In situ delivery of passive immunity by lactobacilli producing single-chain antibodies

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Lactobacilli have previously been used to deliver vaccine components for active immunization *in vivo*. Vectors encoding a single-chain Fv (scFv) antibody fragment, which recognizes the streptococcal antigen I/II (SAI/II) adhesion molecule of *Streptococcus mutans*, were constructed and expressed in *Lactobacillus zeae* (American Type Culture Collection (ATCC) 393). The scFv antibody fragments secreted into the supernatant or expressed on the surface of the bacteria showed binding activity against SAI/II in enzyme-linked immunosorbent assay (ELISA), and surface scFv-expressing lactobacilli agglutinated SAI/II-expressing *S. mutans in vitro* without affecting the corresponding SAI/II knockout strain. Lactobacilli expressing the scFv fragment fused to an E-tag were visualized by scanning electron microscopy (SEM) using beads coated with a monoclonal anti-E-tag antibody, and they bound directly to beads coated with SAI/II. After administration of scFv-expressing bacteria to a rat model of dental caries development, *S. mutans* bacteria counts and caries scores were markedly reduced. As lactobacilli are generally regarded as safe (GRAS) microorganisms, this approach may be of considerable commercial interest for *in vivo* immunotherapy.

Streptococcus mutans is the major pathogen involved in caries development through production of lactic acid[1–4]. Two virulence factors participate in the colonization process. The first, surface antigen SAI/II (also known as B, IF, P1, SR, MSL-1, and Pac[5]), is an adhesin that binds to salivary pellicles. The second is a series of surface glucosyltransferases (GTFs) that catalyze the synthesis of glucan[6], contributing to the later stages of the adherence process.

Local passive immunotherapy can prevent colonization by *S. mutans*, and oral administration of hyperimmune bovine IgG[7] or chicken IgY[8] markedly lowers the caries score in *S. mutans*–infected rats and reduces the bacterial load in humans[9,10]. Similar therapeutic results have been obtained by applying a mouse monoclonal IgG1 antibody, Guy's 13, directed against the SAI/II adhesion molecule on *S. mutans*, to the teeth of both primates[11,12] and humans[13,14]. Guy's 13 antibodies, with the CH3 domain replaced by the CH2 and CH3 domains of a mouse α-chain, have recently been developed and produced in plants[15]. These antibodies effectively prevent colonization of the teeth by *S. mutans* in human volunteers[16], suggesting a new approach in prevention of oral infections using genetically engineered immunoglobulins.

ScFv fragments have been produced in numerous systems[17–20] and used for the treatment of infectious diseases[21–23]. As the antibody fragments have a very short half-life *in vivo*, new methods must be developed to increase their longevity before they can be therapeutically applied at mucosal sites.

Lactobacilli are Gram-positive bacteria that have been used in food fermentation and preservation for centuries[24], and some strains are normal constituents of the human intestinal microflora[25]. Several authors have also suggested that fermented products positively affect the course of certain infectious diseases[26–29]. Selected strains of *Lactobacillus casei* and *Lactobacillus plantarum* have also been shown to exert strong adjuvant effects on the mucosal and the systemic immune response[30–32]. Furthermore, lactobacilli have been suggested as carriers for oral vaccines[33] and expression vectors have previously been constructed for surface expression or secretion of various antigens[32,34–37].

We have expressed Guy's 13 scFv both on the surface of *L. zeae* ATCC 393 (previously named *L. casei* ATCC 393) and as a secreted protein. The recombinant scFv recognized SAI/II and the transformed lactobacilli acted therapeutically in experimental animals, both decreasing the number of *S. mutans* bacteria and reducing the development of caries. Our results thus suggest an approach for *in situ* delivery of passive immunity at mucosal sites.

Results

Construction of scFv-expressing *L. zeae*. The scFv-encoding gene derived from the variable regions (VH and VL) of Guy's 13 (IgG1)[11], with addition of an E-tag–encoding sequence, was inserted into the vector pTUAT[35]. A short or a long anchor sequence of the proteinase P–encoding gene *PrtP* was introduced, generating pTUAT-A (Fig. 1A). The scFv-containing or the scFv-anchor-containing DNA fragments were then cloned into the *Escherichia coli*/*Lactobacillus* shuttle vector pLP402 (Fig. 1B). A terminator from the lactate dehydrogenase gene (T*ldh*) was present between the C-terminal region of amylase and the N-terminal end of the scFv to suppress the expression of the downstream sequences in *E. coli*. The T*ldh* was removed by *Not*I digestion of

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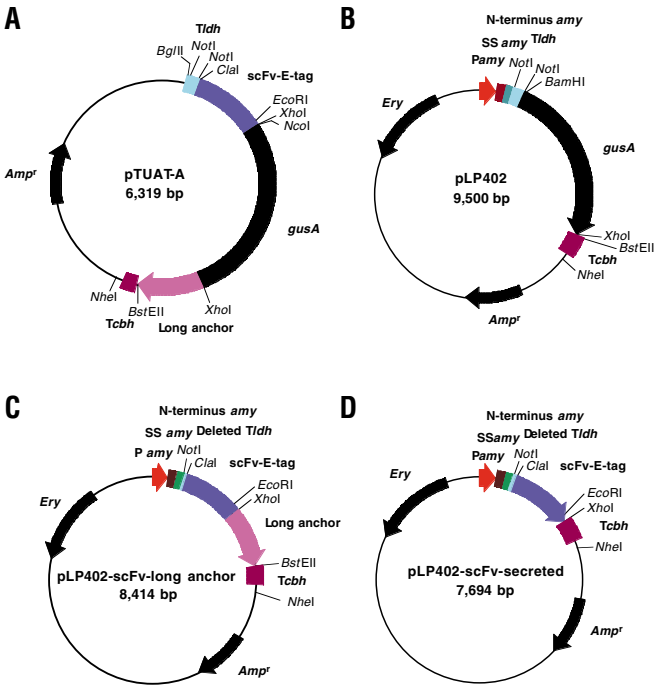

Figure 1. Map of *Lactobacillus* expression vectors. (A) Intermediate vector pTUAT-A, in which the Sc-sense–etag-antisense PCR amplicon was cloned between the *Cla*I and *Nco*I sites of pTUAT[35]. (B) *Lactobacillus* expression vector pLP402. (C) pLP402-scFv–long anchor vector mediating surface-anchored expression of scFv by fusion to the last 244 amino acids of *L. zeae* proteinase P. (D) pLP402-scFv-secreted vector mediating secretion of scFv in the medium. P*amy*, Promoter sequence of the α-amylase gene of *L. amylovorus*; SS *amy*, secretion signal of the α-amylase gene of *L. amylovorus* (36 amino acids); N-terminus *amy*, N-terminus (26 amino acids) of the α-amylase gene of *L. amylovorus*; T*ldh*, transcription terminator of the lactate dehydrogenase gene of *L. zeae*; *gusA*, gene marker encoding β-glucuronidase; long anchor, anchor sequence from the proteinase P gene of *L. zeae* (244 amino acids); T*cbh*, transcription terminator sequence of the conjugated bile acid hydrolase gene of *L. plantarum* 80; *Amp*^{r}, ampicillin-resistance gene; *Ery*, erythromycin-resistance gene; deleted T*ldh*, remaining sequence after deletion of T*ldh*.

the plasmid, and after ligation the resulting vectors were introduced into *L. zeae* ATCC 393. Three different constructs were made: (i) the pLP402-scFv–short anchor vector, mediating cell-surface expression by fusion to the last 117 amino acids of the proteinase P of *L. zeae* ATCC 393; (ii) the pLP402-scFv–long anchor vector mediating cell-surface expression by fusion to the last 244 amino acids of the proteinase P protein (Fig. 1C); and (iii) pLP402-scFv, mediating secretion of scFv into the medium (Fig. 1D). The theoretical molecular masses of the expressed proteins from each vector were 47 kDa, 60 kDa, and 35 kDa, respectively.

Expression of scFv. ScFv expression was analyzed by immunoblotting of *L. zeae*–transformed strains using monoclonal anti-E-tag antibodies. The cell extract of *L. zeae* transformed with the pLP402-scFv–long anchor–encoding vector yielded a 60 kDa protein (Fig. 2A), and the culture supernatant of *L. zeae* transformed with the pLP402S-scFv vector contained a protein of ~40 kDa (Fig. 2B).

Surface expression of scFv in recombinant *L. zeae* strains transformed with the pLP402-scFv–short anchor and pLP402-scFv–long anchor was also analyzed by flow cytometry using an anti-E-tag antibody. The pLP402-scFv–long anchor–transformed lactobacilli showed a strong positive signal when stained by the anti-E-tag antibody, whereas the lactobacilli transformed with the pLP402-scFv–short anchor vector showed a less intense signal (Fig. 2C). This confirms the surface location of scFv in the two recombinant *L. zeae* strains containing the scFv-anchor fusion constructs.

Biological activity of the scFv. The biological activity of the recombinant scFv was analyzed by both ELISA and agglutination assays. ScFvs from homogenates of pLP402-scFv–short anchor– and pLP402-scFv–long anchor–transformed lactobacilli and culture supernatant of pLP402-scFv transformants were tested in ELISA (Table 1). The scFvs from all recombinant strains bound to SAI/II coated to microtiter plates (Table 1), although the pLP402-scFv–short anchor construct bound less well than the pLP402-scFv–long anchor construct.

The streptococci or lactobacilli used in the agglutination assay showed no self-agglutination. *Streptococcus mutans* NG8 (wild type), expressing the SAI/II antigen, was rapidly co-agglutinated with *L. zeae* transformed with the pLP402-scFv–long anchor construct, whereas no co-agglutination occurred when a mutant *S. mutans* 834 strain (SAI/II knockout) was used (Table 2). Mixing of *S. mutans* NG8 and *L. zeae* transformed with the pLP402-scFv–short anchor construct resulted in weak agglutination only (Table 2). Furthermore, the time required for visible aggregation was more than double that of *L. zeae* transformed with the pLP402-scFv–long anchor construct (73 and 33 s, respectively, mean of five experiments).

The transformed lactobacilli were also incubated with magnetic polystyrene beads (Dynal M-450) and coated with anti-E-tag antibodies; a scanning electron microscope was used to visualize the binding. The binding of pLP402-scFv–long anchor–transformed bacteria (Fig. 3A), but not the negative control, *L. zeae* pLP402 (Fig. 3B), to the anti-E-tag–coated beads was determined by SEM. Furthermore, pLP402-scFv–long anchor–transformed lactobacilli bound directly to SAI/II-coated magnetic polystyrene beads (Dynal M-450 Epoxy; Fig. 3C, D).

Therapeutic effect *in vivo*. To be therapeutically effective, antibody-producing lactobacilli must persist in the oral cavity for a prolonged period of time. Desalivated rats were therefore orally inoculated with transformed lactobacilli (pLP402, pLP402-scFv, pLP402-scFv–short anchor, and pLP402-scFv–long anchor), and the presence of bacteria was measured as the presence of erythromycin-resistant colonies containing the scFv insert. When rats were inoculated with the transformed lactobacilli every second day for a period of two weeks, the bacteria persisted for the duration of the experiment (three weeks; data not shown). Thus, bacteria could still be detected one week after the final inoculation. Lactobacilli were eluted

Table 1. Binding activity of the Guy's 13–derived scFv to the SAI/II antigen in ELISA[a]

Sample	scFv	Anti-E-tag	Rabbit anti-mouse Ig	A_{405} [b]
Culture medium	–	+	+	0.26
Culture medium	+	–	+	0.24
L. zeae pLP402-scFv (culture supernatant)[c]	+	+	+	0.63
L. zeae pLP402-scFv–short anchor (bacterial homogenate)[c]	+	+	+	0.41
L. zeae pLP402-scFv–long anchor (bacterial homogenate)[c]	+	+	+	0.69
Guy's 13 (1:1,000)[d]	–	–	+	1.02
Guy's 13 (1:10,000)	–	–	+	0.28
Guy's 13 (1:100,000)	–	–	+	0.17

[a]Three independent experiments were done, and a typical example is given in the table.
[b]Absorbance at 405 nm after 60 min incubation with substrate.
[c]Total protein concentrations were 6.0, 1.3, and 1.5 mg/ml, respectively.
[d]Supernatant of a mouse hybridoma producing the Guy's 13 antibody (concentration 10 μg/ml).

from a cotton swab from the oral cavity and nontransformed bacteria colonized more effectively (approximately 10,000 colonies per swab) than transformants (approximately 1,000 colonies per swab). However, there was no major difference in colonizing capacity between the bacteria harboring the different constructs.

Rats treated with *L. zeae* transformed with the pLP402-scFv–long anchor construct had a significantly lower number of *S. mutans* NG8 in the oral cavity as compared with rats infected with lactobacilli transformed with pLP402 and the *S. mutans* control (Table 3). Significantly fewer animals in the pLP402-scFv–long anchor group developed dental caries lesions as compared with both the pLP402-containing lactobacilli and the *S. mutans* control groups (Table 3). The mean number of caries lesions was also significantly different between the pLP402-scFv–long anchor–expressing *Lactobacillus* group and the *S. mutans* control group (25.6 and 42.3, respectively; $P < 0.05$ (Student's *t*-test)).

Discussion

Most infectious agents enter by the mucosal route, and locally produced IgM and IgA antibodies may readily prevent disease. Oral administration of human, bovine, and chicken immunoglobulins has also effected prophylactic and therapeutic responses against a variety of gastrointestinal infections[38–42].

The majority of antibody preparations used to date have been polyclonal in origin, but a monoclonal antibody will suffice for protection against many infectious diseases or mitigation of an ongoing infection. The latter may be produced by hybridoma technology, in transgenic plants, or by microorganisms such as *E. coli* and yeast. However, these modes of production are fraught with disadvantages and all require purification of the antibodies before administration.

Vaccine antigens and cytokines have been expressed *in vivo* in the gastrointestinal tract by transformed lactobacilli[34] and lactococci[43]. The benefit of using this system for delivery of antibodies or antibody fragments was exploited in our experiments, in which antibodies against a mucosal pathogen, *S. mutans*, were produced by lactobacilli. Theoretically, bacteria colonizing different anatomical sites within the oro-gastrointestinal tract might be selectively used and transformed with vectors encoding antibodies against a variety of pathogens. The long persistence of the transformed lactobacilli in the oral cavity and their therapeutic efficacy suggests that this may be a viable approach for future therapy either prophylactically or therapeutically. *In vivo* production of antibody fragments locally in the intestine circumvents the practical problem of degradation of orally administered antibodies in the stomach.

Table 2. Co-agglutination of pLP402-scFv–long anchor–transformed lactobacilli with wild-type (NG8) and SAI/II-defective (834) *S. mutans*

L. zeae transformants	*S. mutans*	Agglutination
–	NG8	–
–	Mutant (834) (SAI/II ko)	–
pLP402	–	–
pLP402	NG8	–
pLP402	Mutant (834) (SAI/II ko)	–
pLP402-scFv–short anchor[a]	–	–
pLP402-scFv–short anchor[a]	NG8	+
pLP402-scFv–short anchor[a]	Mutant (834) (SAI/II ko)	–
pLP402-scFv–long anchor[a]	–	–
pLP402-scFv–long anchor[a]	NG8	+++
pLP402-scFv–long anchor[a]	Mutant (834) (SAI/II ko)	–

[a]Expression of scFv was induced by growth in LCM medium containing mannitol as described. Agglutination was assayed arbitrarily, where + corresponds to agglutination of 20% of the bateria and +++ corresponds to 90% agglutination. ko, Knockout.

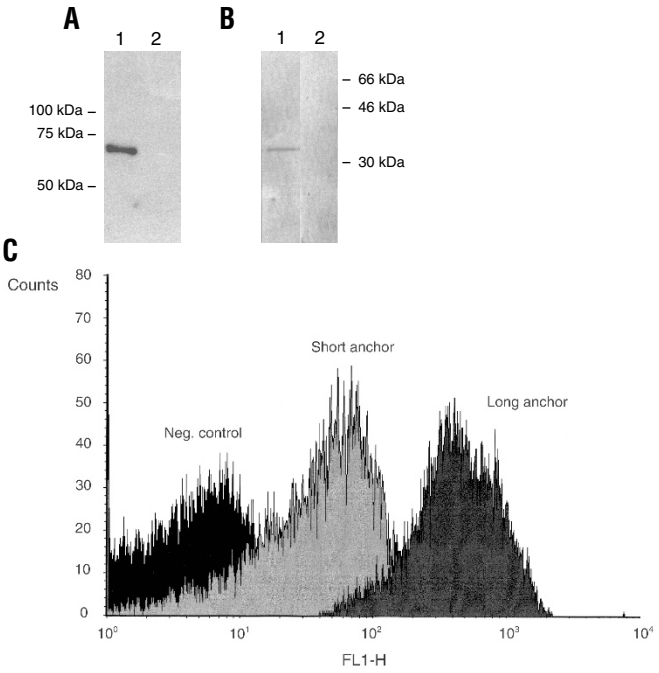

Figure 2. Detection of the scFv expressed by *L. zeae* by immunoblotting and fluorescence-activated cell sorting (FACS). (A) The homogenate of lactobacilli transformed by the pLP402-scFv–long anchor (lane 1) and pLP402 vectors (lane 2). (B) Concentrated supernatant of *Lactobacillus* transformant pLP402-scFv-secreted (lane 1) and pLP402 vectors (lane 2). (C) FACS analysis of lactobacilli transformed with the pLP402, pLP402-scFv–short anchor, or pLP402-scFv–long anchor vectors using a primary mouse E-tag monoclonal antibody and fluorescein isothiocyanate–conjugated goat anti-mouse immunoglobulin.

The vectors used in our experiments contain an inducible amylase promoter, and thus the transformed bacteria, after *in vitro* induction, would be expected to produce scFv fragments only for a limited time *in vivo*, hence the need for repeated administration. As it may be more efficient to use a constitutive promoter, such vectors are currently being constructed.

Our present experiments utilized the Guy's 13 antibody as a parent antibody for the construction of the scFv. This is one of the most well-characterized, therapeutically effective mouse monoclonal antibodies described to date[11–16]. The suggested mechanism of action of the Guy's 13 antibody involves agglutination of the *S. mutans* bacteria, as Fab fragments, in contrast to Fab_2 fragments, do not prevent recolonization[14]. The secreted scFv, also functionally monovalent, would therefore be expected to be therapeutically inferior. Lactobacilli expressing monovalent scFv on their surfaces, however, would be anticipated to work effectively as "biological beads" because of the multivalent binding, resulting in high avidity. In our experiments, this may have promoted agglutination and subsequent clearance of bacteria. The bound *S. mutans* bacteria might also be effectively killed by the high local concentration of bactericidal and bacteriostatic proteins produced by the lactobacilli. Although model scFv fragments have been expressed by Gram-positive bacteria such as staphylococci (anti-IgE)[44] and streptococci (anti-candida)[45], this is the first report to show that antibody fragments can be produced by lactobacilli, thus providing a basis for future application of *in vivo* delivery of passive immunity in humans.

Experimental protocol

Construction of the single-chain antibody gene. The single-chain antibody gene fragment encoding the Guy's 13 monoclonal antibody (scFv, 711 bp) was amplified from cDNA and cloned into the pCANTAB 5E phage vector

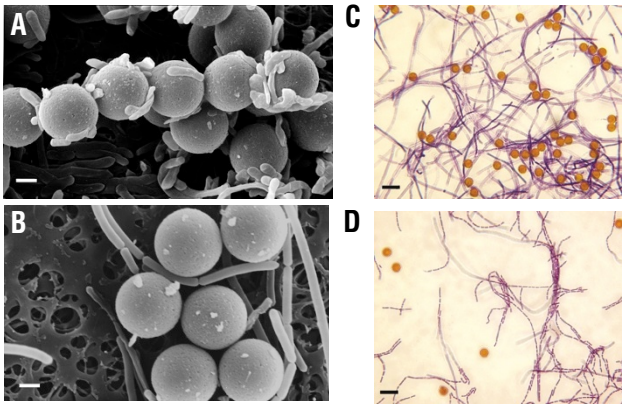

Figure 3. Adherence of transformed lactobacilli to magnetic beads coated with E-tag antibodies or SAI/II. (A) *L. zeae* pLP402-scFv–long anchor transformants adhere to the beads coated with E-tag antibodies (magnification ×5,000; bar, 2 μm). (B) *L. zeae* pLP402 transformants (with empty vector) do not adhere to the beads coated with anti-E-tag antibodies (magnification ×5,000; bar, 2 μm). (C) *L. zeae* pLP402-scFv–long anchor transformants adhere to the beads coated with SAI/II (magnification ×1,000; bar, 11 μm). (D) *L. zeae* pLP402 transformants (empty vector) do not bind to the beads coated with SAI/II and most of them are washed away during the preparation procedure (magnification ×1,000; bar, 11 μm).

(Amersham Biosciences, Uppsala, Sweden) at the *Sfi*I and *Not*I restriction enzyme sites, generating pCANT-scFv. The 3′ terminus of the scFv-encoding gene was fused with an E-tag–encoding gene to allow serological detection. The *Not*I restriction enzyme site between the scFv- and the E-tag–encoding genes was replaced by an *Eco*RI restriction site, as *Not*I digestion was later used to remove the T*ldh* terminator present downstream of the α-amylase gene–derived expression signals in the pLP402 vector (Fig. 1B).

The scFv was amplified from the pCANT-scFv vector using primers Sc-sense (5′-CCCATCGATGCCCAGGTGAAACTG-3′) and Sc-antisense (5′-CGGAATTCCGCC CGTTTTATTTCCA-3′) and cloned into pBluescript II SK (+) (Stratagene, La Jolla, CA) at the *Cla*I and *Eco*RI sites, generating the pBS vector. The E-tag fragment was generated from the same template as above (pCANT-scFv) using the primers tag-sense (5′-CGGAATTCGGTGCGCCGGT-GC CGTAT-3′) and pcant-antisense (5′-TCCCCGCGGTACCAGCGCCAAA-GACATAAGG -3′) and was inserted at the *Eco*RI and *Sac*II sites of pBS, generating the pBSE construct.

Construction of the *Lactobacillus* expression vectors. A series of scFv expression vectors were constructed containing the promoter and secretion signal sequence of the regulated α-amylase gene of *L. amylovorus* as described elsewhere[35,46]. ScFv was produced in *L. zeae* ATCC 393, either anchored on the cell surface or secreted in a free form in the medium. The scFv–E-tag fragment from the pBSE construct was amplified with two sets of primers: Sc-sense and etag-antisense A (5′-CATG*CCATGGCCTCGAGT*GCGGCACGCGGT-3′), and Sc-sense and etag-antisense B (5′-CATG*CCATGGCCTCGAG*CTATGCG-GCACGCGGT-3′), respectively; both of the antisense primers are located downstream of the E-tag and contain *Xho*I and *Nco*I sites. A stop codon (underlined) was introduced in the etag-antisense B primer for the construction of the secretion vector. The PCR products from both amplification reactions were subcloned into an *E. coli* vector, pTUAT, between the *Cla*I and *Nco*I sites, generating pTUAT-A (Fig. 1A) and pTUAT-B, respectively. For the construction of the expression vector encoding an anchored product, the T*ldh*, the scFv, the anchor region, and the T*cbh* were excised from pTUAT-A with *Bgl*II and *Nhe*I and cloned into pLP402 between the *Bam*HI and *Nhe*I restriction sites. The reporter gene *gusA* was deleted from the vector using digestion with *Xho*I followed by ligation with T4 ligase and introduction into *E. coli*. Subsequently, the T*ldh* was deleted from the construct by digestion with *Not*I followed by ligation, generating the pLP402-ScFv–short anchor vector. A construct with an elongated anchor was also made by replacing the existing anchor in pTUAT by the last 732 bp of the coding region of the *PrtP* gene of *L. zeae* ATCC 393 (Fig. 1C). The long anchor is approximately twice the size (244 amino acids) of the short anchor (117 amino acids). The fragment was amplified using primers p6612 (5′-GCCGGAATTC*CTCGAG* AAGAAGACTTCGCTGCTTAACC-3′) and p7343 (5′-GCCGGATCC*GGTCACC*C TATTCTTCACGTTGTTTCCG-3′) and cloned into pTUAT between the *Xho*I and *Bst*EII sites, generating pTUAT-1. The procedure for the generation of the pLP402-scFv–long anchor vector was subsequently the same as that described for the construction of the pLP402-scFv–short anchor vector. For the construction of the secretion vector, the scFv–E-tag fragment was excised from pTUAT-B using *Bgl*II and *Xho*I and inserted between the *Bam*HI and *Xho*I restriction sites of pLP402. The T*ldh* was thereafter deleted as described elsewhere, generating the pLP402-scFv-secreted vector (Fig. 1D).

Escherichia coli JM109 (Stratagene) was used as the host strain for the construction of shuttle vectors and these were introduced by heat shock. Lactobacilli were transfected by electroporation (Gene Pulser II, Bio-Rad Laboratories, Hercules, CA; 25 μF, 100 Ω and 6,250 V/cm)[34] (1 μg of plasmid DNA/100 μl of lactobacilli).

Immunoblot analysis of *Lactobacillus* transformants. To induce expression, cells were grown in *Lactobacillus* carrying medium (LCM) supplemented with 0.5% (wt/vol) mannitol for derepression of the *amy* promoter. Cells were disrupted in 400 μl PBS by sonication (4 × 10 s pulses) and debris was removed by centrifugation. Proteins were separated by SDS–PAGE gel (12% wt/vol) and transferred by electroblotting to 0.2 μm nitrocellulose membranes. The culture supernatant was concentrated 50× using ultrafiltration (Amicon, Beverly, MA) and then processed as above. Immunoblots were developed using mouse monoclonal anti-E-tag antibody (Amersham Biosciences) and horseradish peroxidase (HRP)–conjugated goat anti-mouse IgG Fc antibody (1:1,000; Dako, Glostrup, Denmark). Enhanced chemiluminescence (ECL) buffer (Amersham Biosciences) was used to detect the immunoblotting signal, and the rainbow protein marker (Amersham Biosciences) was used as a molecular mass standard.

ELISA and flow cytometry. Purified SAI/II antigen (3 μg/ml) was coated in 96-well ELISA plates (Nunc, Roskilde, Denmark). Supernatant or cell homogenates containing the scFv was added and anti-E-tag antibody and alkaline phosphatase–conjugated rabbit anti-mouse immunoglobulin (Dako) were subsequently added. Flow cytometry was carried out according to standard protocols and the samples were analyzed using a FACSCalibur (Becton Dickinson, Stockholm, Sweden).

Agglutination assay. *S. mutans* NG8 (SAI/II expressing) and *S. mutans* 834 (SAI/II knockout) strains[47] were cultivated to stationary phase in brain–heart infusion (Difco, Sparks, MD) and harvested by centrifugation. *Streptococcus mutans* (30 μl suspension) was placed on a glass slide (76 × 26 mm, Menzel-Gläser, Braunschweig, Germany), after which 30 μl of *L. zeae* transformants pLP402, pLP402-scFv–short anchor, or pLP402-scFv–long anchor was added. The slide was then rotated vertically by hand and agglutination was arbitrarily scored to determine when visible aggregation first occurred, and then scored again 2 min after the start of the assay.

Electron microscopy. Magnetic polystyrene beads (Dynal M-450 Epoxy; Dynal Biotech, Oslo, Norway) precoated with rat anti-mouse IgG were incubated with the mouse monoclonal anti-E-tag antibody, diluted 1:200, and subsequently incubated with *L. zeae* bacteria transformed with either the pLP402-scFv–long anchor or the pLP402 vector. Beads were analyzed by SEM (JEOL JSM-820, Tokyo, Japan) at 15 kV.

Magnetic polystyrene beads (Dynal M-450 Epoxy; Dynal Biotech) were also coated with SAI/II at a concentration of 4.5 μg/10^7 beads, and 6 × 10^5 beads were mixed with *L. zeae* transformants pLP402 or pLP402-scFv–long anchor and loaded on a glass slide. The slides were rotated for 10 s, Gram-stained, and observed under microscope (Olympus, Hamburg, Germany).

Table 3. Therapeutic *in vivo* effect of transformed lactobacilli in desalivated rats

Lactobacilli	*n*	*S. mutans*[a]	*P* value[b]	Dental caries[c]	*P* value[d]
–	6	2,900	–	4 (67%)	–
pLP402	6	2,350	NS	4 (67%)	NS
pLP402-scFv-long anchor	7	75	<0.05	1 (14%)	<0.05

[a]Summary of two screenings of all individual animals during the final week of the therapeutic experiment. Results are given as median numbers of *S. mutans* colonies.
[b]Mann–Whitney test. NS, Not significant.
[c]Number of animals with dental (slight and moderate) caries at term of the experiment.
[d]χ^2 test.

***In vivo* colonization study.** Rat experiments were all approved by the animal ethics committee of southern Stockholm. Female SPF Sprague–Dawley rats were desalivated through surgery at 21 days of age. This procedure facilitates rapid development of caries[48]. During two successive days, groups of animals were infected with either nontransformed bacteria or lactobacilli transformed with pLP402, pLP402-scFv, pLP402-scFv–short anchor, or pLP402-scFv–long anchor vectors. Rats were inoculated by swabbing the oral cavity after dipping the swab in a bacterial suspension containing 5×10^{10} colony-forming units/ml. Thereafter, the groups were infected every second day until day 14. The rats were screened for growth of lactobacilli every third day until day 14 and thereafter every second day until termination at 20 days after desalivation, using oral swabs plated on Rogosa (Merck, Darmstadt, Germany) agar plates, with erythromycin (3 μg/ml) or without. PCR was used for detection of the inserted scFv gene fragment in erythromycin-resistant colonies.

Caries study. Nineteen SPF Sprague–Dawley rats, 21 days old, were desalivated through surgery[48]. During two successive days, groups of animals were swabbed with sterile water, *L. zeae* pLP402, or *L. zeae* pLP402-scFv–long anchor–transformed bacteria. At 24 and 25 days of age, all groups were infected with *S. mutans* bacteria. Fresh cultures of the *Lactobacillus* transformants were subsequently given every day in the drinking water to the relevant groups, and all animals were screened for numbers of *S. mutans* and *L. zeae* every third day by oral swabbing. The animals were all fed a diet of ground food supplemented with 56% sucrose. At 42 days of age the animals were killed and scored blindly for dental caries by the method of Keyes[49].

Acknowledgments

This work was supported by the Swedish Medical Research Council and by the Foundation for Preventive Caries Research (Patentmedelsfonden).

Competing interests statement

The authors declare that they have no competing financial interests.

Received 5 December 2001; accepted 20 March 2002

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