Genetically engineered probiotics

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Probiotic micro-organisms have been used for many years. Originating as food supplements, they are now most often administered orally and offer an attractive alternative for treating of intestinal disorders. A better understanding of the mechanisms by which these micro-organisms act has now opened up possibilities for designing new probiotic strains. Through genetic engineering, it is possible not only to strengthen the effects of existing strains, but also to create completely new probiotics. These need not necessarily be composed only of bacterial products but can also include elements of regulatory systems or enzymes derived from a foreign—human—source. If designed carefully and with absolute attention to biological safety in its broadest sense, the development of genetically modified probiotics has the potential to revolutionize alimentary health.

Key words: genetic engineering; mechanisms of probiotics; Lactobacillus; Lactococcus lactis; in situ delivery; heterologous gene expression; biological containment.

INTRODUCTION

Conducting reliable double-blind experiments allows researchers to establish a sound knowledge of the effects of probiotic strains. Speculations on mechanisms of action have been developed and tested through detailed microbiological, molecular biological, immunological and physiological analyses. In this chapter, genetically engineered probiotics will be addressed. The title is of dual connotation: the genetic engineering of a previously non-probiotic to acquire probiotic properties and the genetic engineering of a known probiotic to enhance its probiotic properties, both leading to the creation of new, genetically manipulated (GM) probiotics. A knowledge of the mechanism by which defined probiotic strains contribute to health allows the rational design of probiotic micro-organisms through genetic engineering. Existing mechanisms can be strengthened, or mechanistic pathways present in different strains can be combined, to yield more potent strains. From the existing literature, it is clear that completely new concepts can be addressed so one can now create additional mechanisms to enhance probiotic activity.
IS THERE A GENUINE NEED FOR GM PROBIOTICS?

Probiotics used in the treatment of inflammatory bowel disease (IBD) occupy a special place. Not only does oral intake provide excellent targeting of the active biological agent to the affected site, but the use of probiotic therapies in IBD is also especially tempting because of the key role of the intestinal microbiota in the onset and maintenance of mucosal inflammation. Because of the central position of the intestinal microflora in the development of IBD, manipulating this component provides as intriguing new therapeutic approach.

In this context, a number of examples can be cited. Escherichia coli Nissle 1917 and the combined probiotic preparation VSL#3 are, for example used in remission maintenance therapy for ulcerative colitis. The VSL#3 preparation is also effective in preventing flare-ups of chronic pouchitis. Lactobacillus plantarum decreases pain and flatulence in patients with irritable bowel syndrome, and Lactobacillus GG improves the clinical status of children with stable Crohn’s disease. In addition, Bifidobacterium bifidum and Streptococcus thermophilus can reduce the incidence of acute diarrhoea and rotavirus shedding in infants. Saccharomyces boulardii, a non-pathogenic yeast, can be used in the maintenance treatment of Crohn’s disease when combined with mesalamine and shows activity in the treatment of various diarrhoeal diseases. Lactobacillus GG significantly improved dermatitis in infants with atopic eczema and cow’s milk allergy. The uptake of the probiotic was further associated with a drop in fecal tumour necrosis factor and α1-antitrypsin. From this, it can be appreciated that, although very promising, probiotic therapies for the treatment of IBD currently appear to be effective only in therapy to maintain remission.

To allow more active intervention in so-called ‘critical care’ patients, i.e. patients in need of acute immunosuppressive intervention, more powerful probiotics need to be designed and constructed with the aid of genetic engineering tools. Such new strains might utilize mechanisms similar to those used by standard non-engineered strains, but the way in which they are designed may allow for a stronger impact.

NEW PROBIOTIC MECHANISMS ENGENDERED THROUGH GENETIC ENGINEERING

In order to create novel GM probiotic strains, it is essential to understand the different possible mechanisms of action. The effects of probiotics in IBD are manifold and are discussed in detail in other chapters. They involve most of the interactions between the host and its commensal microflora. Some strains alter the microbial content and displace noxious bacteria and toxic compounds. Others, however, show a direct influence on immune cells, thereby mainly lowering the immune response and enhancing tolerance, or increasing epithelial barrier integrity.

In the recent literature, a number of studies have been reported that utilize a diverse combination of tools to design probiotics. Heterologous protein expression is an obvious approach. In this, one can make use of expression systems from native cDNA clones as well as of elaborate products of modern DNA technology such as synthetic genes and designed proteins. One can also expand the range of possible active components beyond protein therapeutics by engineering the metabolism of microorganisms through the integration of foreign enzymes. This so-called metabolic engineering now allows the use of, for example, modified lipopolysaccharide and detoxification via the incorporation of new metabolic pathways.
Sequestration of toxins

Paton co-workers\(^{10}\) have constructed an \textit{E. coli} strain that could answer the need for therapeutics to counteract intestinal infection with Shiga toxigenic \textit{E. coli} (STEC) and other Shiga toxin (Stx)-producing bacteria such as \textit{Shigella dysenteriae}, the causative agent of bacillary dysentery. Stx is a compound toxin, composed of a catalytic A subunit, the actual toxin and a pentameric B subunit that is responsible for binding to its receptor, globotriaosyl ceramide (Gb\(_3\), or Gal\(\alpha_1[1\rightarrow4]\)Gal\(\beta_1[1\rightarrow4]\)Glc-ceramide, found in all human pathogens) or globotetraosyl ceramide depending on the Stx type. Infection and pathology are initiated by a colonization of the gut with STEC that produces Stx locally. The toxin is then taken up in the circulation and concentrated at Gb3-containing tissues such as the kidneys, possibly causing—among other effects—fatal renal failure.

STEC infection can now be rapidly diagnosed, but therapeutic tools against it are not available. Antibiotic treatment leads to a sudden release of surface-associated Stx from the pathogen, which only aggravates the situation. By introducing the genes \textit{lgtC} and \textit{lgtE} from \textit{Neisseria meningitidis} and \textit{N. gonorrhoeae}, respectively, encoding glycosyl transferases that account for the incorporation of the oligosaccharide structure Gal\(\alpha[1\rightarrow4]\)Gal\(\beta[1\rightarrow4]\)Glc into \textit{E. coli}, a strain with an altered LPS structure was obtained. This strain displayed high binding activity for Stx. When administered twice daily to mice infected with STEC, the new strain provided complete protection, the mice in the control population all dying. This research thus provides a recombinant probiotic that has now acquired the potential to sequester an extremely toxic compound. It fills a gap in medicine that has not been met by chemically engineered therapeutics as the authors have demonstrated the superior capacity of their strain over Gal\(\alpha[1\rightarrow4]\)Gal\(\beta[1\rightarrow4]\)Glc covalently linked to silica particles. This is a elegant example of how active components engineered into existing strains need not be limited to polypeptides.

Replacement therapy

A subset of probiotic therapies can be grouped as ‘replacement therapies’, representing an approach whereby a noxious micro-organism is replaced in its ecological niche by a more potent but harmless competitor. Replacement therapy is an innovative approach to preventing microbial diseases. Its origin lies in the complex web of positive and negative interactions that exist between different species of bacteria within the same ecosystem. Some of these therapeutic approaches have been described above when discussing the mechanisms underlying probiotics.

The great potential of genetic engineering to produce such desirable potent yet harmless strains is most elegantly documented in a study conducted by Hillman. Dental caries is caused by \textit{Streptococcus mutans}\(^{11}\), according to the acidogenic theory by the production of lactic acid. Therefore, the \textit{Streptococcus mutans ldh} gene, encoding lactate dehydrogenase, can be considered to be a pathogenic factor. The closely related lactate dehydrogenase-deficient \textit{Streptococcus rattus} is non-cariogenic in gnotobiotic rats\(^{12}\); Hillman therefore cloned the \textit{Streptococcus mutans ldh} gene.\(^{13}\) It was, however, shown that, because \textit{ldh} is an essential gene in \textit{Streptococcus mutans}, only temperature-sensitive lactate dehydrogenase-deficient strains could be obtained.\(^{14}\) Aspects of glucose metabolism appeared to be toxic for growth in non-permissive conditions, but this could be overcome by supplemental alcohol dehydrogenase activity.\(^{15}\) A targeted replacement of the \textit{Streptococcus mutans ldh} gene in strain JHI140 for the \textit{Zymomonas mobilis adh} gene yielded the recombinant \textit{Streptococcus mutans} BCS3-L1, a viable strain.
that makes no detectable lactic acid. The mutant therefore typically produces a higher pH after fermentation of various sugars than its parent. Consequently, BCS3-L1 has dramatically lower cariogenic potential (Figure 1).

Many studies have shown that it is very difficult to displace indigenous \textit{Streptococcus mutans} with laboratory strains. Hillman, however, selected a natural strain that produced a potent lantibiotic bacteriocin, mutacin 1140, which was capable of aggressively displacing indigenous \textit{Streptococcus mutans} and colonizing the oral cavity for prolonged periods. \cite{Hillman1, Hillman2} It was this strain, JH1140, that was used for the gene exchange described above. Trials on the effect of this new probiotic for the prevention of dental caries in humans will be started in 2003 (J. Hillman, pers. comm. 2002). The prevention of dental caries would be an example of biofilm engineering that offered the potential for a highly efficient, cost-effective addition to existing prevention strategies. The use of this approach might also be applied to the prevention of other bacterial diseases.

\textbf{Strategies that involve antibody production}

Neutralizing antibodies that are directed towards a pathogen, toxin, cytokine or other agent have proved very valuable and specific tools in medicine. With the emergence of single chain (ScFv) antibody technology, it has now become possible to produce neutralizing antibodies from recombinant bacteria. Most of the work in this area relates to the production per se of the antibody for downstream processing and use as a purified protein. Owing to their structure, these peptides suffer from very short half-lives in vivo so suitable delivery systems are required to allow their use as therapeutics. A number of applications are now emerging in which the expressor strain itself is used for the in situ production of the antibody fragment especially to control colonization by pathogens.
Next to the earlier discussed replacement therapy, a second strategy to intervene with *Streptococcus mutans* has been presented by Kruger et al. It had been observed that the oral administration of antibodies against *Streptococcus mutans* reduced the incidence of caries. Along the same lines, antibodies that recognize the SAI/II adhesion molecule of *Streptococcus mutans*, so called Guy’s 13 antibodies, are effective by agglutinating the pathogen. *Lb. zeae* have therefore been engineered either to secrete or to surface expose Guy’s 13 ScFv. All ScFv showed the appropriate reactivity with SAI/II adhesin. *Lb. zeae* with surface bound ScFv coagulated with *Streptococcus mutans* suspensions. The oral inoculation of rats with the surface expresser at 2 day intervals resulted in a marked decrease in the *Streptococcus mutans* count and the concomitant development of dental caries.

An even more elaborate approach has been used to combat *Candida albicans*, one of the most frequent causative agents of mucosal inflammation in humans. Infections are seen in the mouth and oesophagus of immunocompromised persons such as HIV-infected subjects. *Candida albicans* also causes acute vaginitis in otherwise healthy women. There is a real need for new therapeutic agents in this area because few adequate agents are known, resistance is increasing, and vaccines are not available. Beninati et al. have produced a recombinant *Streptococcus gordonii*, a species with good vaginal colonization and heterologous expression potential in vivo, for the eradication of *C. albicans* infections. Anti-idiotypic ScFv were produced, the surface of which resembled the structure of a wide-spectrum killer toxin of *Pichia anomala*. Two *Streptococcus gordonii* strains were constructed: one that expressed the ScFv at its surface and a second that secreted the ScFv. Similarity in structure to the killer toxin could be shown by cross-reactivity with specific monoclonal antibodies. Both surface-bound and secreted ScFv showed candidacidal activity over a wide concentration range. Both *Streptococcus gordonii* strains successfully colonized the vagina and cleared experimental *C. albicans* infection in rats, this being dependent on the presence of this ScFv. The secretor, however, showed a 7 days’ faster reduction of the pathogenic load, a result comparable with a full course of treatment with the antifungal fluconazole. This work shows that local production of a designer microbicide is a valid approach for the treatment of a very common mucosal pathology.

**Enzyme deficiencies**

Non-GM probiotics can contribute to the health of individuals suffering from enzyme deficiencies when the enzymatic conversion that is required is common to the metabolism of the bacterium. The treatment of lactose maldigestion by various lactobacilli can serve here as an example. Genetic engineering has, however, the potential to expand dramatically the range of enzyme deficiencies that can be addressed.

Drouault et al. have worked along these lines by constructing *Lactococcus lactis* expressing the lipase from *Staphylococcus hylicus*. The aim was to provide a more adequate treatment of pancreatic insufficiency. The treatment of such diseases suffers at present from the poor performance of the pancreatic enzymatic preparations that are available. Drouault et al thus built a *L. lactis* strain that carried the lip gene under the control of the nisin promoter. Upon induction, the lipase accumulated intracellularly up to a level of 15% of the total protein. The pancreatic duct of pigs was then ligated and cut to simulate disease. This resulted in an approximately 20% decrease in fat absorption (nearly 100% in healthy compared with approximately 80% in the resected animals), as determined from the ratio between ingested and excreted fats. Fat absorption was 10% higher in those animals treated with the lipase expressor strain.
In this study, use was made of the release of intracellular material from *L. lactis* after it had been killed in the upper part of the small bowel.32

**Detoxification**

Over the past century, human activity has caused a rapid alteration in our environment, accompanied by the often-unwanted accumulation of numerous chemical agents. As intrinsic inhabitants of this changing environment, we are obliged to face these changes. This means that humans have become confronted, on a totally different time scale from that of biological evolution, with a varying spectrum of new and difficult to handle residues of toxic compounds. We are therefore often not (yet) equipped with adequate catabolic enzymes to allow for their conversion and elimination. More specifically, such compounds are taken up via nutrition, respiration and skin contact, whereby some may accumulate, mostly as a consequence of their lipophilic nature. Through the genetic engineering of micro-organisms, one can now build systems for in situ detoxification or ‘biodetoxification’. In this way, exotic—either discovered or designed—enzymes that transform noxious chemicals into harmless ones could be applied in human health-care.

An in road in to this area has now been made by adapting existing detoxification systems so that they can be used in a more potent way. Xenobiotics—chemical compounds (drugs, pesticides or carcinogens) that are foreign to living organisms—are detoxified in a two-stage process. This occurs through phase I and II xenobiotic-metabolism enzymes present in the cells of the liver, intestine, kidneys and lung. Phase I XMEs mainly P450 cytochromes, convert the xenobiotics into more reactive compounds that are further metabolized by phase II xenobiotic-metabolism enzymes such as glutathione-S-transferase, N-acetyltransferase and aldehyde dehydrogenase.

Blanquet et al33 have chosen to use genetically engineered *Saccharomyces cerevisiae* that produces the plant cytochrome P450 73A1 (cinnamate-4-hydroxylase) and overexpresses yeast NADPH cytochrome P450 reductase.34 This recombinant strain converts trans-cinnamic acid into *p*-coumaric acid. To investigate whether such system would allow in vivo biodetoxification using P450 cytochromes, they performed a study using two-stage in vitro intestinal simulation of the stomach and small intestine (TIM 135) and large intestine (TIM 223). The recombinant yeast appeared to be very resistant to the conditions in TIM 1 but more sensitive to TIM 2. In both compartments, the conversion of trans-cinnamic acid into *p*-coumaric acid, which accumulated to 41% in TIM1, could be observed.

This shows that, using this technology, a plant P450 cytochrome, i.e. an enzyme of a widely disparate evolutionary origin, might be used in a human intestinal environment for the detoxification of nutrient-borne chemical contamination. Such an approach would intrinsically preclude their accumulation in the cells of the body that normally performed detoxification and thereby avoid the formation of residues. This is therefore an elegant adaptation of existing strategies of detoxification. There are, however, obvious drawbacks to the use of in vitro intestinal simulation machines. The absence of an immune system in particular strongly compromises ad hoc extrapolation to usage in humans.

Metabolic deficiencies resulting from trauma, disease and age may cause the accumulation of waste products that are otherwise removed from the body to levels that are unacceptable for the adequate functioning of the organism. Examples thereof are the accumulation of urea, uric acid or creatine as a result of renal failure, ammonia from liver failure and phenylalanine because of phenylketonuria. Prakash and Chang37 have developed *E. coli* that express *Klebsiella aerogenes* urease. The oral administration
of a formulation of these live bacteria, microencapsulated in alginate–polylysine beads, to rats with experimental renal failure led to a significant and substantial reduction in plasma urea, uric acid and, to a lesser extent, of creatine. In rats, the microcapsules leave the body with the faeces. The microencapsulation shields the recombinant bacteria from the harsh environment in the digestive tract, and it is likely that this physical barrier can also prevent the onset of an immune reaction towards the transgene. The authors estimate that daily administration of 4 g of this formulation could normalize the urea concentration in a 70 kg patient suffering from renal failure.

**Immune intervention**

Immune intervention strategies are those in which the immune system is actively engaged for the acquisition of health benefits. The immune system supervises all the molecules within an individual, its task being to discriminate friend from foe, protecting and maintaining the former and eliminating the latter. Intervention in terms of the immune system may help to establish immunity or readjust inaccurate aspects of immune discrimination. Immunity against previously unencountered pathogens can be achieved through vaccination. A large body of literature exists on the use of lactic acid bacteria (the majority of current probiotics) as vaccine-delivery vehicles (see Ref. 40 for a recent review). This aspect of the use of GM lactic acid bacteria and other microorganisms, falls, however, outside the scope of this article.

The immune system constitutes a complex network of cells that are in constant communication with one another and with the somatic cells of the body. This communication occurs via secreted and diffusible mediators and through cell surface exposed ligands that encounter their cognate receptors on the surface of the target cells. An important class of diffusible mediators is that of relatively small proteins called cytokines, consisting of interleukins (ILs), growth factors, interferons and others. These messengers are often active at a very low concentration and have therefore been used extensively in various attempts to assist the immune system in acquiring immunity or re-establishing homeostasis in cases of malignant inflammation. When, however, they are administered as purified—recombinant—proteins via the systemic route, they diffuse throughout the body and can cause an effect at sites, where these are unwanted, producing side effects. In some cases, such as immune intervention at the mucosa, one could, however, speculate that localized delivery via the in situ synthesis of cytokines by genetically engineered bacteria might prevent side-effects while still allowing the desired biological effect. We have investigated whether genetically engineered \textit{L. lactis} can be used in such a strategy.

Wells et al had reported that, when the C fragment of tetanus toxin (TTFC) was expressed intra-cellularly in \textit{L. lactis}, a protective immunity towards tetanus toxin could be provoked by repeated intra-nasal inoculation of that strain. In general, both IL-2 and IL-6 act as potent stimulators in the onset and maintenance of immune reactions. To investigate whether the immune response to TTFC, responsible for the above-mentioned protection, could be enhanced or modulated via the intra-nasal delivery of cytokines, we constructed strains of \textit{L. lactis} that produced TTFC intracellularly and secreted functional murine IL-2 or IL-6. Immunization was carried out, and the animals were compared with individuals from the control TTFC-expresser strain. The anti-TTFC serum IgG response was significantly (up to 15 fold) higher in mice immunized intra-nasally with the viable \textit{L. lactis} that secreted IL-2 or IL-6. In addition, the concentration of anti-TTFC serum IgA was considerably higher after immunization with the IL-6-secreting strain. This shows that genetically engineered \textit{L. lactis} can produce
IL-2 and IL-6 in the upper airway mucosa and thereby provoke a biological effect that can, because IL-6 is a B-cell growth and IgA secretion-stimulating factor, be steered through the appropriate choice of cytokines.

IL-10 is a strong anti-inflammatory cytokine that has shown promise in clinical trials for the treatment of IBD. The application of this protein suffers, however, from a number of disadvantages that may be overcome through targeted delivery in the intestine. Side-effects are induced following systemic administration, thereby precluding its prolonged use at an elevated concentration. Moreover, IL-10 is extremely acid sensitive, hindering any plans to circumvent the side-effects by targeted delivery via the digestive tract.

IBD can be simulated in a number of mouse models. The repeated addition of dextran sulphate sodium (DSS) to the drinking water of Balb/c mice leads to the induction of chronic colitis. The histology of this condition that of is very similar to ulcerative colitis, but the inflammation immunologically tends towards that of Crohn's disease as it is associated with the upregulation of interferon-gamma, IL-12 and tumour necrosis factor and can be cured by the systemic administration of neutralizing antibodies against these proteins. We constructed \textit{L. lactis} that could secrete bioactive mouse IL-10. The daily ingestion for 2 weeks of these \textit{L. lactis} organisms resulted in 40% of the treated mice reaching a histological score equal to that of healthy control mice. The other animals showed only minor patchy remnants of the inflammation (Figure 2). As killing the IL-10-producing bacteria prior to inoculation abrogated the curative effect, the mechanism of delivery seemed to act via the active in vivo synthesis of IL-10. The observed healing was comparable to systemic treatment with prominent anti-inflammatory drugs (anti-IL12, dexamethasone and IL-10), but the amount of IL-10 required through \textit{L. lactis}-mediated delivery was 10 000-fold lower, which is highly encouraging when the aim is to reduce side-effects.

Our current work focuses on experiments in humans. For this, we have now constructed a biologically contained \textit{L. lactis} strain that can produce human IL-10. This will enable us to investigate whether \textit{L. lactis}-mediated IL-10 delivery has the potential to lead to the development of a new therapeutic for the treatment of IBD in humans.

\textbf{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 major focus attention in the discussion of genetically engineered probiotics is therefore the fact that, when administering of a live recombinant micro-organism to a human, one is in fact deliberately releasing a genetically modified organism into the environment. The design of the new probiotic should therefore be such that safety is guaranteed often its usage. This point relates essentially to the absence of antibiotic selection markers, preventing the of accumulation of the GM micro-organism in the environment and preventing of lateral dissemination of the genetic modification to other bacteria. It is best and most elegant if these concerns can be addressed through a biological system that is propagated along with the host, such
Figure 2. Haematoxylin and eosin-stained histological slides of colonic tissue: representative images of untreated mice (normal), mice in which colitis was induced by the addition of dextran sulphate sodium to the drinking water and mice that underwent the same induction followed by treatment with a mouse IL-10-producing Lactobacillus lactis strain.
systems being termed ‘biological containment’ systems. These systems or combinations thereof should address all of these concerns. As no reports have yet been released on the use of GM probiotics in human or veterinary medicine, the literature on the specific area of the biological containment probiotics is scarce. The strategies that are likely to be adopted will, however, be similar to those used in other disciplines in which proficient use is made of GM organisms.

Biological containment systems can be subdivided in active and passive. The former essentially provide control through the conditional production of a bacterial toxin—via tightly regulated gene expression—which is controlled by an environmentally responsive element. Passive systems render growth dependent on the 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 et al. designed contained GM bacteria that degraded a model pollutant, 3-methylbenzoate, using the *Pseudomonas putida* TOL plasmid for aromatic hydrocarbon metabolism. In *Azoarcus Pseudomonas* putida, a background necessary to enforce the system, fusion with the Pm promoter controls lacI. *Escherichia coli* Gef, a porin-inducing protein, is produced under the control of the Plac 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 a decrease in LacI Gef production and killing of the host. This is an elegant system, but its application is obviously very restricted because of the high degree of integration of the various components. In a streptavidin-based suicide system, streptavidin expression was induced in a similar way by the absence of 3-methyl benzoate, which resulted in a 1000-fold reduction of *Pseudomonas putida* viability within 8 hours.

*Escherichia coli* K12 relA mutants die faster than wild-type organisms. When supplemented with a system in which the alkaline phosphatase gene promoter drives the phage T7 lysozyme gene, the rate of killing still increases further following phosphate depletion. Positioning the *E. coli* alkaline phosphatase gene promoter so that it controls the parB locus of plasmid R1 leads to stable inheritance of the resulting plasmids and death of the carrier strains as a consequence of phosphate starvation. The activity of the *E. coli* rimB 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 are encountered upon its release into the environment.

The toxin in an active biological containment system can also be an endonuclease, counteracted by a methylase, as in the type II EcoRI restriction—modification system. The ecoRIR lethal gene is present on a plasmid, and the ecoRIM gene encoding the cognate EcoRI methylase is situated on the chromosome. Lateral dissemination of the plasmid to the 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 furthermore acquire the methylase from the 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. A combination of the genes for both colicin production and colicin immunity produces a viable strain. When the E3 gene in such a strain is closely linked to a genetic modification, lateral gene transfer of the latter decreases by several orders of magnitude.
A plasmid containment system using the lethal *E. coli* relF gene has been shown to be effective in vitro and also in rat intestine.\(^5\)

**Passive systems for biological containment**

A number of food-grade selection markers have been described, and these can be used to eliminate the need for antibiotic selection markers. Similarly, some of the deletion mutants utilized in such system are essentially dependent for their growth on the presence of a particular compound, which can be used in the development of contained strains.

D-Alanine is an essential component in the biosynthesis of the cell wall of many lactic acid bacteria. Alanine racemase (Alr) catalyses the interconversion of D-alanine and L-alanine, so *L. lactis* and *Lb. plantarum* strains with a detection of alr show auxotrophy for D-alanine.\(^5\) The alr genes of both species have been used as food-grade selection markers to complement for the deletion of alr. Plasmids carrying a heterologous alr mutation were stably inherited in a alr deleted background.\(^6\) The deletion of alr has been used by Hillman et al to contain of the GM *Streptococcus mutans* designed for their anticaries therapy, described above (J. Hillman, pers. Comm., 2002). Fu and Xu\(^6\) have described a similar containment system for *Lb. acidophilus* using the thymidilate synthase gene (*thyA*) from *Lb. casei* as selective marker for plasmid maintenance. In this cases, a foreign gene is used to avoid the reversion of the mutation by back-recombination of the marker gene.

Platteeuw and co-workers\(^6\) used the soluble carrier enzyme IIA Lac, encoded by the 300 bp lacF, as a selection marker in an lacF-deleted *L. lactis*. 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.\(^6\) 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.\(^6\) The integration of expression units into the chromosome of the carrier strain is often highly desirable because the GM character is then stably inherited and lateral gene transfer is reduced strongly when compared with plasmid-borne systems. Frequently, however, the level of expression of the transgene decreases significantly as a consequence of reduced copy number. Leenhouts et al have described a method of obtaining multiple integrations in a way that is compatible with use in humans. The integrated plasmid is devoid of 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*.\(^6\) Intermediate engineering can be carried out *L. lactis* strains that produce the pWV01 RepA protein. Single-crossover integration can be strongly enhanced on medium containing sucrose as the only carbon source.

**CONCLUSIONS**

Although they have been in use for a long time and described for almost a century, we are only now seeing the onset of a medical use for probiotics. Especially in the area of intestinal disorders, in which lifelong treatment is often required, they promise elegant
alternatives to high-impact chemical therapeutics. Mechanisms of action have been gradually described, and reliable clinical studies have demonstrated efficacy. It is conceivable that the creation of new probiotic organisms with the help of genetic engineering will revolutionize this field. With the advent of GM probiotic strategies, mechanistic tools can be applied from foreign sources and combined to make new organisms. Very much like traditional pharmacology extracts and copies specific compounds from nature for their interesting properties, active components, even derived from pathogens, can now be placed in harmless carrier strains through genetic engineering and thus provide a safe vehicle for such elements. Molecular medical research has furnished a large collection of ingenious therapeutic compounds. Their application can, however, be complicated by their sensitivity in vivo and the cost of their production. The use of GM probiotics can circumvent the short half-life and fragility of the therapeutics and produce very cost-effective access to such expensive therapeutics.

It is clear that GM probiotics open up a plethora of applications. It is, however, also clear that the uninhibited spread of GM micro-organisms in the environment is highly undesirable. Researchers working in the field must therefore proactively engage in discussions on the biosafety of the strains they have created. When designing or applying GM probiotics, one should further realize that they are in many ways similar to newly synthesized chemical therapeutic agents and require caution while testing them in vivo. Antigenic reactions especially in particular are a key consideration. If a strain is designed to introduce in a foreign protein, this may well provoke an immune reaction, not only jeopardizing the future use of this medication but also possibly even causing bystander recognition and allergy. It is appreciated that man, as a consequence of lifelong 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 or 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 breath of potential applications and demand caution when considering the clinical use of these systems. This need not, however, be an unsurmountable hurdle as similar problems in other areas have been overcome through the 'humanization' of the foreign proteins.

The GM probiotics that have so far been designed are the first attempts in what has the potential to become a new avenue of pharmacology. Although the goals are clearly ambitious, the problems encountered in real-life application are often immense. But this is also true of any cutting-edge development. The challenge is now to creative molecular biologists and pioneering physicians to conceive a new generation of powerful tools to combat what are among the most malicious of diseases.

**SUMMARY**

Probiotic microorganisms can be effective in IBD therapy via a number of mechanistic pathways. Some strains can displace noxious bacteria by competitive binding. This can also be achieved through death or growth inhibition by the production of antibacterial compounds or a lowering of the pH. Some probiotics have a defined influence on the host immune cells, for example the induction of cytokine production or an increase in local IgA secretion. Some can sequester toxins and remove these from the intestine or counteract their biological effects. Probiotics can influence epithelial and tissue integrity by low-dose nitric oxide synthesis, stimulating mucus production, enhancing
proliferation of the gut epithelium, inhibiting endogenous carcinogen production and providing nutrients by short chain fatty acid production.

The current probiotic treatment of IBD is still, however, limited to the maintenance of remission. To obtain more powerful strains, genetic engineering to adapt current or establish new mechanistic pathways may open up new perspectives. Examples of such GM probiotics include strains that sequester toxins through altered lipopolysaccharide structure or compete with and displace pathogens. Further strategies involve the production of antibodies and antiidiotypic ScFv that resemble a toxin, the production of enzymes for detoxification and the complementation of enzyme deficiencies, and production of cytokines for immune intervention.

The biological safety of GM probiotics must be assured through systems for biological containment that are either active—providing control through the conditional production of a bacterial toxin—or passive—in which growth depends on the complementation of an auxotrophy or other gene defect.

**Practice points**
- some selected probiotics can be used in the remission maintenance of IBD
- no GM probiotics are currently use in medicine

**Research agenda**
- a molecular description of probiotic activity should be provided
- mechanistic pathways need to be applied to create new strains
- the construction of biologically contained GM probiotics is of vital importance
- new strains must be evaluated in animal models
- clinical experimentation will allow an assessment of the efficacy of GM probiotics

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