nature biotechnology

Biological containment of genetically modified *Lactococcus lactis* for intestinal delivery of human interleukin 10

Lothar Steidler[1], Sabine Neirynck[1], Nathalie Huyghebaert[2], Veerle Snoeck[3], An Vermeire[2], Bruno Goddeeris[3], Eric Cox[3], Jean Paul Remon[2] & Erik Remaut[1]

Genetically modified *Lactococcus lactis* secreting interleukin 10 provides a therapeutic approach for inflammatory bowel disease. However, the release of such genetically modified organisms through clinical use raises safety concerns. In an effort to address this problem, we replaced the thymidylate synthase gene *thyA* of *L. lactis* with a synthetic human *IL10* gene. This *thyA*$^-$ *hIL10*$^+$ *L. lactis* strain produced human IL-10 (hIL-10), and when deprived of thymidine or thymine, its viability dropped by several orders of magnitude, essentially preventing its accumulation in the environment. The biological containment system and the bacterium's capacity to secrete hIL-10 were validated *in vivo* in pigs. Our approach is a promising one for transgene containment because, in the unlikely event that the engineered *L. lactis* strain acquired an intact *thyA* gene from a donor such as *L. lactis* subsp. *cremoris*, the transgene would be eliminated from the genome.

Genetically modified food-grade microorganisms provide a possible means for targeting therapeutic proteins to the mucosa. By *in situ* synthesis, genetically modified *Lactococcus lactis* can deliver mouse interleukin 2 (mIL-2) and mIL-6 to the upper airways[1] and mIL-10 to the intestine[2]. *Streptococcus gordonii*[3] and *Lactobacillus zeae*[4] have been used in this way to deliver single-chain antibodies. However, the use of genetically modified organisms in medicine raises legitimate concerns about their survival and propagation in the environment and about the dissemination of antibiotic selection markers or other genetic modifications to other microorganisms.

Delivery of the anti-inflammatory cytokine mIL-10 to the intestine by genetically modified *L. lactis* cures or prevents experimental enterocolitis in mice[2]. The mouse experimental colitis model mimics the severe, chronic intestinal inflammation in humans known as inflammatory bowel disease (IBD). IBD requires lifelong medication, so the development of inexpensive, easily administered therapeutics with minimal side effects is highly desirable. Although IL-10 is a good candidate for IBD therapy[5], the administration of IL-10 by injection induces side effects[6–8] that prohibit its long-term use at high concentrations. This problem cannot be solved by delivery to the intestine, as IL-10 is acid sensitive[9,10]. Localized IL-10 synthesis by genetically modified *L. lactis* may address these shortcomings.

In this study we investigated a strategy for preventing escape into the environment of the transgene and the genetically modified organism. The thymidylate synthase gene, *thyA*, which is essential for the growth of *L. lactis*, was replaced with the expression cassette for *hIL10*. As expected, the resulting strain was dependent on thymidine or thymine for growth and survival. We assessed the resulting genetically modified strains *in vitro* and in pigs, animals whose digestive systems are very similar to those of humans in both physiology and size. Transgene escape through acquisition of an intact *thyA* gene is very unlikely and, in the event it did occur, would recombine the transgene out of the genome, resulting in reversion of the genetically modified organism to the unmodified state.

RESULTS

Gene exchange between *thyA* and *hIL10*

The sequence of the *thyA* gene in *L. lactis* MG1363 is published[11]. We expected, from the literature on bacterial recombination[12], that the known flanking fragments were too short to allow efficient crossover using our genetic modification protocol. We therefore cloned the *thyA* locus with longer flanking sequences. Comparison with the *thyA* locus of *L. lactis* IL1403 (ref. 13) showed that the two *thyA* sequences are 88% identical. The sequences upstream of *thyA* are completely unrelated and a 1,276-bp sequence downstream from MG1363 *thyA* is absent in IL1403. The putative *rma*1 genes further downstream from *thyA* is 86% identical. We confirmed the structure of the MG1363 *thyA* locus by Southern blot analysis (data not shown).

We used conditionally nonreplicative plasmids[14], in which 1 kb of the regions upstream and downstream of MG1363 *thyA* flank a series of *hIL10* expression cassettes, for targeted gene exchange by double homologous crossover (**Fig. 1a**). The structure of the resulting *L. lactis* strains was confirmed by PCR (**Fig. 1b**), Southern blotting (**Fig. 1c**) and DNA sequencing. The recombinant strains were called Thy11, Thy12, Thy15 and Thy16 (**Fig. 2a**) and are here referred to collectively as Thy11–16. Growth of these strains was dependent on the addition of

[1]Department of Molecular Biomedical Research, Vlaams Interuniversitair instituut voor Biotechnologie, Ghent University, KL. Ledeganckstraat 35, B-9000 Ghent, Belgium. [2]Laboratory of Pharmaceutical Technology, Ghent University, Harelbekestraat 72, B-9000 Ghent, Belgium. [3]Laboratory of Veterinary Immunology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium. Correspondence should be addressed to L.S. (l.steidler@ucc.ie).

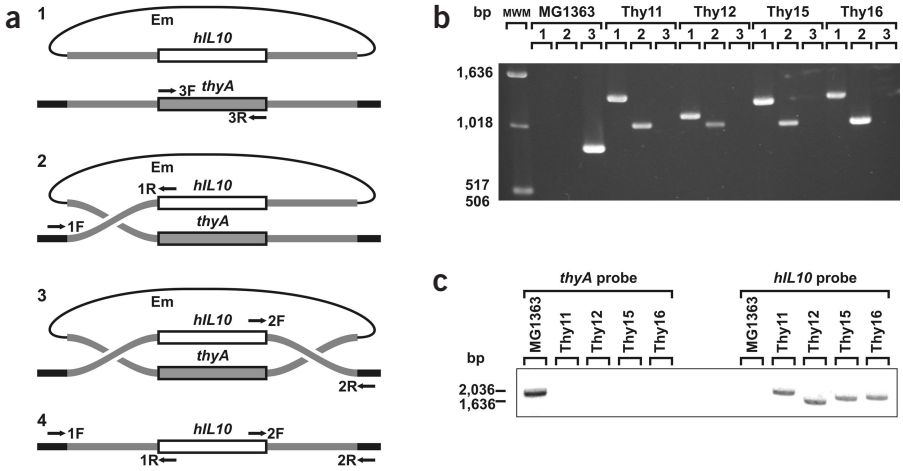

Figure 1 Exchange between *thyA* and *hIL10* genes. (**a**) Overview of the strategy used. Gray lines represent target areas for recombination, thick black lines represent nontarget MG1363 chromosome fragments and thin black lines represent the exchange vector. 1, 2 and 3 represent PCR primer pairs (F and R), designed in such a way that PCR using primer pair 1 shows collinearity between chromosomal DNA located 5′ of the target area and *hIL10* (PCR1), that PCR using primer pair 2 shows collinearity between chromosomal DNA located 3′ of the target area and *hIL10* (PCR2), and that PCR using primer pair 3 shows the presence of *thyA* (PCR3). Stages include (1) introduction of the nonreplicative vector; (2) 5′ crossover, forced by erythromycin selection and identified by PCR1; (3) second crossover in the absence of Em, identified through screening by PCR2; and (4) acquisition of desired transgenic chromosome organization. (**b**) PCR identification of Thy11–16 by three rounds of PCR, 1, 2 and 3, as in **a** (see **Fig. 2a** for strain differences). (**c**) Southern blot analysis of chromosomal DNA of the indicated strains, cut with *Spe*I and *Nde*I and revealed with digoxygenin-labeled probes for identifying *thyA* (*thyA* probe) or *hIL10* (*hIL10* probe). The fragments detected by the *hIL10* probe differ in size because of differences in the promoter region.

thymidine or thymine to the growth medium, with a strong preference for thymidine. Supplementation with 20 μM thymidine allowed the cultures to grow to full saturation, whereas thymine was ~1,000-fold less effective (data not shown).

Expression of *hIL10*

Thy11–16 contain different promoter regions. Thy15 contains the P1 promoter[15] from *hIL10*. Thy12 contains the *thyA* promoter (P*thyA*). Thy 11 and Thy16 contain regions of both promoters; Thy11 includes the *thyA* ATG start codon and Thy16 does not (**Fig. 2a**). In all strains *hIL10* replaces *thyA* exactly down to its stop codon, which was expected to prevent polar effects on downstream genes. The *hIL10* gene is synthetic—codon-optimized for *L. lactis* (see ref. 16) and fused to the *usp45* secretion leader[17]. As replacing proline with alanine at the N terminus of a heterologous protein has been shown to increase secretion[18], we constructed a P2A mutant hIL-10, which indeed displayed markedly greater secretion (**Fig. 2b**). N-terminal protein sequencing of the secreted protein indicated that it was correctly processed, and an hIL-10 bioassay[19] showed that the recombinant protein had full biological activity. Thus, Thy11–Thy16 had acquired the capacity to produce functional hIL-10 (**Fig. 2c,d**; analysis done as in ref. 2).

In vivo production of hIL-10

We tested *in vivo* production of hIL-10 using strain Thy12, which contained the least amount of foreign DNA and showed the highest hIL-10 expression *in vitro* (14 ng/ml, **Fig. 2c**). After $1.44 \times 10^{11} \pm 1.72 \times 10^{10}$ Thy12 bacteria were injected into an ileal loop of a pig and incubated for 4 h, the loop of the pig's intestine contained 4.44×10^{9} colony-forming units (cfu)/ml ± 1.98×10^{8} cfu/ml (mean ± standard deviation (s.d.)) Thy12 and 470.62 pg/ml (± 13.98 pg/ml) hIL-10. The intestinal tissue of the loop contained 4.17×10^{8} cfu/ml (± 1.84×10^{8} cfu/ml) Thy12 and 58.27 pg/ml (± 1.42 pg/ml) hIL-10. Thus, the Thy12 strain produced hIL-10 *in vivo* in pigs and was able to acquire the necessary nutrients from the pig host.

In vitro viability of *thyA*-deficient strains

The survival of *thyA*-deficient strains depends on the presence of thymidine or thymine in the growth medium. To assess Thy12 growth under thymidine limitation, we inoculated 15 single, purified Thy12 colonies in rich thymidine-free medium containing 0.5% (wt/vol) glucose. The suspension was divided in three, and 0 μM, 0.4 μM (low concentration) and 10 μM (high concentration) thymidine were added, respectively. In the absence of thymidine (**Fig. 3a**), the cfu of Thy12 decreased over 6 orders of magnitude in ~60 h. Beyond 72 h of incubation, no viable cells could be detected. At low thymidine concentration (**Fig. 3b**), a slight increase in cfu was immediately followed by a rapid decrease, similar to that seen in the absence of thymidine. The high concentration of thymidine (**Fig. 3c**) supported growth of the culture until saturation, which was immediately followed by a steep decrease in viable bacteria. No viable bacteria could be detected in cultures initiated with low or high concentrations of thymidine after 175 and 250 h, respectively. This decrease was not associated with substantial lysis of bacterial cells, as the culture turbidity remained unchanged. The thymidine dependence of Thy11, Thy15 and Thy16 was identical to that of Thy12.

Thymidine auxotrophs are self-limiting because depletion of the essential component immediately induces cell death. Thymidine auxotrophy is thus different in nature from any auxotrophy that does not affect DNA metabolism. For example, growth of *L. lactis* MG1363 is dependent on the addition of glucose. In contrast to other bacteria, *L. lactis* manages glucose starvation stress—much as it does stress caused by such varied agents as heat, ethanol, osmotic pressure and $CdCl_2$—by induction of the central stress response and survival factor YtgH[20]. To assess the glucose stress response of strain MG1363, we inoculated 15 individual colonies in glucose-free rich growth medium. The suspension was split and 0%, 0.1% (low concentration) or 0.5% (high concentration, wt/vol%) glucose was added, respectively (**Fig. 3**). In contrast to the results of thymidine limitation described above, in the absence of glucose, no change in MG1363 cfu was detected until after 72 h (whereas in the absence of thymidine, all 3.2×10^{6} Thy12 had died by that time point) (**Fig. 3a**). Addition of low or high concentrations of glucose to MG1363 led to increased cfu and stagnation over the course of 28 h, followed by a slow decrease in cfu (**Fig. 3b,c**). The low-glucose culture did not reach the same plateau as the high-glucose culture, demonstrating glucose depletion (**Fig. 3b**). This is in marked contrast with the viability profiles of Thy12 under thymidine limitation, which showed a rapid decrease in cfu. The cfu of strain MG1363 under glucose limitation never decreased by more than 4 orders of magnitude from the highest level reached during growth, markedly different from the decrease of 9 orders of magnitude seen for Thy12 at thymidine concentrations as high as 10 μM (**Fig. 3c**).

In vivo viability of strain Thy12

Even in low-thymidine conditions, Thy12 showed a substantial reduction in viability after *in vivo* passage through porcine intestine. HPLC analysis showed ~1 μM thymidine in total extracts of porcine ileum. Thymidine could also be detected, albeit at (as expected) a lower concentration (up to 0.075 μM), in ileal lavages from human patients. To protect *L. lactis* from nonspecific lysis by bile in the duodenum and jejunum, we used gelatin capsules filled with equal amounts of either lyophilized Thy12 or MG1363 and coated with an enteric coating designed for pig ileal release. Because pigs chew their food and would thereby destroy the coating, we created a fistula to access the proximal duodenum. A second fistula was created to sample the ileal content (method adapted from ref. 21). The capsules were inserted through the duodenal fistula and time samples were taken from both the ileal content and the feces. Lactococci were detected by capturing them from suspensions of ileal or fecal material with anti-lactococcal IgG bound to the surface of a plastic Petri dish. We then determined bacterial cfu by washing, overlaying the Petri dishes with low-melting agar and incubating the agar at 30 °C. Before the administration of *L. lactis*, none of the pigs showed any signal in this assay. From the pig that was given MG1363, we detected 3.64×10^8 *L. lactis* in the ileal samples and 2.94×10^8 in the feces (ratio 0.80). From the pig that was given Thy12, we detected 2.69×10^8 *L. lactis* in the ileum and 0.125×10^8 in the feces (ratio 0.04) (**Fig. 3d**). The viability of Thy12 therefore decreased ~20-fold more rapidly after intestinal passage than did the viability of MG1363.

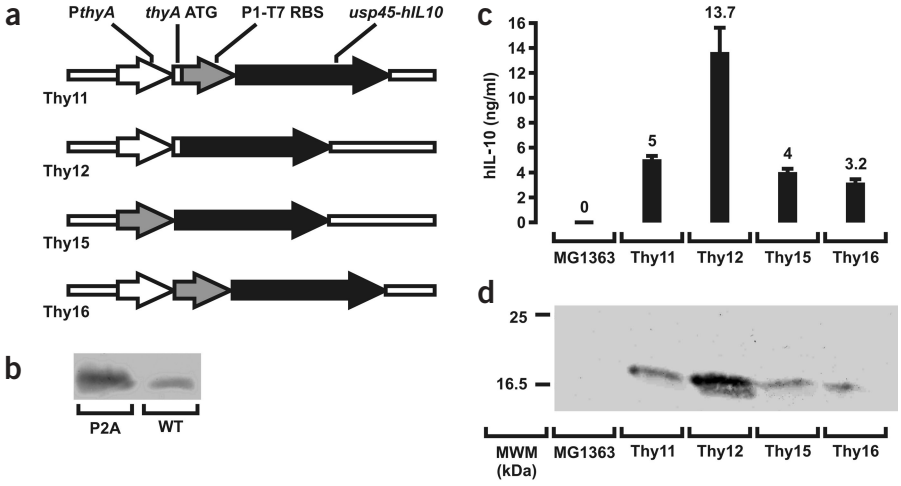

Figure 2 hIL-10 production by Thy11–16. (**a**) Schematic representation of the structure of Thy11–16. White, nonforeign DNA fragments, including the *thyA* promoter (P*thyA*) and the *thyA* ATG start codon; gray, lactococcal P1 promoter; black, *usp45-hIL10* fusion gene. (**b**) Western blot of crude culture supernatant showing higher hIL-10 secretion after replacement of proline by alanine at position 2 of the mature hIL-10 (lane P2A). The two expression strains are otherwise identical. (**c**) hIL-10 secreted in the culture supernatant of 10^9 bacteria of the indicated *L. lactis* strains as determined by ELISA. Bars, average amount of hIL-10 produced; error bars, standard deviation of one experiment representative of at least three independent experiments. (**d**) Western blot of hIL-10 secreted in the culture supernatant of 10^9 bacteria of the indicated *L. lactis* strains.

Acquisition of foreign *thyA* genes

To examine whether Thy12 can spontaneously acquire a *thyA* gene from an exogenous source, we produced mixed colonies of Thy12 and potential *thyA* 'donor' bacteria by superposed inoculation on solid agar plates. In these mixed colonies, very high bacterial densities of up to 5×10^{11} cfu/ml were obtained. The colonies were plated on solid agar plates containing thymidine-free medium, thereby possibly selecting for *thyA* gene transfer to Thy12. Potential donor bacteria tested were *L. lactis* MG1363, *L. lactis* subsp. *lactis* and subsp. *cremoris*, *Lactobacillus casei*, *Escherichia coli* DH5α and O157, and *Salmonella choleraesuis*. Thy12 was transformed with an erythromycin (Em) resistance plasmid and donor strains were transformed with a chloramphenicol (Cm) resistance marker. We were unable to isolate any Em^R *L. lactis* strain that showed thymidine-independent growth. In contrast, mixed colonies on thymidine-free-medium solid agar plates supplemented with thymidine were composed of comparable numbers of Em^R and Cm^R bacteria.

DISCUSSION

Here we describe a system for the containment of live genetically modifed bacteria intended for therapeutic use in humans. The *L. lactis thyA* gene was replaced with expression constructs driving a synthetic *hIL10* gene, resulting in strains that produce hIL-10 and are strictly dependent on thymidine or thymine for growth and survival. These strains are self-limiting because they die rapidly in the absence of their essential growth component.

Biological containment systems can be subdivided into active and passive forms. Active containment provides control through the conditional production of a toxic compound whose expression is tightly controlled by an environmentally responsive element or suppressed by an immunity factor[22]. Some well-integrated active systems of this type have been developed. Homologs of the *Hok* plasmid maintenance factor, Gef and RelF were used for the control of *Pseudomonas putida*[23] and *E. coli*[24], respectively. Other active containment systems for *E. coli* include the phage T7 lysozyme[25], the type II *Eco*RI restriction-modification system[26] and colicin E3 (ref. 27). Although active containment systems provide actual killing of the host, they have notable drawbacks. First, these systems often involve the introduction of a large amount of foreign DNA, which impedes their use in humans. Second, many are plasmid borne, and it remains to be demonstrated that function is maintained when the plasmids become integrated in the bacterial chromosome to reduce lateral dissemination.

In passive systems, which circumvent these limitations, growth is dependent on complementation of an auxotrophy or other gene defect by supplementation with either the intact gene or the essential metabolite. For example, plasmids carrying a heterologous alanine racemase (*alr*) are stably inherited in a Δ*alr* background[28], and experiments are presently ongoing using an *alr* deletion mutant for containment of a genetically modifed strain of *Streptococcus mutans* designed for anti-carie therapy (J. Hillman, personal communication). The *lacF* gene can be used as a selection marker in Δ*lacF L. lactis*[29]. An amber suppressor, supD, has been used as a selectable marker for plasmid maintenance to complement suppressible pyrimidine auxotrophs[30]. However, passive systems have the drawback that they are often bacteriostatic rather than bactericidal.

The choice of *thyA* as a target gene combines the advantages of passive and active containment systems. 'Thymine-less death'[31], which was described as early as 1954 (ref. 32), involves activation of the SOS

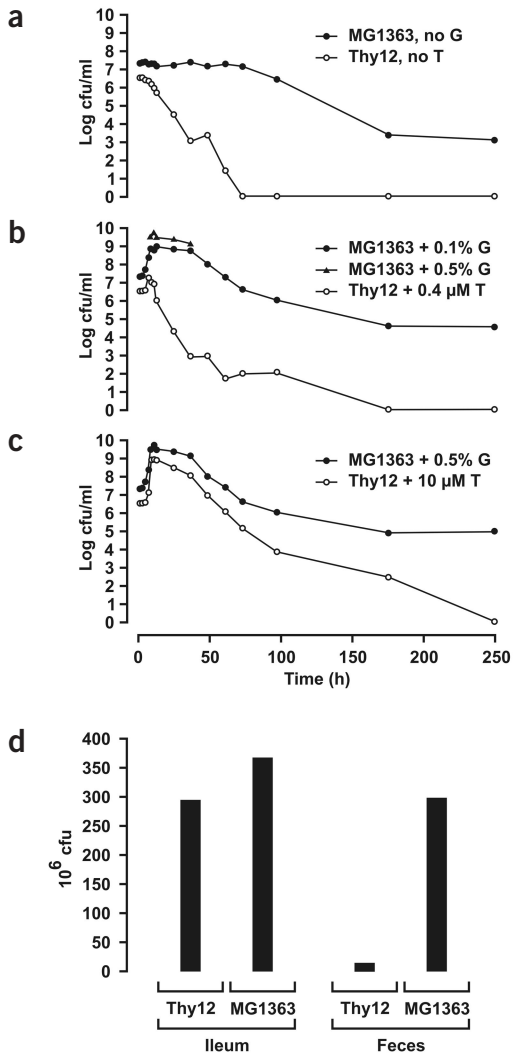

Figure 3 Growth and survival of Thy12 and MG1363. (**a**–**c**) *Lactococcus* growth and survival in the absence (**a**) of an essential component for growth of Thy12 (thymidine, T) or MG1363 (glucose, G, % w/v) and in the presence of low (**b**) or high (**b**) concentrations of these components. (**d**) Survival of Thy12 and MG1363 after passage through the porcine colon. The respective strains were released in the ileum and recovered from the feces at the indicated cfu counts.

repair system and DNA fragmentation, thereby constituting an indigenous suicide system. Thymine and thymidine growth dependence differs from most other auxotrophies[31] in that absence of the essential component is bactericidal in the former and bacteriostatic in the latter. Thus, *thyA*-deficient bacteria cannot accumulate in the environment. *thyA* from *L. casei* has been used as a selective marker for plasmid maintenance in *thyA*-deficient *Lactobacillus acidophilus*[33]. Researchers isolated the *thyA* gene of *L. lactis* MG1363 (ref. 11) but were unable to isolate *thyA*-deficient strains by use of conventional selection methods (P. Ross, personal communication). Such a strain was recently generated by targeted gene deletion[34].

Thy12 provides a satisfactory solution to concerns about biosafety for several reasons. First, only the absolute minimal amount of foreign DNA—the gene of interest—is present in the genetically modified organism. No resistance marker is required to guarantee stable inheritance of the transgene. Second, accumulation of the genetically modified organism in the environment is very unlikely, as rapid death occurs upon thymidine starvation. We observed a 10^7-fold decrease in cfu after 72 h in the absence of thymidine. Even after prolonged incubation in rich growth medium devoid of thymidine, we failed to isolate revertants or suppressors. In addition, we did not observe acquisition of *thyA* from other microorganisms. Owing to high sequence diversity at *thyA* loci, successful acquisition would probably be possible only from the homologous *L. lactis* subsp. *Cremoris cremoris*. Third, were the intact *thyA* acquired by homologous recombination, the transgene would be removed. Fourth, the risk of disseminating the genetic modification through lateral gene transfer is minimized because the *hIL10* gene is integrated in the *L. lactis* chromosome. Several mechanisms for lateral gene transfer do not function in Thy11–16: *L. lactis* MG1363 is deficient for Tn916 and Tn919 conjugative transposition[35] and phage replication is severely impaired in *thyA*-deficient *L. lactis*, thereby disabling phage-mediated transduction of host genetic material[34].

Our approach thus provides a simple and robust system for biological containment. One of the strains reported here, Thy12, has been approved by the Dutch authorities as an experimental therapy for humans with IBD. This clinical trial will be the first to use a live genetically modified bacterial therapeutic.

METHODS

Thymidine-free medium. Thymidine-free medium was obtained by growing *L. lactis* Thy12 to saturation in M17 broth (Difco) containing 0.5% (wt/vol) glucose. Bacteria were then removed by centrifugation, filtration and sterilization.

Genetically modified strains of *L. lactis* MG1363. Strains were constructed as described in the Results section and as shown in **Figure 1**. Further details are available upon request.

Animal experiments. Eight-week-old Piétrain/Landrace crossbred pigs were fasted overnight and anesthetized with zoletil 100 (Virbac, France) (2% (wt/vol) xylazine; 0.22 ml per kg body weight) administered intramuscularly (i.m.)).

Ileal loop incubations were performed as described[36]. Care was taken to minimize surgical trauma and to maintain an adequate blood supply to the ligated segments. Lyophilized Thy12 culture (3.0751 g, $1.44 \times 10^{11} \pm 1.72 \times 10^{10}$) was resuspended in 11.3 ml M9 buffer[2] without nutrients, thymidine or thymine, and 5 ml was injected with a 26-G needle into an ileal loop of ~5 cm length. The volume of loop contents before injection could not be determined. The small intestine was returned to the abdominal cavity and the abdomen was closed. During the 4-h incubation period, the pig was conscious and received finadyne as analgesic (1 ml per 45 kg body weight, i.m.).

The sample was left to incubate in the conscious pig for 4 h. The contents of the loop were isolated with a syringe and the tissue was washed and homogenized.

Duodenal and ileal fistulae were created as described[21]. After laparotomy, a duodenal fistula and an ileal fistula were created. After a 10-d recovery, the capsules were inserted in the duodenal fistula. Euthanasia was performed by intravenous injection of pentobarbital (24 mg per kg body weight). All animal experimentation was carried out in accordance with Belgian law (KB 14/11/1993; approval was obtained from the ethical committee of the Faculty of Veterinary Medicine, Ghent University, file no. EC 2002/06).

Counting of *L. lactis* in ileal and fecal material. Ileal or fecal material was resuspended in M9N (M9, as in ref. 2, containing 0.05% (wt/vol) NaN_3; at 4 °C, this solution did not affect lactococcal viability over a period of 2 weeks) at a ratio of 1 g per 9 ml. Large particles were removed by filtering through a 80-µm cell strainer (Becton Dickinson). All particulate material was collected from the suspension by centrifugation, resuspended in 1:10 vol of M9N and further diluted, as appropriate, in M9N. Bacterial Petri dishes (Bibby Sterilin) were coated with 3 ml of 10 µg/ml rabbit anti-MG1363 IgG in M9N and incubated overnight at 4 °C. The non bound antiserum was removed and the plates were blocked overnight at 4 °C with 10 ml

0.1% (wt/vol) casein in M9N. The blocking solution was removed and the plates were overlaid with 3 ml of the suspensions of fecal or ileal material and incubated for 3 h at 22 °C. Plates were washed three times with water, overlaid with M17 containing 0.5% (wt/vol) glucose, 50 μg/ml thymidine and 0.8% (wt/vol) agar, incubated for 24 h at 30 °C and counted.

Accession number. The GenBank accession number for the *thyA* locus is AF462070.

ACKNOWLEDGMENTS

The authors thank Inge Bruggeman, Hilde Devlies Koen Van Laer and Elena Yancheva for expert technical assistance, Karen Madsen, Claude Cuvelier and Frans Van Roy for critically reviewing the manuscript, and Jan Kok and Emmanuelle Maguin for providing genetic tools. This study was financed by the Vlaams Interuniversitair instituut voor Biotechnologie, Ghent University (GOA project nos. 12050700 and 12051501) and the commission of the European Communities, specific research, technological development and training activities program "Quality of Life and Management of Living Resources," QLK1-2000-00146 "Probiotic strains with designed health properties."

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 10 December 2002; accepted 17 April 2003
Published online 15 June 2003; doi:10.1038/nbt840

1. Steidler, L. *et al.* Mucosal delivery of murine interleukin-2 (IL-2) and IL-6 by recombinant strains of *Lactococcus lactis* coexpressing antigen and cytokine. *Infect. Immun.* **66**, 3183–3189 (1998).
2. Steidler, L. *et al.* Treatment of murine colitis by *Lactococcus lactis* secreting interleukin-10. *Science* **289**, 1352–1355 (2000).
3. Beninati, C. *et al.* Therapy of mucosal candidiasis by expression of an anti-idiotype in human commensal bacteria. *Nat. Biotechnol.* **18**, 1060–1064 (2000).
4. Kruger, C. *et al.* In situ delivery of passive immunity by lactobacilli producing single-chain antibodies. *Nat. Biotechnol.* **20**, 702–706 (2002).
5. van Deventer, S.J., Elson, C.O. & Fedorak, R.N. Multiple doses of intravenous interleukin 10 in steroid-refractory Crohn's disease. Crohn's Disease Study Group. *Gastroenterology* **113**, 383–389 (1997).
6. Fedorak, R.N. *et al.* Recombinant human interleukin 10 in the treatment of patients with mild to moderately active Crohn's disease. The Interleukin 10 Inflammatory Bowel Disease Cooperative Study Group. *Gastroenterology* **119**, 1473–1482 (2000).
7. Schreiber, S. *et al.* Safety and efficacy of recombinant human interleukin 10 in chronic active Crohn's disease. Crohn's Disease IL-10 Cooperative Study Group. *Gastroenterology* **119**, 1461–1472 (2000).
8. Tilg, H., Ulmer, H., Kaser, A. & Weiss, G. Role of IL-10 for induction of anemia during inflammation. *J. Immunol.* **169**, 2204–2209 (2002).
9. Schotte, L., Steidler, L., Vandekerckhove, J. & Remaut, E. Secretion of biologically active murine interleukin-10 by *Lactococcus lactis*. *Enzyme Microb. Technol.* **27**, 761–765 (2000).
10. Masood, R. *et al.* Interleukin-10 is an autocrine growth factor for acquired immunodeficiency syndrome-related B-cell lymphoma. *Blood* **85**, 3423–3430 (1995).
11. Ross, P., O'Gara, F. & Condon, S. Cloning and characterization of the thymidylate synthase gene from *Lactococcus lactis* subsp. *lactis*. *Appl. Environ. Microbiol.* **56**, 2156–2163 (1990).
12. Biswas, I., Gruss, A., Ehrlich, S.D. & Maguin, E. High-efficiency gene inactivation and replacement system for gram-positive bacteria. *J. Bacteriol.* **175**, 3628–3635 (1993).
13. Bolotin, A. *et al.* The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* ssp. *lactis* IL1403. *Genome Res.* **11**, 731–753 (2001).
14. Law, J. *et al.* A system to generate chromosomal mutations in *Lactococcus lactis* which allows fast analysis of targeted genes. *J. Bacteriol.* **177**, 7011–7018 (1995).
15. Waterfield, N.R., Le Page, R.W., Wilson, P.W. & Wells, J.M. The isolation of lactococcal promoters and their use in investigating bacterial luciferase synthesis in *Lactococcus lactis*. *Gene* **165**, 9–15 (1995).
16. Stemmer, W.P., Crameri, A., Ha, K.D., Brennan, T.M. & Heyneker, H.L. Single-step assembly of a gene and entire plasmid from large numbers of oligodeoxyribonucleotides. *Gene* **164**, 49–53 (1995).
17. van Asseldonk, M. *et al.* Cloning of usp45, a gene encoding a secreted protein from *Lactococcus lactis* subsp. *lactis* MG1363. *Gene* **95**, 155–160 (1990).
18. Barthelemy, I. *et al.* Production and secretion of human interleukin 6 into the periplasm of Escherichia coli: efficient processing of N-terminal variants of hIL6 by the *E. coli* signal peptidase. *J. Biotechnol.* **27**, 307–316 (1993).
19. Thompson-Snipes, L. *et al.* Interleukin 10: a novel stimulatory factor for mast cells and their progenitors. *J. Exp. Med.* **173**, 507–510 (1991).
20. Giard, J.C., Verneuil, N., Auffray, Y. & Hartke, A. Characterization of genes homologous to the general stress-inducible gene gls24 in *Enterococcus faecalis* and *Lactococcus lactis*. *FEMS Microbiol. Lett.* **206**, 235–239 (2002).
21. Decuypere, J.A., Vervaeke, I.J., Henderickx, H.K. & Dierick, N.A. Gastro-intestinal cannulation in pigs: a simple technique allowing multiple replacements. *J. Anim. Sci.* **45**, 463–468 (1977).
22. Molin, S. *et al.* Suicidal genetic elements and their use in biological containment of bacteria. *Annu. Rev. Microbiol.* **47**, 139–166 (1993).
23. Molina, L., Ramos, C., Ronchel, M.C., Molin, S. & Ramos, J.L. Construction of an efficient biologically contained *Pseudomonas putida* strain and its survival in outdoor assays. *Appl. Environ. Microbiol.* **64**, 2072–2078 (1998).
24. Knudsen, S. *et al.* Development and testing of improved suicide functions for biological containment of bacteria. *Appl. Environ. Microbiol.* **61**, 985–991 (1995).
25. Schweder, T., Hofmann, K. & Hecker, M. *Escherichia coli* K12 relA strains as safe hosts for expression of recombinant DNA. *Appl. Microbiol. Biotechnol* **42**, 718–723 (1995).
26. Torres, B., Jaenecke, S., Timmis, K.N., Garcia, J.L. & Diaz, E. A gene containment strategy based on a restriction-modification system. *Environ. Microbiol.* **2**, 555–563 (2000).
27. Diaz, E., Munthali, M., de Lorenzo, V. & Timmis, K.N. Universal barrier to lateral spread of specific genes among microorganisms. *Mol. Microbiol.* **13**, 855–861 (1994).
28. Bron, P.A. *et al.* Use of the alr gene as a food-grade selection marker in lactic acid bacteria. *Appl. Environ. Microbiol.* **68**, 5663–5670 (2002).
29. MacCormick, C.A., Griffin, H.G. & Gasson, M.J. Construction of a food-grade host/vector system for *Lactococcus lactis* based on the lactose operon. *FEMS Microbiol. Lett.* **127**, 105–109 (1995).
30. Sorensen, K.I., Larsen, R., Kibenich, A., Junge, M.P. & Johansen, E. A food-grade cloning system for industrial strains of *Lactococcus lactis*. *Appl. Environ. Microbiol.* **66**, 1253–1258 (2000).
31. Ahmad, S.I., Kirk, S.H. & Eisenstark, A. Thymine metabolism and thymineless death in prokaryotes and eukaryotes. *Ann. Rev. Microbiol.* **52**, 591–625 (1998).
32. Cohen, S.S. & Barner, H.D. Studies on unbalanced growth in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **40**, 885–893 (1954).
33. Fu, X. & Xu, J.G. Development of a chromosome-plasmid balanced lethal system for *Lactobacillus acidophilus* with thyA gene as selective marker. *Microbiol. Immunol.* **44**, 551–556 (2000).
34. Pedersen, M.B., Jensen, P.R., Janzen, T. & Nilsson, D. Bacteriophage resistance of a ΔthyA mutant of *Lactococcus lactis* blocked in DNA replication. *Appl. Environ. Microbiol.* **68**, 3010–3023 (2002).
35. Bringel, F., Van Alstine, G.L. & Scott, J.R. A host factor absent from *Lactococcus lactis* subspecies *lactis* MG1363 is required for conjugative transposition. *Mol. Microbiol.* **5**, 2983–2993 (1991).
36. Moon, H.W., Sorensen, D.K. & Sautter, J.H. *Escherichia coli* infection of the ligated intestinal loop of the newborn pig. *Am. J. Vet. Res.* **27**, 11317–11325 (1966).