L. lactis mediated TFF-secretion cures established acute DSS colitis: Acute DSS-colitis was induced during seven days. Concomitant with the return to normal drinking water, treatment with various LL-mTFF was initiated and maintained for 5 days. No mortality was observed during the 5-day period of treatment. There were no significant differences in body weight between the various groups and the controls.

LL-mTFF treatment reduced MPO accumulation in the DSS-induced inflamed colon by 30%. Again this effect was specific for administration of live LL-mTFF1, LL-mTFF2 or LL-mTFF3 and was not observed with either UV-killed LL-mTFF1 or live LL-mIL10. Similarly, histological analysis of the middle colon showed an LL-mTFF specific reduction of epithelial damage and lymphoid infiltration by at least 30%. Treatment with either UV-killed LL-mTFF1 or live LL-mIL10 did not show any histological improvement of the pathology.

We conclude that daily intragastric inoculation of mice with $2 \times 10^9$ cfu of LL-mTFF1, LL-mTFF2 or LL-mTFF3 for five days leads to impressive acceleration of the process of healing established acute colitis.

L. lactis mediated TFF delivery versus administration of purified recombinant TFF in prevention or treatment of acute colitis: As indicated above, an intragastric inoculum of $2 \times 10^9$ cfu LL-mTFF can produce approximately 32 ng TFF1 or TFF3 and up to 40 ng TFF2 in the colon (Table 2). We compared the effects of purified recombinant mTFF1, given either orally or rectally during or after DSS administration.

During DSS administration, daily oral treatment with 5 µg or 50 µg mTFF1 protein did not lower the MPO levels in the inflamed colon nor did it improve the histological score (Fig. 4). Rectal administration of mTFF1 did improve histological scores in a statistically significant and dose-dependent manner (Fig. 4).

Although purified TFF was administered in doses that were up to 1200-fold higher than those expressed in situ by LL-mTFF, improvement following treatment with purified TFF never reached levels comparable to those observed following LL-mTFF treatment. Clearly, delivery of mTFF to the colon epithelia through in situ secretion by L. lactis is considerably more effective for

Fig. 4. Representative histology of the middle colon in the prophylactic LL-mTFF experiment on day 8, representative for healthy control mice (A) and mice with acute DSS colitis either mock-treated (B) or treated with LL-pTREX1 (C), LL-mTFF1 (D), LL-mTFF2 (E), LL-mTFF3 (F), LL-mIL10 (G), UV-killed LL-mTFF1 (H), purified mTFF1 administered orally at 5 µg (I) or 50 µg (J) per day, purified mTFF1 administered rectally at 5 µg (K) or 50 µg per day (L).
treatment and prevention of acute DSS colitis than through administration of the purified protein to the intestinal tract.

Therapeutic effect of TFF-secreting L. lactis on established chronic colitis: We evaluated the effect of LL-mTFF2 on spontaneous chronic enterocolitis in IL-10 knockout mice. Daily administration of $2 \times 10^9$ cfu LL-mTFF2 for a period of 7 or 14 days decreased MPO by 25% and 34% respectively. Also, the histological score of the distal colon was significantly improved (28% and 36% respectively).

To the best of our knowledge this observation is the first indication that trefoil factors may be effective as therapeutics in reducing pathology, not only in acute colitis but also in established chronic colitis.

**DISCUSSION AND THERAPEUTIC OUTLOOK**

We report on the development of a bacteria-mediated delivery technology for biologically active proteins. The method involves oral administration of bacteria, genetically engineered for synthesis of immunomodulatory proteins and topical delivery thereof, preferentially at mucosal surfaces. Our research focuses on the application of genetically engineered Lactic Acid Bacteria (LAB) and aims at obtaining proof of principle for the therapeutic efficacy of the delivery system, particularly in inflammatory bowel disease (IBD).

The bacterial strain, used throughout this, work was *Lactococcus lactis* subsp. cremonis MG1363, transformed with specific expression plasmids. In all constructs the expression cassette consisted of an in frame fusion between the secretion leader sequence of the lactococcal *usp45* gene and the sequence encoding the mature protein of the gene of interest, preceded by the strong, constitutive *P1* promoter.

Mice that are disabled in the synthesis of IL-10 (knockouts) spontaneously develop enterocolitis with aging (19) suggesting an important role for this natural anti-inflammatory cytokine in protection against intestinal inflammation. Another line of evidence derives from the finding that in DSS-induced colitis, next to an increase in TNF and IL-1β, there is also a marked increase of IL-10, whose neutralization abolishes the downregulation of inflammation (62).

IL-10 is known as a major endogenous anti-inflammatory cytokine and can be produced by most of the body’s immune cells (53). It controls and suppresses inflammation essentially by down-regulating pro-inflammatory cytokine production, most likely through its NF-κB blocking activity (45). Apart from its clear downregulatory role on cellular immunity, IL-10 is known as a B-cell growth and differentiation factor, involving class switching to IgA and differentiation of B-cells to antibody producing plasma cells. This is an especially interesting feature in view of the observation that IgA antibodies that react with the commensal microflora provide a primitive, T-cell independent mechanism of tolerance (25). Further, abundant model studies have shown the efficacy of IL-10 for the curing of enterocolitis. This has lead to the knowledge that IL-10 has a very distinct role in the surveillance of the intestinal mucosa, especially for maintenance of tolerance and non-T cell-mediated immunity.

The intestinal epithelial lining is a principal barrier against the uncontrolled influx of luminal antigen in the mucosa. It is therefore not surprising that intrusion of the epithelium is a key aspect in the development of IBD. IL-10 is involved in the maintenance of epithelial barrier integrity as IL-10 gene-deficient mice show an increase in ileal and colonic permeability prior to the development of mucosal inflammation (24). Most experimental models for IBD cannot be established in germ-free animals and IL-10 knockout mice show the appearance of mucosal adherent colon bacteria causative for the development and maintenance of the inflammation (23).

From the above cited properties and actions of IL-10 this cytokine emerged as a prime candidate for topical delivery at the site of intestinal inflammation. We evaluated the effect of *L. lactis*-mediated delivery of mIL-10 on inflammation of the colon in two murine models for IBD, (i) a model in which colitis is induced by chemical treatment and (ii) a model in which colitis develops spontaneously in IL-10 knockout mice (48). The daily ingestion of mIL-10-secreting *L. lactis* prevented the onset of spontaneous colon inflammation in IL-10 knockout mice. Also, treatment of mice, in which DSS-induced chronic colitis had been installed, reduced typical mean histological score of five points to a score of approximately one in 40% of the treated mice, which is a status equal to that of healthy control mice. Most other animals from the treated group only showed minor patchy remnants of the inflammation. Killing of
the IL-10-producing bacteria by UV irradiation immediately prior to inoculation abrogated this curative effect. Therefore it can be attributed to the active in vivo delivery of IL-10. We further documented this by demonstrating in situ de novo synthesis of mIL-10 in the colon of IL-10 knockout mice. The degree of the observed healing is comparable to systemic treatment with prominent anti-inflammatory drugs such as dexamethasone, neutralizing anti-IL-12 antibodies or mIL-10. However, the amount of IL-10 delivered through in situ secretion by L. lactis is estimated to be 10,000 fold lower than the amount of systemically administered IL-10 protein. Reduction of the required amount of therapeutic is a major objective in the search for methods that bear less risk to side effects.

While IL-10 holds promises for the treatment of chronic colitis it is not effective in acute colitis and may even aggravate the inflammation. It would seem that, though apparently contradictory, anti-inflammatory treatment in chronic colitis is not per se indicated in acute colitis (14, 18). Acute colitis is caused by a breakdown of the intestinal epithelial barrier as a result of bacterial infection, chemical contamination or during relapses of Crohn’s disease (5). The inability of the body to sense acute bacterial infection was shown to be a risk factor in the development of Crohn’s disease. Therefore, treatment of acute intestinal inflammation may be a means to prevent inflammatory bowel disease. Trefoil Factors (TFF) are a class of non-mitogenic peptides that play important roles in the protection and repair of the intestinal epithelium (32, 33). This makes them good candidate therapeutics for the treatment of acute colitis. We constructed recombinant L. lactis that produce biologically active murine TFF1, mTFF2 or mTFF3. Following intragastric administration we could demonstrate in situ delivery of the proteins in the colon. Daily administration of the bacteria resulted in significant amelioration of the detrimental effects associated with the DSS-induced acute colitis, such as reduced mortality, gain of body weight and prominent restoration of epithelial integrity. Moreover, the healing process of established acute colitis was significantly accelerated by the treatment (65).

Prostaglandin-endoperoxide synthase (Ptgs2), a known target of TFF signaling (39, 54), has been reported to contribute to the healing and down-regulation of the inflammatory responses in the gastrointestinal tract (9, 29). Ptgs2 is strongly induced in the intestines of mice treated with TFF-secreting L. lactis. This finding proves that the bacteria produced TFF that were biologically active in situ in the colon. Inhibition of Ptgs2 by meloxicam substantially abrogated the prophylactic effect on acute DSS colitis. This indicates that, although Ptgs2 is probably not the only TFF-induced factor that is involved, the upregulation of Ptgs2 is important in prevention of colitis through L. lactis mediated topical delivery of TFF (65).

Remarkably, L. lactis mediated TFF treatment also ameliorated established chronic colitis in IL-10 knockout mice; a finding that broadens the potential therapeutic application of trefoil factors in IBD. Mice treated with UV-killed bacteria were not protected against DSS-induced acute colitis. This indicates that the protective effect is dependent on in situ secretion of TFF.

Oral administration of high amounts of purified mTFF1 protein did not ameliorate acute colitis. Although rectal administration scored a weak and dose dependent activity, it was far less effective than orally administered TFF-secreting L. lactis, particularly when one takes into account that the amount of TFF was about 1200 times higher in the former than in the latter case. How can the small amount of mTFF secreted by L. lactis provoke such outspoken preventive and therapeutic effects on acute colitis, considering a fortiori that the entire epithelium of the intestine is lined with TFF-containing mucus (56). A possible explanation for this apparent paradox is that the large majority of TFF molecules introduced as soluble protein in the lumen are immediately incorporated in the mucus and so become metabolically inert upon their interaction with the VWFC cysteine-rich domains of the abundantly present mucins. We hypothesize that intimate basolateral contact between colonocytes and L. lactis cells - as could occur following L. lactis transport by M cells (allowed by both bacterial size and shape) or through ruptures in the epithelium - would enable TFF to accumulate out of reach of complexing mucins and allow them to interact with the putative basolateral TFF receptors on enterocytes (57).

In the above paragraphs we have detailed several embodiments of the Lactococcus lactis mediated delivery technology to direct potentially therapeutic proteins uniquely at the desired target site, i.e. the gastrointestinal tract. The examples include the cytokine, IL-10 and the three trefoil factors, which have proven curative effects in murine models for IBD. Our ultimate goal, of course, is to apply this proof of principle approach for treatment of gastrointestinal disorders in humans.

However, the use in the clinic raises a legitimate concern about the deliberate release of genetically modified (GM) bacteria in the environment. The strategy
we chose was to insert the therapeutic gene in place of an essential bacterial gene thus creating a conditionally lethal strain. We replaced the coding region of the thymidylate synthase gene (thyA), essential for DNA replication, with the coding region of human IL-10. In the absence of thymine, the resulting bacteria not only fail to grow but also are in fact suicidal because of active DNA fragmentation trough a process known as ‘thymineless death’. As thymine is scarce in the environment outside the human body, the engineered bacteria will not survive long after passage. The strain does not contain plasmids or antibiotic selection markers and the transgene is stably incorporated in the bacterial chromosome, as a consequence of which lateral dissemination is reduced to a very low probability. Because of the thyA deletion the strain also cannot spontaneously revert to a thyA+ phenotype. In the unlikely event that the bacteria were to acquire thyA DNA from another source, restoration of the thyA+ phenotype would require homologous recombination with simultaneous removal of the hiIL-10 gene. Such a recombinant would in fact no longer be a GM organism.

In a pilot clinical trial we administered this strain, in enteric-coated capsules containing freeze-dried bacteria, to Crohn’s disease patients. We found treatment to be safe for the patients with only minor temporary side effects being observed. The biological containment strategy was effective and the results obtained suggest a clinical effect. We conclude from these limited data that the use of GM L. lactis for mucosal delivery of proteins is a feasible strategy in humans. This novel strategy avoids systemic side effects and is therefore suitable as maintenance treatment for chronic intestinal disease.

A major fundamental aspect of the L. lactis mediated topical delivery of therapeutic proteins concerns the mechanism of action. In fact, whereas we collected experimental evidence that allows us to understand in some detail the downstream mode of action of the delivered proteins, we cannot but speculate on the primary role and mechanism of action of the bacteria themselves. One unequivocal prerequisite for the system to work is the necessity for delivering live bacteria to the target site. Indeed, all biological effects, e.g. curing of inflammation in colitis, observed with either IL-10 or TFF are completely abrogated when UV-killed bacteria are administered. Therefore, it seems fair to conclude that the protective effects are dependent on in situ secretion of the therapeutic protein. Another common feature relates to the very small amounts of active compounds, IL-10 or TFF, actually delivered in the colon, yet apparently equally effective as the much larger amounts that are required when administered systemically. One can envisage two major routes for the therapeutic protein to reach its effector tissue: transport of the secreted soluble protein or transport of the bacteria themselves through the epithelium. The lactococci may indeed secrete the protein in the lumen and the protein may travel towards its responsive cells in the epithelium or the lamina propria. The major route of uptake for dissolved proteinaceous material occurs by pinocytosis through the epithelial cells, with the possible consequence of intracellular breakdown. If there were receptor-mediated binding, then transport of macromolecules more likely would result in intact translocation through the epithelial cells. The problems of soluble protein transport are particularly well illustrated when considering the case of TFF delivery. It is difficult to imagine how the small amount of mTFF secreted by L. lactis can provoke such outspoken preventive and therapeutic effects on acute colitis, considering that the entire epithelium of the intestine is lined with TFF-containing mucus. Alternatively, the lactococci may be taken up in the mucosa and the major part of the effect may be scored through in situ production in localized or more downstream lymphoid tissue. Particulate material can only be taken up from the lumen through the M cells. The size and shape of lactococci are compatible with this type of transport. Consequently, a particulate delivery system may prove more efficient in the delivery of a given quantity of recombinant protein at the lamina propria as it will circumvent breakdown in the epithelium. Again, the TFF case lends support to the hypothesis of transport or transit of intact bacteria. Intimate basolateral contact between colonocytes and TFF-secreting L. lactis cells - as could occur following L. lactis transport by M cells or through ruptures in the epithelium - would enable TFF to accumulate out of reach of complexing mucins and allow them to interact with the putative basolateral TFF receptors on enterocytes. It should be noted that the integrity of the intestinal lining can be severely impaired during inflammation. Increased paracellular transport may thus provide an additional alternative route of transit, applicable to the soluble protein as well as to the protein-secreting L. lactis cells.

In conclusion, we have presented evidence that the L. lactis mediated topical delivery of therapeutic proteins, illustrated by in situ secretion of IL-10 and trefoil factors, may hold great promise for clinical application in humans, particularly for the treatment of gastrointestinal disorders and more specifically for IBD. A most interesting observation is that each of the named
compounds has its specific and mutually complementary niche of action.

Due to the chronic nature of IBD, life long intake of anti-inflammatory drugs is very often the fate of patients. Therefore it is desirable that the pharmaceuticals should be low risk for side effects, easy to take in and preferably inexpensive. Especially in view of the decrease in incidence age, the necessity to develop "mild" therapeutics is high. Lactococcal mediated delivery has by its very nature the desired properties for sustained presence of the drug at localized concentrations higher than achievable or even desirable, with regard to latent side effects, when given through systemic delivery. Since L. lactis is a non-colonising microorganism, accurate dosage and timing during treatment can easily be obtained. This method is effective via the oral route, which is far most desirable for pharmacological formulations. Moreover, lactococci can be cultivated on cheap growth media so the method is intrinsically very cost effective.

One could speculate that a vast multitude of protein therapeutics - not necessarily restricted to cytokines – could be delivered through L. lactis. The spectrum of activities would therefore not need to be limited to intestinal inflammatory disorders but could be as broad as all diseases treatable through mucosal application of protein drugs.

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