

Flow Cytometric Testing of Green Fluorescent Protein-Tagged *Lactobacillus rhamnosus* GG for Response to Defensins

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Received 4 November 2005/Accepted 15 April 2006

Lactobacillus rhamnosus GG is of general interest as a probiotic. Although *L. rhamnosus* GG is often used in clinical trials, there are few genetic tools to further determine its mode of action or to develop it as a vehicle for heterologous gene expression in therapy. Therefore, we developed a reproducible, efficient electroporation procedure for *L. rhamnosus* GG. The best transformation efficiency obtained was 10⁴ transformants per µg of DNA. We validated this protocol by tagging *L. rhamnosus* GG with green fluorescent protein (GFP) using the nisin-controlled expression (NICE) system. Parameters for overexpression were optimized, which allowed expression of *gfp* in *L. rhamnosus* GG upon induction with nisin. The GFP⁺ strain can be used to monitor the survival and behavior of *L. rhamnosus* GG in vivo. Moreover, implementation of the NICE system as a gene expression switch in *L. rhamnosus* GG opens up possibilities for improving and expanding the performance of this strain. The GFP-labeled strain was used to demonstrate that *L. rhamnosus* GG is sensitive to human beta-defensin-2 but not to human beta-defensin-1.

Increasing consumer awareness of health-promoting intestinal bacteria has increased the use of probiotic bacteria as functional ingredients in certain foods, most commonly dairy products (19). Probiotics are defined as live microorganisms which, when adequate amounts are administered, provide a health benefit to the host beyond basic nutrition (11, 12). Although the concept of probiotics is becoming established in medicine and the food and feed industry, the underlying scientific framework needs to be strengthened (44). A great number of trials have suggested beneficial effects of probiotics (10, 22, 24, 32, 41, 45). Nevertheless, the basic molecular mechanisms underlying the observations have not been completely determined yet (40, 44). A prerequisite for thoroughly addressing the mode of action of a probiotic strain is amenability to genetic analysis (46).

Lactobacillus rhamnosus GG (= ATCC 53103) (53), which was isolated from a naturally occurring healthy human gut flora, is one of the most extensively studied probiotic organisms (16, 23, 49). However, our understanding of the physiology and genetics of this bacterium is still limited, mainly due to the absence of a reliable transformation procedure. In the past decade electroporation has become the method of choice for transforming lactobacilli (34). The efficiencies of electroporation between different *Lactobacillus* species and even between strains belonging to the same species can vary significantly (34). Another difficulty in the development of a transformation procedure is choosing the plasmid used. Indeed, the ability of a plasmid to replicate and to express its selectable marker in a given species cannot be predicted and influences the transformation frequency (52). The objective of the present study was

to develop a reproducible electroporation procedure for *L. rhamnosus* GG. We adapted the previously described procedure for electrotransformation of *L. rhamnosus* 1/6 (56). The optimized protocol was used to increase the arsenal of genetic tools for *L. rhamnosus* GG with the green fluorescent protein (GFP). The first report describing GFP labeling of *Lactobacillus* was made by Geoffroy et al. in 2000 (15). Since then, other GFP-labeled lactic acid bacteria have been described (17, 25, 43). A GFP-labeled *Lactobacillus plantarum* strain was obtained by using the nisin-controlled expression (NICE) system initially developed for *Lactococcus lactis* (4). After addition of subinhibitory amounts of the signaling molecule nisin (7), the two-component NisRK system drives expression of GFP under control of the nisin-inducible *nisA* promoter (4). The NICE system has been used successfully for a variety of lactic acid bacteria (9, 26, 29, 42). In this study, we used the optimized electroporation protocol to implement the NICE system in *L. rhamnosus* GG in order to tag *L. rhamnosus* GG with the green fluorescent protein. The GFP-tagged strain was used to determine the sensitivity of *L. rhamnosus* GG to human beta-defensins.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. All strains were grown at 37°C. *L. rhamnosus* GG was inoculated from a glycerol stock (−80°C) into Man-Rogosa-Sharpe (MRS) medium (Difco) and grown in static conditions. *Escherichia coli* was grown with aeration in Luria-Bertani broth (47). When appropriate, antibiotics were added to the culture medium. For *Lactobacillus*, erythromycin and chloramphenicol were used at final concentrations of 5 and 10 µg/ml, respectively. In case of *E. coli*, antibiotics were supplied at following final concentrations: ampicillin, 100 µg/ml; kanamycin, 30 µg/ml; erythromycin, 250 µg/ml; and chloramphenicol, 30 µg/ml.

Molecular methods. Standard protocols were used for buffer preparation, cloning, plasmid isolation, preparation of *E. coli* competent cells, and transformation (47). Restriction enzymes were used according to the manufacturer's instructions. Southern hybridization was carried out using a nonradioactive

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TABLE 1. Bacterial strains, plasmids, and primer sequences used for PCR

Strain or plasmid	Relevant genotype and/or phenotype ^a	Source and/or reference
Strains		
<i>E. coli</i> TOP10F ^r	F ^r [<i>lacI</i> ^r Tn10(Tet ^r)] <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>lacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>deoR</i> <i>recA1</i> <i>araD139</i> Δ(<i>ara-leu</i>)7697 <i>galU</i> <i>galk</i> <i>rpsL</i> (Str ^r) <i>endA1</i> <i>nupG</i>	Invitrogen
<i>E. coli</i> GM2163	Dam ⁻ /Dcm ⁻ ; F ⁻ <i>ara-14</i> <i>leuB6</i> <i>hhuA31</i> <i>lacY1</i> <i>tsx78</i> <i>glnV44</i> <i>galk2</i> <i>galt22</i> <i>mcrA</i> <i>dcm-6</i> <i>hisG4</i> <i>rfbD1</i> <i>rpsL136</i> (Str ^r) <i>dam13::Tn9</i> (Cam ^r) <i>xylA5</i> <i>mtl-1</i> <i>thi-1</i> <i>mcrB1</i> <i>hsdR2</i> ; Cm ^r	New England BioLabs Inc.
FAJ1905	<i>L. rhamnosus</i> GG with chromosomal insertion of pMEC10 in the <i>attB</i> sequence of phage mv4; Ery ^r	This study
FAJ1906	<i>L. rhamnosus</i> GG with chromosomal insertion of pFAJ1934 in the <i>attB</i> sequence of phage A2; Ery ^r	This study
<i>L. casei</i> ATCC 393	Wild type; cheese isolate	American Type Culture Collection, Rockville, MD
<i>L. rhamnosus</i> 1/6	Wild type; cheese isolate	Valio Ltd.
<i>L. rhamnosus</i> GG	Wild type; human isolate	ATCC 53103 (53)
Plasmids		
pEM40	pUC19E-derived integration vector (<i>attB</i> located at the 3' end of the tRNA ^{Leu} locus) containing a 1.6-kb <i>int-attP</i> cassette of phage A2; Ery ^r Amp ^r	1
pFAJ1934	2,634-bp PCR fragment (flanking EcoRI sites) carrying the <i>nisRK</i> genes of pMEC10 cloned into the EcoRI site of pEM40 downstream of the <i>ery</i> gene in the opposite orientation; Ery ^r Amp ^r	This study
pGK13	Shuttle vector between <i>E. coli</i> and <i>Lactococcus lactis</i> ; pWV01 replicon; Ery ^r Cm ^r	27
pLAB1301	<i>L. hilgardii</i> pLAB1000 replicon; shuttle vector between <i>E. coli</i> and <i>L. plantarum</i> ; Ery ^r Cm ^r	21
pMEC10	Integration plasmid (<i>attB</i> located at the 3' end of the tRNA ^{Ser} locus) containing <i>int-attP</i> cassette (of phage mv4) of pMC1 cloned into pNZ9500 (pUC19 derivative carrying a 2.7-kb chromosomal DNA fragment of <i>L. lactis</i> NZ9700 containing the 3' end of <i>nisP</i> and <i>nisRK</i>); expression driven by <i>ery</i> read-through; Ery ^r	42
pMEC30	<i>L. hilgardii</i> pLAB100 replicon; <i>gfp_{uv}</i> cloned in expression vector containing the constitutive <i>ldhL</i> promoter from <i>L. plantarum</i> ; Ery ^r Amp ^r	15
pMEC45	<i>L. lactis</i> pSH71 replicon; pNZ8037 derivative with <i>gfp_{uv}</i> cloned downstream of the <i>nisA</i> promoter from <i>L. lactis</i> NZ9800; Cm ^r	15
pNZ8008	pNZ273 (pSH71 replicon) carrying the <i>E. coli</i> <i>gusA</i> reporter gene transcriptionally fused to the <i>nisA</i> promoter; Cm ^r	28, N1Z0
pRV85	<i>gfp_{uv}</i> cloned downstream of the <i>L. sakei</i> <i>ldhL</i> promoter in the replicative plasmid pG+host5; Ery ^r	17

^a *gfp_{uv}*, synthetic GFP gene (with improved codon usage) that was subsequently subjected to recursive cycles of DNA shuffling, thereby selecting for bright fluorescence that could be observed under UV light.

labeling and detection system, as previously described (47). DNA sequences were determined by the dideoxynucleoside triphosphate chain termination method (48) with an AutoRead sequencing kit (Pharmacia-LBK) and an automated sequencer (ALX; Pharmacia-LBK). PCR primers were purchased from Eurogentec. PRO-12 (5'-ATGAATTCGGTCAGGATGATGCTATTAACC-3') and PRO-13 (5'-ATGAATTCGCACCGTTAACTCCTAATAAACC-3') (EcoRI recognition sites are underlined) were used for construction of pFAJ1934 (see below); RHI-144 (5'-ATTAGAAATGAGAACTATGAAGTTGC-3') and RHI-145 (5'-TACTCCTAATCTGATAAATATACTCCG-3') are homologous to the 5' and 3' regions of *nisR*; RHI-146 (5'-AATTTTCAAGCCGTTCAAAGATTG C-3') and RHI-147 (5'-ATTAACAAAGATATTGATAAGTGCTCG-3') are homologous to the 5' and 3' regions of *nisK*; and RHI-148 (5'-AACTTTTCAC TGGAGTTGTCC-3') and RHI-149 (5'-TAATGGTCTGCTAGTTGAACG-3') are homologous to the 5' and 3' regions of *gfp_{uv}*. If single colonies of *L. rhamnosus* GG were used, the colony smeared in a PCR tube was heated in a microwave oven at full power for 3 min before the PCR mixture was added. The PCR was carried out with a Personal Mastercycler (Eppendorf) used according to the manufacturer's instructions. Integration of pMEC10 at the tRNA^{Ser} locus and integration of pFAJ1934 at the tRNA^{Leu} locus were confirmed by PCR and Southern blotting, as previously described (1, 8). The following primers were used: PRO-567 (5'-GTCGACACAGGATTTGAACC-3'; corresponding to primer T2 [8]) and PRO-570 (5'-CAAGCCAACAGACGTGCAAGCA-3'; binding to the 3' end of the *int* gene [8]) for tRNA^{Ser} integration and PRO-564 (5'-AGCAGGACGAGAAAGCAATGAATGT-3'; corresponding to primer att1 [1]) and PRO-565 (5'-GCCGGTGTGGCGGAATTGGCAG-3'; corresponding to primer att7 [1]) for tRNA^{Leu} integration.

Construction of pFAJ1934. The *nisRK* genes were amplified by PCR with the proofreading *Pfx* enzyme (Invitrogen) used according to the manufacturer's instructions and primers PRO-12 and PRO-13, using pMEC10 as the template DNA. The 2,634-bp fragment obtained, containing the *nisR* promoter, was digested with EcoRI and ligated into the corresponding unique site of pEM40. PCR, restriction, and sequence analysis confirmed the localization of *nisRK* downstream of the erythromycin resistance (*ery*) gene. The plasmid was designated pFAJ1934 and introduced into *E. coli* for propagation and subsequent plasmid DNA isolation. In pFAJ1934, the *nisRK* genes are localized downstream of the *ery* gene of pEM40, in the opposite orientation. This orientation of the *nisRK* genes prevents read-through transcription of *ery*, which lacks a functional transcription terminator (42) and allows low levels of expression of *nisRK*, which is required for a functional inducible expression system. Indeed, in order to retain the inducibility to nisin without a significant increase of basal activity, low levels of expression of *nisRK* are necessary (42).

Preparation of *L. rhamnosus* GG electrocompetent cells. An overnight culture of *L. rhamnosus* GG was serially diluted (10²- to 10⁶-fold) into freshly prepared prewarmed MRS medium supplemented with 2% glycine and was incubated without agitation at 37°C. After overnight growth, 5 ml of the culture in the exponential growth phase (optical density at 600 nm [OD₆₀₀], 0.8 to 1) was inoculated into 100 ml of freshly prepared, prewarmed MRS medium supplemented with 2% glycine. The resulting culture, which was kept in a tightly closed 100-ml Duran bottle (to minimize oxygen transfer), was incubated without agitation at 37°C. When the OD₆₀₀ was 0.2 to 0.3, ampicillin was added to obtain a final concentration of 10 μg/ml (57). Then the cells were incubated until the OD₆₀₀ was 0.4 to 0.5. This optical density should have been reached within 3 to

4 h after the initial inoculation of *L. rhamnosus* GG in order to obtain efficient competent cells. Cells were harvested by centrifugation at room temperature (10 to 15 min, 6,000 × g). The cells were washed twice at room temperature with electroporation buffer (0.5 M sucrose, 7 mM potassium phosphate [pH 7.4], 1 mM MgCl₂) (2), resuspended in 1 ml of the same buffer, and placed on ice. The electrocompetent cells were used immediately for electroporation. After 16 h of freezing at -80°C, a slight decrease in the electrocompetency of the cells was noticed, but after 1 week of storage, no transformants could be obtained.

Electroporation of *L. rhamnosus* GG. A mixture containing 100 µl of a cooled cell suspension and 400 ng of DNA (maximum volume, 4 to 5 µl) was transferred into a precooled electroporation cuvette (Eurogentec) with a 0.2-cm electrode gap and immediately electroporated (Gene Pulser; Bio-Rad Laboratories) by using the following settings: peak voltage, 1.7 kV; capacitance, 25 µF; and parallel resistance, 200 Ω. Following the pulse, the cells were immediately diluted with 5 ml of MRS medium containing 2 mM CaCl₂ and 20 mM MgCl₂ and incubated (without agitation) at 37°C for 3 h before they were plated onto MRS agar containing the appropriate antibiotic. The transformation efficiency was assessed by determining the number of transformants per microgram of plasmid DNA after anaerobic incubation (BBL GasPak system) at 37°C for 48 to 72 h. Transformants were validated either by restriction digestion (after plasmid isolation and back-transformation to *E. coli*) or by colony PCR.

Isolation of *L. rhamnosus* GG DNA. Total DNA of *L. rhamnosus* GG was isolated as previously described for *Rhodococcus* (36), with following modifications. Prewarmed MRS medium (freshly prepared) was inoculated with ca. 2 × 10⁸ CFU of an overnight culture of *L. rhamnosus* GG. After 2 to 3 h of growth without agitation at 37°C, 600 µg/ml ampicillin was added to the culture, which was then incubated for another 2 to 3 h. Bacterial cells were washed with Tris-EDTA buffer (10 mM Tris, 1 mM EDTA; pH 8.0) and treated with lysozyme (2 mg/ml; Sigma) at 37°C for 30 min. The cells were lysed further by addition of sodium dodecyl sulfate (final concentration, 1%) and NaCl (final concentration, 1 M). RNase A (final concentration, 35 µg/ml; Sigma), prepared as described by Sambrook et al. (47), was added, and this was followed by 15 min of incubation at 55°C. The remainder of the protocol has been described previously (36). The precipitated DNA was resuspended at 4°C for 48 to 72 h. Total DNA was stored at 4°C. For isolation of plasmid DNA from *L. rhamnosus* GG, the protocol used for isolation of total DNA was used, with some minor adaptations. After sodium dodecyl sulfate and NaCl were added, the mixture was incubated at -20°C for 1 h. Cell wall components and degraded total DNA were removed by centrifugation, after which the procedure used for isolation of total DNA was used.

Microtiter plate β-glucuronidase assay to optimize nisin induction. Nisin-induced expression of *gusA* (pNZ8008) (28) was optimized essentially as described by Pavan et al. (42), except that strain *L. plantarum* NCIMB8826 was replaced by *L. rhamnosus* GG FAJ1905. pNZ8008 (28) was introduced into FAJ1905 by using the optimized electroporation protocol described above. β-Glucuronidase assays were performed by the method described by de Ruyter et al. (4), modified as described by Miller (35), which resulted in the following optimized microtiter plate-based protocol. After appropriate induction, 1 ml of cells was harvested by centrifugation (5 min, 5,000 × g) and resuspended in 500 µl of NaPi buffer (50 mM NaHPO₄, pH 7.0). After the OD₅₉₅ of 135 µl was determined, cell suspensions were frozen at -80°C in 10-µl aliquots in 96-well plates until they were assayed for β-glucuronidase activity. To each 10-µl cell suspension, 90 µl of GUS buffer (50 mM NaHPO₄ [pH 7.0], 14.3 mM β-mercaptoethanol, 1 mM Na₂EDTA, 0.1% Triton X-100, 0.1% sodium laurylsarcosine, 25 mM *p*-nitrophenyl-β-D-glucuronic acid [PNPG] [Sigma]) was added. The mixture was incubated at 37°C. The reaction was stopped by adding 35 µl of a 1 M Na₂CO₃ solution after sufficient yellow color had developed. The reaction was stopped at at least three different times (replicates) to ascertain that enzyme activity was still linearly increasing with incubation time. The reaction time was recorded. Both OD₄₁₅ and OD₅₉₅ were determined. Identical treatments were performed with NaPi buffer but without cells as controls to correct measured sample values. Miller units (MU) of β-glucuronidase activity were defined as 1,000 times the increase in absorbance at 415 nm per minute per unit of optical density at 595 nm of the cell suspension, as follows: MU = 1,000 × {(OD_{415-PNPG} - 1.75 × OD_{595-PNPG}) × v₁}/(t × v_t × OD₅₉₅), where *t* is the time of the reaction (in minutes), OD₅₉₅ is a measure of cell density just before the assay, OD_{415-PNPG} is the absorbance by PNPG measured after the reaction, OD_{595-PNPG} is a measure of the cell density after the β-glucuronidase reaction (used as a correction for light scattering by cell debris), 1.75 is the corresponding correction factor, v₁ is the volume (in µl) of cells used in the reaction mixture, and v_t is the total volume (in µl) of the reaction mixture. The statistical analysis was based on at least three independent repetitions, as designed in the optimization experiment. Optical densities were determined with a microtiter plate reader (VERSAmax; Molecular Devices).

Expression of *gfp* under control of the nisin promoter. Expression of *gfp* driven by the *nisA* promoter (pMEC45) in *L. rhamnosus* GG was optimally induced as follows. An overnight culture of *L. rhamnosus* GG FAJ1905/pMEC45 or FAJ1906/pMEC45 was used to inoculate fresh, prewarmed MRS medium diluted 1:50. After 30 min of incubation at 37°C without shaking, 500 ng/ml nisin was added to the culture, which was then incubated for 2.5 to 3 h. Nisin was prepared as previously described (9).

Epifluorescence microscopy and flow cytometric analysis. Fluorescence microscopy was performed as previously described (58). Flow cytometric analysis was performed with a FACSCalibur (Becton Dickinson, Erembodegem, Belgium) equipped with a 15-mW, air-cooled argon ion laser excitation light source (488 nm), as previously described (31, 55). For two-color analysis, fluorescence compensation was used to correct for spectral overlap of the green and red signals. Data were analyzed using CellQuestPro (Becton Dickinson).

Human beta-defensin sensitivity assay. *L. rhamnosus* GG cells were induced with nisin as described above and collected by centrifugation, and ca. 10⁶ CFU was resuspended in 45 µl of sixfold-diluted Dulbecco's modified Eagle's medium (Gibco, Invitrogen) supplemented with 16.67 mM glucose. Subsequently, 5 µl of human beta-defensins (Peptides International, Inc., Louisville, KY) dissolved in 10 mM acetic acid was added. For human beta-defensin-2 (hBD2), final concentrations ranging from 0 to 12 µg/ml were tested. For hBD1, a final concentration of 12 µg/ml was used. After 3 h of incubation at 37°C, bacterial viability was measured either by plating serial dilutions on MRS agar or by flow cytometry using the green fluorescent protein-propidium iodide (PI) assay as previously described (31). Each experiment was performed in triplicate.

RESULTS AND DISCUSSION

Optimization of the electroporation protocol. The optimization strategy used for electroporation is shown in Fig. 1. Initially, pGK13 (27) was used as the DNA source, which resulted in an efficiency of 10³ CFU/µg DNA after some optimization (Fig. 1). The optimal pulse strength was 8.5 kV cm⁻¹, which is higher than the optimal pulse strength in the protocol described by Varmanen et al. (7.5 kV cm⁻¹) (56) but lower than the optimal pulse strength described by Wei et al. (12.5 kV cm⁻¹) (57), indicating that optimization of conditions for electrotransformation is necessary for each species or even for each strain of lactic acid bacteria. However, pGK13 seemed to be segregationally unstable in *L. rhamnosus* GG, and the results were not reproducible (data not shown). Plasmid pLAB1301 (21), which contains the replicon of a native plasmid isolated from *Lactobacillus hilgardii*, was used subsequently. Different electrocompetence-promoting protocols were tried, as shown in Fig. 1. Adding the cell wall weakener glycine (25) at a concentration of 2% to the preculture medium increased the transformation efficiency 10-fold. Addition of ampicillin, also a cell wall weakener (57), during the early exponential growth phase of *L. rhamnosus* GG led to a 20-fold increase in the efficiency compared to the efficiency of an identical protocol without addition of ampicillin. Addition of ampicillin was first described by Wei et al. (57) for electroporation of *Lactobacillus* isolates, and this protocol was also used by McCracken et al. (33) for electroporation of *L. rhamnosus* GG. However, in our hands, the protocol described by Wei et al. (57) did not result in efficient electroporation of *L. rhamnosus* GG (data not shown). Compared to the protocol of Wei et al. (57), we included in our optimization protocol an extra cell wall weakener, glycine, during the two culture phases, and we also changed the composition of the washing and electroporation buffer, the temperature at which the washing step was performed, and the electrical parameters used for electroporation (33, 57).

With this optimized protocol, a transformation efficiency of

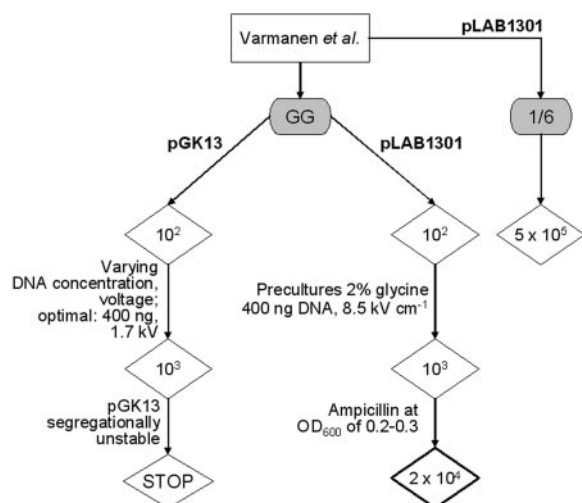


FIG. 1. Optimization of the electroporation protocol. The protocol described by Varmanen et al. (56) for electrotransformation of *L. rhamnosus* 1/6, a cheese isolate, was used as a starting point. Using the originally described protocol with *L. rhamnosus* GG resulted in an efficiency of only 10^2 CFU/ μ g DNA. By gradually varying the DNA concentration (0.2 to 3 μ g DNA) and the voltage applied (1.4 to 2.0 kV) we increased the efficiency 10-fold, and the optimal parameter combination was 400 ng DNA and 1.7 kV. However, pGK13 seemed to be segregationally unstable in *L. rhamnosus* GG and was not used further. Electroporation of *L. rhamnosus* GG and *L. rhamnosus* 1/6 using pLAB1301 resulted in reproducible efficiencies of 10^2 and 5×10^5 CFU/ μ g DNA, respectively. The resilience of the peptidoglycan layer in gram-positive bacteria complicates the development of electroporation protocols (54). The composition of the growth medium directly affects the cell wall density and thickness. Therefore, weakening the cell wall by addition of cell wall weakeners, such as glycine (18), can improve the transformation efficiency. We included two culture steps with 2% glycine in the growth medium. When 2% glycine was added to the serial dilutions of an overnight culture of *L. rhamnosus* GG, the electroporation efficiency increased 10-fold compared to the electroporation efficiency of an identical protocol without an overnight preculture supplemented with glycine. Addition of ampicillin, also a cell wall weakener (57), during the early exponential growth phase of *L. rhamnosus* GG led to a 20-fold increase in the efficiency compared to the efficiency of an identical protocol without addition of ampicillin.

10^4 *L. rhamnosus* GG transformants per μ g of pLAB1301 DNA was obtained. A similar efficiency was obtained for *Lactobacillus delbrueckii* subsp. *bulgaricus* (52), but this efficiency was still less than the efficiency obtained for *L. plantarum* (10^6 to 10^7 CFU/ μ g) (14). Perhaps the efficiency can still be improved, but 10^4 CFU/ μ g DNA is sufficient to select for double homologous recombination events, as described by Varmanen et al. (56) for the construction of a *L. rhamnosus* 1/6 *pepR* mutant. Here, we validated the protocol by overexpressing *gfp* under control of the NICE system, resulting in GFP-labeled *L. rhamnosus* GG that could be sorted by fluorescence-activated cell sorting and be used in other applications.

Expression of *gfp* in *L. rhamnosus* GG driven by constitutive promoters. Using the optimized transformation protocol, we introduced pMEC30 (15) (a pLAB1301 derivative containing *gfp_{uv}* under control of the constitutive *ldhL* promoter from *L. plantarum*) into *L. rhamnosus* GG (transformation efficiency, 10^4 CFU/ μ g DNA). pMEC30 could be successfully maintained in *L. rhamnosus* GG, as verified by PCR and plasmid isolation, and subsequently reelectroporated into *E. coli*. Although

pMEC30 was functional in *E. coli*, no fluorescent phenotype was observed in *L. rhamnosus* GG.

pRV85 was used previously to tag *Lactobacillus sakei* with GFP (17). pRV85 (17) could be successfully transferred to *L. rhamnosus* GG, as confirmed by PCR and Southern analysis (data not shown). *L. rhamnosus* GG cells bearing pRV85 appeared as green fluorescent bacteria in a fluorescence microscopic analysis, albeit with a low intensity (data not shown). However, pRV85 is a replication-thermosensitive derivative of pWV01 and replicates in gram-positive bacteria at 28°C and is lost at temperatures above 37°C (3). Expression of *gfp* from *pldhL* in *L. sakei* was reported to increase when the culture temperature was decreased (17). In view of future applications, optimization of *gfp* expression by altering the growth temperature of *L. rhamnosus* GG, which prefers 37°C for growth, was not pursued.

Also, in *L. plantarum* *gfp* expression from a constitutive promoter appeared not to be feasible (15). We attempted to express *gfp* under control of a nisin-inducible promoter, which was previously reported to be successful in *L. plantarum* (15).

Expression of *gfp* in *L. rhamnosus* GG through implementation of the NICE system. The *nisRK* genes with their own promoter were integrated into the chromosome of *L. rhamnosus* GG. Site-specific integration was accomplished by electroporating the suicide plasmid pMEC10 (42) into *L. rhamnosus* GG. It is noteworthy that successful integration was obtained only with pMEC10 DNA isolated from *E. coli* GM2163, a *Dam*⁻ *Dcm*⁻ strain (unmethylated DNA). Putative integrants, which were able to grow on erythromycin, were analyzed by PCR and Southern hybridization (data not shown). No plasmid DNA containing *nisRK* could be reisolated. One integrant, FAJ1905, exhibited the expected tRNA^{Ser} integration patterns and was used for further manipulation.

In parallel, we integrated *nisRK* into the *L. rhamnosus* GG chromosome by an alternative method using plasmid pFAJ1934. The *nisRK* genes were cloned in pEM40, a nonreplicative integration vector containing the A2-specific recombination cassette *int-attP* (1). Bacteriophage A2 integrates at the 3' end of the tRNA^{Leu} locus. This attachment site allowed integration of A2 *attP*-containing vectors into the genomes of *Lactobacillus casei* and *Lactobacillus paracasei* (1), both of which are closely related to *L. rhamnosus* GG. Transformation of *L. rhamnosus* GG with pFAJ1934 and selection for erythromycin-resistant colonies yielded putative integrants. PCR and Southern hybridization confirmed that there was chromosomal integration of pFAJ1934 at the corresponding tRNA loci in these clones (data not shown). One integrant that had the correct structure was selected and designated FAJ1906.

The two integration plasmids supported successful chromosomal insertion at permissive sites based on the integration properties of a bacteriophage. This method provides an efficient way to generate stable heterologous DNA integration in *L. rhamnosus* GG.

Subsequently, pMEC45 (15), the expression vector containing *gfp* under control of the *nisA* promoter, was electroporated into FAJ1905 and FAJ1906. Transformants were verified by PCR analysis (data not shown).

In previous applications of the NICE system in different species the workers stressed the need for optimization of nisin induction conditions in each host separately (5, 9, 42). The

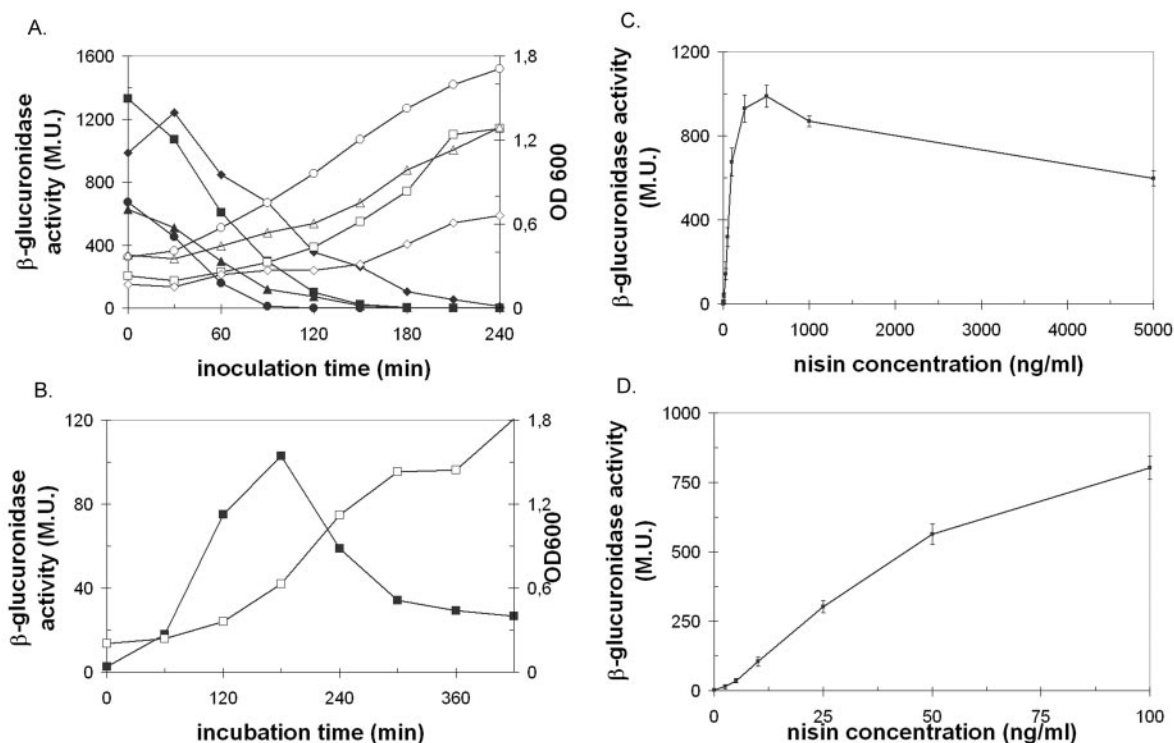


FIG. 2. β -Glucuronidase activity of FAJ1905/pNZ8008 cell extracts. (A) β -Glucuronidase activity (solid symbols) was determined during growth (open symbols) in different conditions (squares, 1/50 inoculum and incubation at 37°C; triangles, 1/20 inoculum and incubation at 30°C; circles, 1/20 inoculum and incubation at 37°C; diamonds, 1/20 inoculum and incubation at 30°C) after induction with 50 ng of nisin/ml at different times after inoculation. The β -glucuronidase activity was measured after 2.5 h of contact with nisin. (B) β -Glucuronidase activity (■) after different periods of contact with 50 ng nisin/ml. The OD₆₀₀ (□) was also determined. (C) Dose-response curve for *gusA* expression in FAJ1905 harboring pNZ8008 induced with different concentrations of nisin (0 to 5,000 ng/ml). (D) β -Glucuronidase activity with nisin concentrations ranging from 0 to 100 ng/ml. β -Glucuronidase activity is expressed in Miller units (M.U.), as described in Materials and Methods. The error bars indicate standard deviations.

optimal growth phase for nisin induction, the optimal contact time with the inducer, and the optimal nisin concentration for induction were determined as described previously (42). To do this, we used the β -glucuronidase activity of FAJ1905/pNZ8008 as a reporter for nisin induction, concomitantly optimizing a user-friendly protocol for analysis of *gusA* expression in *L. rhamnosus* GG (see Materials and Methods). The results are summarized in Fig. 2. As Fig. 2 shows, the maximal levels of β -glucuronidase activity were obtained when the inducer was added early in the exponential phase, 30 min after inoculation (1/50 inoculum; 37°C), after 2.5 to 3 h of induction. Similar to the situation in *L. plantarum* (42), *L. rhamnosus* GG was most receptive to nisin when it was added very early in the exponential growth phase; in contrast, in *L. lactis* addition in the middle of the exponential growth phase was more effective (4).

The inoculation and incubation times were optimized as described by Pavan et al. (42), using 50 ng/ml nisin. However, the maximum levels of β -glucuronidase activity were observed after induction with 500 ng/ml (final concentration) nisin. In retrospect, this concentration may have been more appropriate in our study for optimizing the inoculation and incubation times. Remarkably higher doses of the inducer are necessary for *L. rhamnosus* GG (250 to 500 ng/ml) than for *L. lactis* (1 to 5 ng/ml) (5) or *L. plantarum* (20 to 25 ng/ml) (42). The optimal

nisin concentrations for *L. rhamnosus* GG are in the same range as the concentrations used for induction in *Streptococcus* species (1 to 5 μ g/ml) (9). In *Bacillus subtilis* and *Enterococcus faecalis*, concentrations as high as 20 μ g/ml are necessary, and these concentrations are just below the inhibitory level (9). In addition, the optimal contact times with the inducer are dif-

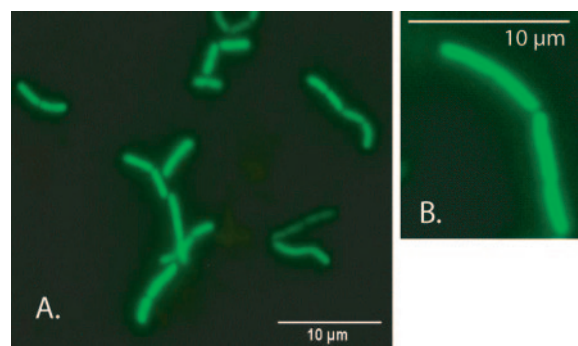


FIG. 3. GFP-tagged *L. rhamnosus* GG, based on implementation of the NICE system: epifluorescence microscopic analysis of bacterial cells grown in liquid broth. (A) FAJ1905/pMEC45 induced with nisin as described in Materials and Methods. (B) FAJ1906/pMEC45 induced with nisin as described in Materials and Methods.

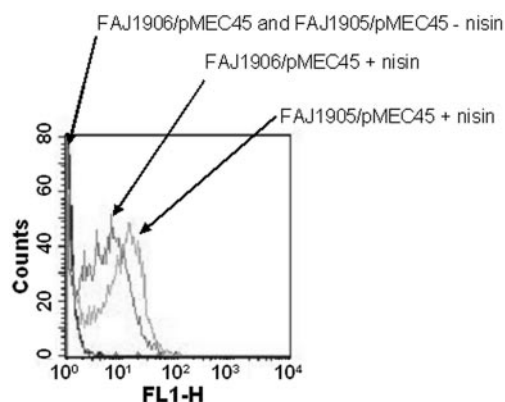


FIG. 4. Flow cytometric analysis of induced (GFP⁺) and uninduced (GFP⁻) *L. rhamnosus* GG. FAJ1905/pMEC45 and FAJ1906/pMEC45 were induced with nisin as described in Materials and Methods (FAJ1905/pMEC45 + nisin and FAJ1906/pMEC45 + nisin). Cultures without nisin were used as negative controls (FAJ1906/pMEC45 and FAJ1905/pMEC45 - nisin). Green fluorescence intensities (FL1-H) are indicated on the x axis, and cell counts are indicated on the y axis. The cytometric analysis was performed for 2×10^4 events.

ferent for *L. plantarum* and *L. rhamnosus* GG and are 5 h (42) and 2.5 h (this study), respectively. It has been reported previously that the optimal contact time with the inducer, leading to maximal protein production, can vary greatly with the host (26, 42). The induction factor, defined as the ratio of the β -glucuronidase activity observed after maximal induction to the basal activity with no inducer, is 500. This value is similar to values reported for *L. plantarum* (42), which in turn were only two- to threefold lower than the values reported for *L. lactis* (4).

Using these inducing conditions with FAJ1905/pMEC45 and FAJ1906/pMEC45 resulted in bright green fluorescent bacteria, as determined by fluorescence microscopy (Fig. 3) and flow cytometric analysis (Fig. 4). FAJ1905/pMEC45 exhibited slightly enhanced fluorescence intensity compared to FAJ1906/pMEC45 (Fig. 4). This could have been due to the two different locations of *nisRK* (i.e., in the tRNA^{Ser} and tRNA^{Leu} loci, respectively).

Use of GFP-tagged *L. rhamnosus* GG to determine sensitivity to human beta-defensins.

The combination of GFP and PI has been shown to have some advantages for analysis of bacterial viability compared to the frequently used LIVE/DEAD BacLight bacterial viability kit (31). Here we used the GFP-PI-based assay for flow cytometric measurement of the viability of *L. rhamnosus* GG after contact with human beta-defensins. Human beta-defensins are antimicrobial peptides expressed by epithelial cells (30, 50). It is now recognized that beta-defensins are integral components of the constitutive and regulated innate host defense; hBD1 is a constitutively expressed molecule, while hBD2, hBD3, and hBD4 are upregulated by bacterial challenge and proinflammatory cytokines (38, 39). It has been reported that constitutively expressed hBD1 can mediate epithelial interactions with the commensal flora, whereas hBD2 may participate in the host defense response to enteric microbes that can breach the epithelial barrier. Human beta-defensins are antimicrobial peptides that are particularly efficient in destroying gram-negative bacteria (38), while it has been reported that a bifidobacterial probiotic strain is not sensitive to human beta-defensin-mediated killing by recombinant hBD1, hBD2, and hBD3 (13). We investigated the effect of hBD1 and hBD2 on GFP-labeled *L. rhamnosus* GG using flow cytometry (Fig. 5). While hBD1 did not kill *L. rhamnosus* GG (Fig. 5A), *L. rhamnosus* GG was very sensitive to hBD2 (Fig. 5A). These results were confirmed by plate counting (Fig. 5B). The sensitivity was directly related to the defensin concentration (Fig. 5C). Subsequently, a time course experiment was performed in order to determine the defensin contact time required to kill the bacterial cells. At an hBD2 concentration of 6 μ g/ml, a decrease in cell viability was observed 2 h after addition of the defensin to the culture medium. However, at a concentration of 12 μ g/ml, *L. rhamnosus* GG was killed almost immediately after addition of the defensin (data not shown). Hudault et al. (20) reported that the number of *L. rhamnosus* GG cells in the gut of *L. rhamnosus* GG-monoassociated mice was dramatically decreased after a challenge with *Salmonella*, but the mechanism behind this phenomenon is unknown. It could be speculated that *Salmonella* infection upregulates defensin production (37), which leads to a decrease in the number of viable *L. rhamnosus* GG cells and reduced protection

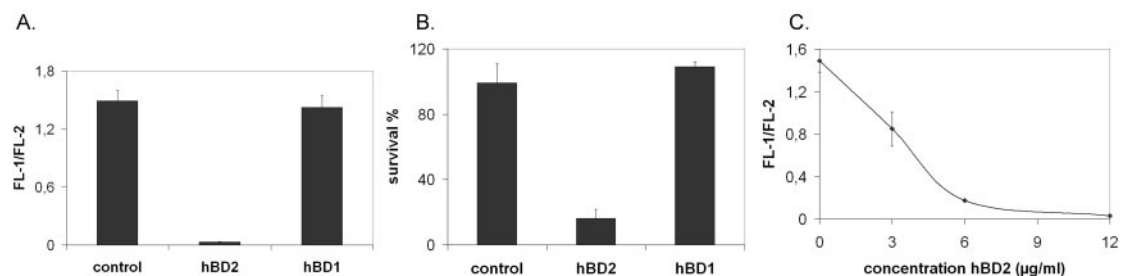


FIG. 5. Sensitivity of *L. rhamnosus* GG to human beta-defensins. (A) Bacterial viability of *L. rhamnosus* GG after 3 h of contact with 12 μ g/ml hBD1 or hBD2, as determined by GFP-PI-based flow cytometric analysis. The values are the ratios of the mean intensities of the green (FL-1) and red (FL-2) signals. The error bars indicate standard deviations of three independent measurements. No human beta-defensin was added to the control. (B) Bacterial viability of *L. rhamnosus* GG after 3 h of contact with 12 μ g/ml hBD1 or hBD2, as determined by plate counting. The values are percentages of survival of the initial number of *L. rhamnosus* GG cells. The error bars indicate standard deviations of three independent measurements. (C) Concentration-dependent sensitivity of *L. rhamnosus* GG to hBD2, as determined by GFP-PI-based flow cytometric analysis as described above.

against the pathogen. Therefore, incorporation of a screen for defensin resistance in the selection of probiotic strains could be appropriate.

Concluding remarks. Although there have been many studies of *L. rhamnosus* GG and *L. rhamnosus* GG has been used in numerous clinical trials, molecular genetic analysis of *L. rhamnosus* GG is poorly developed. This fact can be explained in part by the lack of a reliable transformation procedure. In this paper, we describe a reproducible electroporation protocol for *L. rhamnosus* GG. This protocol enabled us to implement the NICE system in *L. rhamnosus* GG and subsequently label this strain with GFP. As previously shown (15), preloaded fluorescent bacteria can be used for a variety of in vitro and in vivo experiments, including studies of the interaction of *L. rhamnosus* GG with members of the microbiota and with specific members of the immune system. Here, we used preloaded fluorescent bacteria to show *L. rhamnosus* GG sensitivity to hBD2.

The flexibility of the *L. rhamnosus* GG NICE system should allow expression of other genes of interest under control of the *nisA* promoter. This could be used to increase the technological suitability of *L. rhamnosus* GG by food-grade (nisin is a food-grade, approved antibiotic peptide) genetic manipulation approaches (46), as shown by overexpression of the GroESL heat shock protein chaperones in *Lactobacillus paracasei* NFBC338 (6). Moreover, this system could be used for development and design of live recombinant vaccines (51) in which *L. rhamnosus* GG is used as a live delivery vehicle. Thus, the implementation of the NICE system in *L. rhamnosus* GG provides an approach for improving and expanding both the technical performance and the probiotic performance of this strain.

ACKNOWLEDGMENTS

S.D.K. was a research associate of the Belgian Fund for Scientific Research (FWO-Vlaanderen) when this study was conducted. P.H. is a research associate at FNRS. This work was supported by the IWT through research projects STWW-00162 and GBOU-20160.

We gratefully acknowledge Valio Ltd., A. Mercenier, M. Alvarez, W. de Vos, and M. Zagorec for kindly providing strains and plasmids used in this study.

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