

Worst-case scenarios for horizontal gene transfer from *Lactococcus lactis* carrying heterologous genes to *Enterococcus faecalis* in the digestive tract of gnotobiotic mice

Carl-Alfred ALPERT, Denis D.G. MATER, Marie-Claude MULLER, Marie-France OURIET, Yvonne DUVAL-IFLAH and Gérard CORTHIER*

Unité d'Ecologie et de Physiologie du Système digestif, INRA, 78352 Jouy-en-Josas, France

Since genetically modified (GM) lactic acid bacteria (LAB) might be released in open environments for future nutritional and medical applications, the purpose of this study was to determine an upper limit for the horizontal gene transfer (HGT) in the digestive tract (DT) from *Lactococcus lactis* carrying heterologous genes (*lux* genes encoding a bacterial luciferase) to *Enterococcus faecalis*. Two enterococcal wide host-range conjugative model systems were used: (i) a system composed of a mobilizable plasmid containing the heterologous *lux* genes and a native conjugative helper plasmid; and (ii) a Tn916-*lux* transposon. Both systems were tested under the most transfer-prone conditions, *i.e.* germfree mice mono-associated with the recipient *E. faecalis*. No transfer was observed with the transposon system. Transfers of the mobilizable plasmid carrying heterologous genes were below 10^2 transconjugants per g of faeces for a single donor dose and reached between 10^3 and 10^4 transconjugants per g of faeces when continuous inoculation of the donor strain was used. Once established in mice, transconjugants persisted at low levels in the mouse DT.

Key words: *Lactococcus lactis* / *Enterococcus faecalis* / horizontal gene transfer / conjugative transposon / conjugative and mobilizable plasmids / digestive tract / luciferase / genetically modified bacteria / gnotobiotic mice

Abbreviations: DT: digestive tract; GM: genetically modified; GMO: genetically modified organism; HGT: horizontal gene transfer; LAB: lactic acid bacteria.

INTRODUCTION

Modern molecular biological methods now allow directed genetic modifications of many living organisms for nutritional and medical purposes. This applies especially to lactic acid bacteria (LAB), (Mercenier et al., 2003; Renault, 2002). Living genetically modified (GM) bacteria are designed to be used in open systems including the digestive tract (DT) and will finally be released in the environment (Bumann et al., 2000; Corthier and Renault, 1999; Mercenier et al., 2000; Robinson et al., 1997; Steidler et al., 2000). These developments raise concerns about safety. The transfer of heterologous genes to the digestive microflora must thus be considered.

Horizontal gene transfer (HGT) was described in complex environments. Germfree mice, associated with a

known microflora, were previously used to follow plasmid transfers from *L. lactis* to various bacterial species (Gruzza et al., 1992; Gruzza et al., 1994). Transfers were only observed into *Enterococcus faecalis* (Gruzza et al., 1994). Otherwise, conjugative transposition from *Bacillus subtilis* to a mixed species oral biofilm developed in a fermenter was first demonstrated recently (Roberts et al., 1999). Such findings encourage studying the transfer of GM transposons or plasmids between LAB in the DT.

Conjugative plasmids and genetic elements like the enterococcal conjugative transposon Tn916 (Bertram et al., 1991; Poyart et al., 1995) represent, in nature, an important mode for HGT to a broad host-range of recipients. They may actually have wide evolutionary

* Corresponding author:

Tel.: 33-1 34 65 24 67; fax: 33-1 34 65 24 62; e-mail: corthier@jouy.inra.fr

consequences (Davison, 1999). A general trend in the construction of recombinant bacteria intended for environmental release is the removal of unnecessary heterologous DNA, especially of those elements which could contribute to the further dissemination of the newly inserted genes (Davison, 2002). This can be done *e.g.* by the use of a conjugative “helper” plasmid that can mobilize a second plasmid carrying the heterologous gene of interest. In the final construct the helper plasmid is eliminated, thus preventing the direct transfer of the mobilizable GM plasmid to secondary hosts. However, conjugative plasmids are present in nature and might be recovered by GM cells carrying the mobilizable plasmid, thereby re-establishing the complete transfer mechanism. The use of a complete helper/mobilizable system would lead to establish an upper rate limit at which *in vivo* mobilization might occur. Some available systems would be useful to assess this point. For instance, a helper/mobilizable plasmid system has been described for recombination in lactococci (Langella et al., 1993) and was recently used to clone genes in non-transformable LAB (Thompson et al., 2001).

Transposons used for strain construction have been modified by the elimination of the transposase from the mobile element and provision of the transposase *in trans* in such a way that it can be eliminated after transfer (Davison, 2002). Experimental data are needed to ensure that exogenous natural transposons might not unlock the minitransposon which, in turn, might escape GM cells and possibly spread. In this situation, an upper limit for *in vivo* transposition must also be determined. A transposon approach using Tn916 was proposed to modify *L. lactis* (Goupil-Feuillerat et al., 2000). The interest of this model system is highlighted by the recent finding that Tn916 is unable to transfer *in vitro* from *L. lactis* to other bacteria (Marra et al., 1999). This system is thus similar to the situation created by a locked minitransposon and, if non-transferable *in vivo*, might be suitable to prevent GM-LAB spread.

In this study, we consider that these systems (helper/mobilizable system and Tn916 transposon in *L. lactis*) are useful as models for plasmidic and chromosomal constructions to determine an upper limit for the HGT from GM *L. lactis* to *E. faecalis* in the DT. We expand here the genetic model by the insertion of the *Vibrio harveyi* luciferase reporter genes (*lux*) as an example for heterologous genes of interest. Luciferase activity is frequently used as an easily detectable reporter function in LAB (Corthier et al., 1998; Drouault et al., 2002). Moreover, *V. harveyi* is not a member of the DT and the *lux* genes are absent from the DT flora. Their detection,

both by activity measurements or PCR, is therefore unambiguously an indicator for the presence of these heterologous genes and is independent of antibiotic selection.

In this work, we chose *E. faecalis* as the recipient strain since (i) previous plasmid transfer experiments showed that *E. faecalis* transconjugants could be recovered from the mice DT (Gruzza et al., 1994); (ii) it is closely related to the natural sources of the conjugative systems used and should thus easily allow the selection of stable transconjugants; and (iii) it is present in the human DT flora in subdominant numbers and therefore of significant ecological importance. In order to enhance even more the interaction and thus the transfer possibilities between donor *L. lactis* and recipient *E. faecalis* cells, we use here the gnotobiotic mono-associated mouse model, where *E. faecalis* is known to establish in the DT at titers far higher than in a complete complex DT flora.

Overall, we used an animal model and genetically modified constructs that are considered to be very favorable for transfer, *i.e.* we created a worst-case scenario, in order to estimate an upper rate limit at which HGT might occur.

RESULTS

Mobilizable plasmid

In vitro conjugation

In conjugation experiments with the donor *L. lactis* CA33 and the recipient *E. faecalis* Z1666, transfer of the mobilizing pIL205 occurred at 1.3×10^{-2} per donor, while co-mobilization of pCAC4 was significantly lower with a transfer rate at 4.6×10^{-4} per donor. Using CA31 as the donor, co-mobilization of pCAC2 was even lower since the transfer rate reached only 4.8×10^{-8} . When the plasmid extracted from the transconjugant was reintroduced in *L. lactis* 1403 by electroporation, luciferase activity levels as in the original strain were observed, indicating that there was a genuine transfer of the plasmid between *L. lactis* and *E. faecalis*.

In vivo conjugation

We performed conjugation experiments in mice, which were associated with *E. faecalis* (Fig. 1). In a single dose experiment, the titer for the donor *L. lactis* CA33 in the faeces dropped within four days by approximately 6 logs, while the titer for the enterococci remained quite stable at

Conjugative DNA transfer in the mouse digestive tract

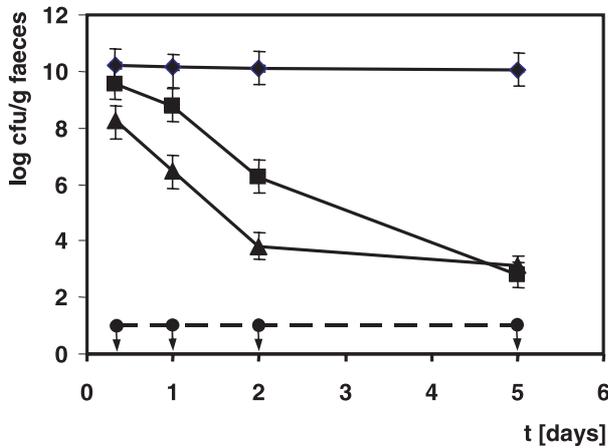


Figure 1. *In vivo* conjugation of pIL205/pCAC4 from *L. lactis* CA33 in mice associated with *E. faecalis* Z1666, single dose experiment. Diamonds indicate *E. faecalis* Z1666, squares *L. lactis* CA33, triangles *E. faecalis* Z1666/pIL205, circles *E. faecalis* Z1666/pIL205/pCAC4. Down arrows indicate values below the detection limit.

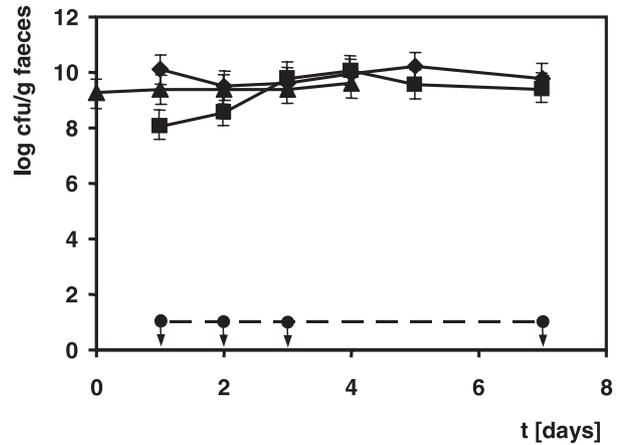


Figure 2. *In vivo* conjugation of pIL205/pCAC4 from *L. lactis* CA33 in mice associated with *E. faecalis* Z1666, continuous dose experiment (during 4 days). Diamonds indicate *E. faecalis* Z1666, squares *L. lactis* CA33, triangles *E. faecalis* Z1666/pIL205, circles *E. faecalis* Z1666/pIL205/pCAC4.

or above 1×10^{10} CFU.g⁻¹ of faeces. One day after inoculation with the donor strain, *E. faecalis* transconjugants containing pIL205 were observed at titers between 1×10^3 and 1×10^4 CFU.g⁻¹ of faeces (Fig. 1). This titer remained stable throughout the experiment. *E. faecalis* transconjugants containing pIL205 as well as pCAC4 were found on days 1 through 4 after inoculation, then decreased to a non-detectable level on day seven (Fig. 1). In order to permit the donors to interact longer or at higher numbers with the recipients, transfer was assessed with continuous dosage of the donor bacteria during 4 days (Fig. 2). By this means, the titer of *L. lactis* detected in the faeces rose from 10^8 CFU.g⁻¹ at day 1 to about 10^{10} CFU.g⁻¹ at day 4, where it leveled off. *E. faecalis* transconjugants containing pIL205 reached levels between 1×10^4 and 1×10^5 CFU.g⁻¹ faeces, while transconjugants containing pIL205 and pCAC4 remained about two logs below this titer (Fig. 2).

Conjugation experiments in mice were also made with CA31 as the donor (data not shown). A single transconjugant *E. faecalis* (CA61) was detected on day 4, harboring both the conjugative pIL205 and the mobilizable pCAC2. This low transfer frequency of the co-mobilizable plasmid was also confirmed when, in two additional series of continuous dosage of the donor strain, no further transconjugant was observed.

Plasmid stability of the CA61 *E. faecalis* transconjugant obtained *in vivo* was assessed in germfree mice. The

strain established at the same levels as observed before for the *E. faecalis* recipient. It did not lose pIL205 as well as pCAC2 plasmids for a period of two months.

Conjugative transposon

In vitro transfer

Since it is known that *L. lactis* is unable to transfer Tn916 to other hosts *in vitro*, the transferability of the modified transposon was first ascertained with a *B. subtilis* donor. This step was also useful to obtain a variety of hosts harboring Tn916-lux for subsequent experiments. The conjugation from *B. subtilis* to *L. lactis* IL1403 occurred at rates of up to 6.4×10^{-7} transconjugants/donor, which confirms that Tn916-lux can still conjugate at a detectable rate after its modification. However, when the *L. lactis* transconjugant (CA3) was used as the donor in a consecutive mating experiment with *E. faecalis* TTC00.0395 as a recipient, no transconjugant was obtained ($< 2.2 \times 10^{-10}$ transconjugants/donor).

In vivo transfer

Germfree mice were associated with *E. faecalis* TTC00.0395, which established at a titer about 10^{10} CFU.g⁻¹ of faeces. When *L. lactis* CA3 was given by a single oro-gastric intubation, more than 10^9 CFU.g⁻¹ of faeces were detected for the lactococci

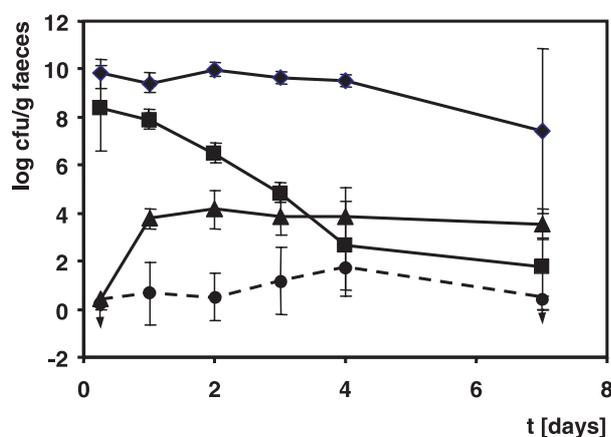


Figure 3. *In vivo* conjugation of Tn916-lux from *L. lactis* in mice associated with *E. faecalis*, single dose experiment. Diamonds indicate *E. faecalis* TTC00.0395, squares *L. lactis* CA3, triangles spores of thermoresistant *B. stearothermophilus*, circles *E. faecalis* TTC00.0395::Tn916-lux. Down arrows indicate values below the detection limit.

after an 8-hour transit (Fig. 3). This number decreased by approximately seven logs within a five-day period, similar to the titer of the *B. stearothermophilus* spores, which were used as indicators for passive transit. No transconjugant *E. faecalis* was detected during this time. Similarly, *L. lactis* was given orally continuously during 4 days (Fig. 4). Even under these potentially more promiscuous circumstances than the single inoculation, no enterococcal transconjugant was detected (Fig. 4). These observations were also true for an identical experiment where *E. faecalis* Z1666 was established as an alternative recipient strain (data not shown).

DISCUSSION

Our purpose was to determine an upper limit for HGT from GM LAB to a potential recipient in the DT. The two basic genetic elements used in this work (*i.e.* the helper/mobilizable plasmid system and the conjugative transposon Tn916 derivative) have a broad host-range which makes them attractive tools for the construction of GM bacteria into which heterologous genetic material cannot be introduced by other current techniques (Bertram et al., 1991; Langella and Chopin, 1989; Poyart et al., 1995). Similar genetic elements could be used in the future for some applications leading to a release into open environments.

The helper/mobilizable plasmid system first investigated in this study was recently proposed for genetic engineering of non-transformable bacteria

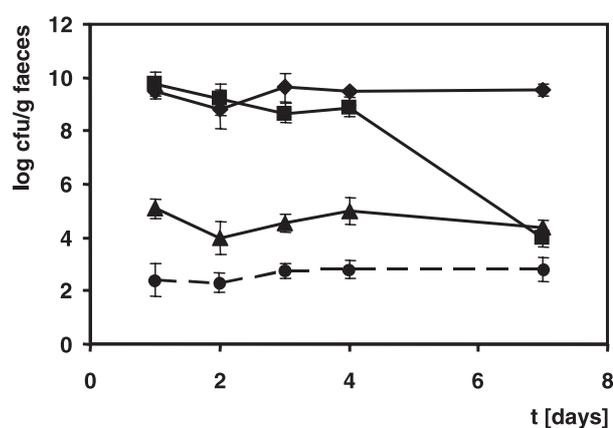


Figure 4. *In vivo* conjugation of Tn916-lux from *L. lactis* in mice associated with *E. faecalis*, continuous dose experiment (during 4 days). Diamonds indicate *E. faecalis* TTC00.0395, triangles *L. lactis* CA3 in the drinking bottle, squares *L. lactis* CA3 in the faeces, circles *E. faecalis* TTC00.0395::Tn916-lux. Down arrows indicate values below the detection limit.

(Langella et al., 1993; Hickey et al., 2001; Thompson et al., 2001). The conjugative helper plasmid encodes all functions necessary for the transfer to a wide variety of recipient bacteria (Bougueleret et al., 1981; Buu-Hoi et al., 1984; Clewell, 1981; Engel et al., 1980; Gonzalez and Kunka, 1983; Pucci et al., 1988). The results from our *in vitro* experiments with this system indicate that it is indeed active, even when the co-mobilizable plasmid has been modified by the insertion of the *lux* genes. The mobilization rates observed in our transfer experiments to *E. faecalis* are lower than the rates observed in intraspecies conjugations with *L. lactis* (5.2×10^{-3} transconjugants per donor; Langella et al., 1993). Although differences in the experimental conditions may be at stake, this could also be due to the transfer to a heterologous host or to the insertion of the foreign luciferase genes into the mobilizable construct. Our observations for the *in vivo* experiments showed that the native helper plasmid pIL205 can be introduced in all instances in to the enterococcal recipient. It is noteworthy that the fraction of the recipient population containing the conjugative plasmid, stays, once established, rather stable. It appears that there is either no secondary transfer from the enterococcal subpopulation containing the plasmid to the cells which have not acquired pIL205, or the transfer rate is in equilibrium with the loss of cells carrying the plasmid. The *E. faecalis*, which pick up the mobilizable plasmid, were found to be stable in mono-associated gnotobiotic mice, suggesting that the transconjugants have no ecological disadvantage.

The differences in transfer for the 2 constructed mobilizable plasmids are quite remarkable, although a similar effect has previously been observed in intraspecies transfers in *L. lactis*, where a 1.5 kb fragment of the *mob* region inserted in two different orientations into the mobilizable plasmid was used (2.8×10^{-4} / donor vs. 5.8×10^{-7} / donor (Langella et al., 1993). This orientation dependent effect may explain the difficulty to observe the transfer of pCAC2, which is evidently less frequently transferred than pCAC4. If the reduction is, as in this instance, also on the order of one to two logs, transfer of pCAC2 would be at the limit of detection as is here the case.

The second transfer system investigated in this work is the conjugative transposon Tn916-*lux*. Here, we establish a situation similar to a transposase-deficient minitransposon which is unable to escape its host cell. Indeed, we take advantage of *in vitro* observations that Tn916, once it has entered *L. lactis*, is unable to transfer actively to other cells due to the lack of an as yet unidentified host factor (Marra et al., 1999). Such a construct would permit an estimation of the transfer of chromosomal GM genes under optimized transfer-prone conditions. Since this situation is similar to the transfer of the transposase-less minitransposons, an upper limit for this type of constructs might be established.

No transconjugation from *L. lactis*::Tn916-*lux* to *E. faecalis* was observed *in vitro*, which is conform to the findings of Marra et al. (1999) and might lead to consider *L. lactis*::Tn916-*lux* as a suitable model for a "stable" genetic modification. The question was, if this would also apply to the situation in the DT of the *E. faecalis* associated mice. In this particular environment, the donor bacteria might eventually produce the lacking transfer factor and be able to support conjugation. However, the *in vivo* experiments did not give any transconjugants, even in continuous dosage experiments. But even if the construct is not self-transmissible out of *L. lactis*, genetic elements could be released in the environment since lysis of *L. lactis* has been described in the DT (Drouault et al., 1999). The release of the transposon DNA into the intestinal lumen may represent a risk if the naked DNA persists long enough to be taken up by other competent bacteria (Kharazmi et al., 2002), which at least was not observed for *E. faecalis* in our experiments. This might be different in a complex natural flora setting, where bacteria which can develop natural competence are also present.

CONCLUSION

We have studied the HGT from genetically modified *L. lactis* in the DT of mice under conditions considered to

be some of the most favorable for the transfer in order to determine an upper limit of transfer rates for genetically modified organisms (GMOs) in this environment, *i.e.* we have created a worst-case scenario. However, in the case of the conjugative transposon, mimicking a mini transposon-derived GMO, no transfer of the element to *E. faecalis* was observed. For the plasmid model, a complete donor system (conjugative and mobilizable plasmids) was developed. Only few transconjugants were actually observed *in vivo*, but the population remains stable in the DT without any selection by antibiotic pressure. The risk of transfer thus appears very low but could not be excluded. It will now be necessary to study the transfer of these plasmids in mice associated with a complex human flora in order to find out if the gene transfer is reduced or enhanced by the autochthonous human flora.

MATERIALS AND METHODS

Bacterial strains and genetic constructions are listed in Table 1. The system for HGT by plasmid conjugation consists of two plasmids: the *tra*⁺ *mob*⁺ Cm^r helper plasmid pIL205, a derivative of the *Enterococcus agalacticae* wide host range plasmid pIP501 (Langella et al., 1993) and the following construct. The P170 promoter from *L. lactis* was amplified from pAMJ295, a derivative of pAMJ752 (Madsen et al., 1999) with the primers D170 (TCCCCCGGGGACAAGCTTTTCGCGAGCTCGAG) and F170 (GCTCTAGAGCCGATCCTACTAGACAACAAAATAG) (Madsen et al., 1999) and cloned as a *Sma*I-*Bam*HI fragment in the pBSIIKS- vector (Stratagene). The resulting plasmid was inserted after linearization with *Sa*II into pNLter7 (which carries *lux* genes), giving pNL2951a. The pBS vector of this plasmid was removed by digestion with *Sac*I and replaced by the *Sac*I linearized plasmid pIL1001, which carries the 2.2 kb *mob* region from pIP501 on a pBS KSII+ backbone. Plasmids with the two different relative orientations of the fragments were obtained and named pCAC2 and pCAC4, which were transferred by electroporation into *L. lactis* IL1403. The constructs carrying the *luxAB* genes are co-mobilizable but not self transmissible pAMβ1 derivatives (Renault et al., 1996). *L. lactis* containing the non-self transmissible but co-mobilizable pCAC2 or pCAC4 respectively were mated with *L. lactis*/pIL205 in order to construct strains, which contain the complete transfer system, *i.e.* both the helper and the mobilizable plasmids. This was achieved at rates of 1.3×10^{-3} transconjugants per donor for *L. lactis*/pCAC2 as recipient and at 2.3×10^{-3} transconjugants per donor for *L. lactis*/pCAC4 as

Table 1. Bacterial strains and genetic constructions.

Strain/Plasmid	Description	Source or reference(s)
JIM5245	<i>B. subtilis</i> ::Tn916- <i>lux</i>	Goupil-Feuillerat et al., 2000
IL1403	<i>L. lactis</i> wild type	Chopin et al., 1984
IL3567	<i>E. coli</i> TG1/pIL1001	Langella et al., 1993
IL1776	<i>L. lactis</i> 1403 /pIL205	Langella et al., 1993
TTC00.0395	<i>E. faecalis</i>	J. van der Vossen, TNO, The Netherlands
Z1666	<i>E. faecalis</i>	P. Tailliez, INRA, France
pIL1001	2.2 kb <i>mob</i> from pIL205 in pBS	Langella et al., 1993
pNL2951a	P170 _{<i>L. lactis</i>} :: <i>luxAB</i> _{<i>Vibrio harveyi</i>} in pNL ter7S	this work and Madsen et al., 1999 Renault et al., 1996
CA3	<i>L. lactis</i> 1403::Tn916- <i>lux</i>	this work
CA25	<i>L. lactis</i> 1403/pCAC2	this work
CA27	<i>L. lactis</i> 1403/pCAC4	this work
CA31	<i>L. lactis</i> 1403/pIL205/pCAC2	this work
CA33	<i>L. lactis</i> 1403/pIL205/pCAC4	this work
CA61	<i>E. faecalis</i> Z1666/pIL205/pCAC2	this work

recipient, rates, which are comparable to the range reported earlier for the transfer of pIL205 in intraspecies conjugations with *L. lactis* strains (Langella et al., 1993). The Tn916-*lux* luciferase-targeted transposon contains the transcriptional fusion of the promoter for the acetolactate decarboxylase gene from *L. lactis* and the luciferase genes from *Vibrio harveyi* (*aldBp*::*luxAB* Em^r) inserted into the *Hind*III site of the *tetM* gene from Tn916 (Corthier et al., 1998; Goupil-Feuillerat et al., 2000; Renault et al., 1996). *Bacillus subtilis* JIM 5245 containing Tn916-*lux* was used as initial donor of conjugative transposons.

Luciferase tests

Luciferase activity was measured on liquid samples as previously described (Corthier et al., 1998). Alternatively, the presence of the *lux* genes was confirmed by PCR using the primers CA203 (5' CTCACCTATCAGCCACCTGAG 3') and CA204 (5' CATTACGTATGAGTCGTACC 3'), which hybridized to *luxA* to give a product of 748 nt.

Culture conditions

Lactococcus lactis was grown at 30 °C either in static liquid cultures or on plates with M17glc (5 g glucose per liter) as growth medium (Terzaghi and Sandine, 1975). Cultures of *E. faecalis* were similarly grown as static

liquid cultures or on plates at 42 °C either on the same medium or MRS (DIFCO). When necessary, *L. lactis* or *E. faecalis* were incubated in anaerobic jars (GENbox anaer, bioMérieux, France); otherwise, all plates were incubated aerobically. Media for plates were solidified with 12 g of agar per liter. Selection for the presence of plasmids was done at 10 µg.mL⁻¹ of chloramphenicol (Cm) (pIL205) or erythromycin (Em) (pCAC2 and pCAC4) respectively and with 5 µg.mL⁻¹ of Em for Tn916-*lux*. Liquid *E. coli* and *B. subtilis* cultures were grown with agitation in LB at 37 °C. Thermoresistant *B. stearothermophilus* spores were used as transit markers and activated at 60 °C in G-spores medium according to the procedure described by Contrepois and Gouet (1969) for enumeration.

Conjugation procedures

Plasmid conjugation

For plasmid transfer from *L. lactis* to *E. faecalis*, 1 mL each of a fresh overnight culture was harvested by centrifugation and washed once with LCY (Liquid Casein Yeast extract; Raibaud et al., 1966). The pellets were suspended in 100 µL of media and subsequently mixed. 100 µL of the cell suspension was deposited in a square of 0.86 cm² on a GN-6 Metricel gridded cellulose filter (Pall Corporation, Michigan, USA) placed on a MRS plate. Following incubation at 30 °C for 18 h, cells

were recovered from the filter by vortex mixing in 1 mL LCY, and appropriate dilutions were plated for selection and enumeration of parent and transconjugant cells. Titters for donor cells were determined on M17glc-Em-Cm plates with aerobic incubation at 30 °C. In contrast, recipient and transconjugant titers were determined on MRS selection plates aerobically incubated at 42 °C, a non-permissive growth condition for *L. lactis*. Selection conditions for transconjugants were as follows: Cm-containing medium was used to evaluate the transfer of pIL205 only, Em-containing medium for the transfer of the mobilizable plasmid, and Cm-Em-containing medium for the transfer of both plasmids.

Conjugative transfer of the Tn916-lux

Transposon transfer and selection for transconjugants were essentially performed as for plasmid conjugation except that no filter system was used, *i.e.* the parent cell mix was directly deposited on the plate in a spot of about 20 mm diameter. Transfers from *B. subtilis* to *L. lactis* were performed on M17glc plates. Titters for donor cells were determined on LB-Em and aerobic incubation at 42 °C, while recipient titers were determined on M17glc plates with anaerobic incubation at 30 °C. Transconjugants were selected on M17glc-Em plates anaerobically incubated at 30 °C. For transfers from *L. lactis* to *E. faecalis*, the selection for transconjugants was on MRS-Em at 42 °C in aerobic conditions, while for transfers from *B. subtilis* to *E. faecalis* the selection for transconjugants was on MRS-Em at 42 °C in anaerobic jars.

Animal model

Germfree C3He/J mice were reared in sterile Trexler-type isolators (La Calhène, Vélizy, France) fitted with a rapid transfer system, in an environmentally controlled room (21 °C) with a 12 h light-dark cycle. Mice had free access to irradiated food (UAR, Villemoisson, France) and sterilized water. Germfree adult male mice were used throughout the investigations. Bacteria were given to the mice for single dose experiments by oro-gastric intubations of 0.4 mL of washed concentrated overnight cultures resuspended in LCY, while for continuous dosage experiments the drinking water supply was replaced by bacterial solutions in LCY, which keeps the bacteria alive in the drinking bottle, but permits no multiplication. The bacterial suspensions were replaced daily by fresh solutions.

For each experiment four mice were inoculated per experiment and the faeces were collected and plated individually from at least three animals. Results are presented in the Figures with the standard error to the mean.

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