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Adaptation of the Nisin-Controlled Expression System in *Lactobacillus plantarum*: a Tool To Study In Vivo Biological Effects

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The potential of lactic acid bacteria as live vehicles for the production and delivery of therapeutic molecules is being actively investigated today. For future applications it is essential to be able to establish dose-response curves for the targeted biological effect and thus to control the production of a heterologous biopeptide by a live lactobacillus. We therefore implemented in *Lactobacillus plantarum* NCIMB8826 the powerful nisin-controlled expression (NICE) system based on the autoregulatory properties of the bacteriocin nisin, which is produced by *Lactococcus lactis*. The original two-plasmid NICE system turned out to be poorly suited to *L. plantarum*. In order to obtain a stable and reproducible nisin dose-dependent synthesis of a reporter protein (β-glucuronidase) or a model antigen (the C subunit of the tetanus toxin, TTFC), the lactococcal *nisRK* regulatory genes were integrated into the chromosome of *L. plantarum* NCIMB8826. Moreover, recombinant *L. plantarum* producing increasing amounts of TTFC was used to establish a dose-response curve after subcutaneous administration to mice. The induced serum immunoglobulin G response was correlated with the dose of antigen delivered by the live lactobacilli.

Lactic acid bacteria (LAB) are used worldwide in the preparation of fermented foods, including dairy products. They are also known for the potentially beneficial effects they may exert on the health of humans and animals (see, for example, reference 25). Their "generally recognized as safe" status (1), linked to their metabolic and technological properties, has recently led to their development as potential live-vaccine vehicles. *Lactobacillus plantarum* NCIMB8826 (17, 33) has been chosen for this purpose in our laboratory on the basis of its capability to persist in the mouse gastrointestinal and urogenital tracts (38). The ability to control the expression level of foreign proteins in LAB may offer certain advantages. However, while several controlled expression systems have been developed for *Lactococcus lactis* (9, 23), very few inducible promoters are available for lactobacilli: the *xylR* promoter from *Lactobacillus pentosus* (29), the α-amylase promoter from *L. amylovorus* (31), and the *p*-coumarate decarboxylase promoter from *L. plantarum* (4). One of the most promising lactococcal controlled expression systems is based on the autoregulatory properties of the *L. lactis* nisin gene cluster (7, 23). Nisin is an antimicrobial peptide belonging to the family of lantibiotics (19) and is used as a natural preservative in the food industry (5). Nisin induces the transcription of the genes under control of the *nisA* and *nisF* promoters, via a two-component regulatory system (34, 37) consisting of the histidine protein kinase NisK and the response regulator NisR (14, 21, 22). A transferable nisin-controlled expression (NICE) system (24) based on the combination of the *nisA* promoter and the *nisRK* regulatory genes has recently been developed (7, 20). It consists of two compatible replicons, a plasmid carrying the *nisRK* regulatory genes (regulatory plasmid) and an expression vector carrying the gene of interest under control of the *nisA* promoter. This system offers several advantages. (i) The level of expression can be controlled by the amount of nisin used for induction (6, 20, 21). (ii) Recombinant protein synthesis can reach very high levels (up to 60% of the total intracellular proteins [7]). (iii) The expression of the gene of interest is reported to be undetectable at the uninduced state, which allows the production of lethal proteins (8). (iv) This expression system has already been successfully transferred to other gram-positive bacteria (13, 20).

In this report, we describe the implementation of the NICE system in *L. plantarum* NCIMB8826, using the β-glucuronidase (*gusA*) reporter gene. This system was then used to design *L. plantarum* strains producing increasing amounts of TTFC (C subunit of tetanus toxin) at regulated levels, which allowed us to establish a dose effect of TTFC after subcutaneous administration to mice of recombinant lactobacilli bearing different loads of the antigen.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* MC1061 was cultured as described by Sambrook et al. (32). *L. plantarum* was grown at 30 or 37°C in MRS medium (Difco) without shaking. Antibiotics (Sigma) were used at the following final concentrations: for *Escherichia coli*, chloramphenicol (20 μg/ml), erythromycin (250 μg/ml), and ampicillin (100 μg/ml); and for *L. plantarum*, chloramphenicol (10 μg/ml) and erythromycin (5 μg/ml) in association with lincomycin (10 μg/ml). Double transformants of *L. plantarum* were grown in the presence of erythromycin (5 μg/ml) and chloramphenicol (5 μg/ml).

Transformation and DNA manipulations. *E. coli* and *L. plantarum* were electrotransformed as described by Dower et al. (10) and Josson et al. (18), respectively. *E. coli* MC1061 was used as an intermediate host for cloning. Molecular biology techniques were performed essentially as described by Sambrook et al. (32). Restriction endonucleases, T4 DNA ligase, and *Taq* polymerase were purchased from Boehringer Mannheim and were used following the recommendations of the manufacturer. *L. plantarum* plasmid DNA was extracted according

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Characteristics	Source or reference
Strains		
E. coli MC1061	*araD139* Δ(*ara-leu*)*7696 lacX74 galV galK hsr-hsm rpsL*	3
L. plantarum NCIMB8826	Isolated from human saliva	NCIMB[a]
L. plantarum NCIMB8826 Int-1	NCIMB8826 containing the *nisRK* genes stably integrated to the $tRNA^{Ser}$ locus; obtained by transformation with pMEC10	This work
Plasmids		
pNZ9520	Ery^r, *nisRK* cloned in pIL253, expression of *nisRK* driven by *rep* read-through, high copy number	20
pNZ9521	Ery^r, *nisRK* cloned in pIL253, expression of *nisRK* driven by *nisR* promoter and *rep* read-through, high copy number	20
pNZ9530	Ery^r, *nisRK* cloned in pIL252, expression of *nisRK* driven by *rep* read-through, low copy number	20
pNZ9531	Ery^r, *nisRK* cloned in pIL252, expression of *nisRK* driven by *nisR* promoter and *rep* read-through, low copy number	20
pNZ9500	Amp^r, Ery^r, pUC19 derivative containing a 2.7-kb chromosomal DNA fragment from *L. lactis* NZ9700, which contains the 3′ end of *nisP* and *nisRK*	20
pNZ8008	Cm^r, pNZ273 carrying the *gusA* reporter gene transcriptionally fused to the *nisA* promoter	21
pNZ8032	pNZ8008 derivative, carrying the *gusA* gene translationally fused to the *nisA* promoter	7
pNZ8037	pNZ8008 derivative carrying a multiple-cloning site; allows translational fusion to the *nisA* promoter	7
pMC1	pRC1 derivative, containing the ϕmv4 *int-attP* sequence	12
pMEC3	pZErO -2 derivative, containing the TTFC gene cloned into the multiple cloning site	Chagnaud et al., unpublished results
pMEC10	Integration plasmid, *int-attP* cassette of pMC1 cloned into pNZ9500, expression driven by *ery* read-through	This work
pMEC46	Replicative plasmid derived from pNZ8037, which contains the TTFC gene from pMEC3 translationally fused to the *nisA* promoter	This work

[a] NCIMB, National Collection of Industrial and Marine Bacteria, Terry Research Station, Aberdeen, Scotland.

to the method of Posno et al. (30). Chromosomal DNA of *L. plantarum* was isolated according to the method of Ferain et al. (15). For Southern blotting, chromosomal DNA was extracted, digested, and transferred onto a nitrocellulose membrane after agarose gel electrophoresis. DNA was hybridized with digoxigenin-labeled pMEC10 (see below) using the DIG DNA labeling kit supplied by Boehringer Mannheim.

Plasmid constructions. The plasmid allowing integration of *nisRK* in the $tRNA^{Ser}$ chromosomal locus of *L. plantarum* was constructed as follows. An *Eco*RV-*Sca*I fragment containing the mv4 bacteriophage *int-AttP* cassette from pMC1 (Table 1) was inserted into the *Sma*I site (130 bp downstream of the Em^r marker) of pNZ9500, which carries *nisRK* under control of its own promoter, giving rise to pMEC10 (Table 1). The replicative plasmid carrying the TTFC coding sequence under control of the *nisA* promoter was obtained by cloning the *Nco*I-*Bam*HI fragment from pMEC3 (P. Chagnaud et al., unpublished results) into pNZ8037 (Table 1) digested with the same enzymes. The resulting plasmid, pMEC46, harbors the TTFC gene fused to the ATG of the *nisA* gene.

Plasmid stability. The integrant *L. plantarum* containing pNZ8008 or pNZ8032 was grown at 37°C in MRS medium without antibiotic. Serial subcultures were performed by inoculating 10 μl of the previous culture in 10 ml of fresh medium. After 10 subcultures, the number of chloramphenicol-resistant CFU was determined from the total number of CFU by plating equal numbers of bacterial dilutions on control MRS agar plates and MRS agar plates supplemented with 10 μg of chloramphenicol/ml. To determine the structural plasmid stability, six randomly chosen colonies plated from the 10th subculture were examined by plasmid DNA extraction and restriction analysis.

Nisin induction and β-glucuronidase activity. Nisin induction of recombinant *L. plantarum* NCIMB8826 strains was first performed as described for *L. lactis* (6), except for the induction time. Briefly, bacteria were grown at 30°C to an A_{600} of 0.5, after a 1/20 inoculation (vol/vol) of fresh medium with an overnight culture. Nisin was added at different concentrations (0 to 100 ng/ml), and cultures were further grown for 3 h at 30°C. β-Glucuronidase assays were performed as described previously (6).

Optimization of the protocol for *L. plantarum* NCIMB8826 (see Results) led to the following modifications. Bacteria were grown at 37°C by inoculating fresh medium 1/50 (vol/vol) with an overnight culture. After 1 h of growth (beginning of the exponential growth phase), nisin was added at a concentration of 25 ng/ml and cells were propagated for 5 h at 37°C before harvest and the β-glucuronidase assay.

Protein analysis. TTFC production was examined by Western blotting (32) after induction of 10-ml cultures by nisin. Cells were harvested by 10 min of centrifugation at 3,000 × *g* at 4°C and were washed with 10 mM Tris-HCl (pH 7.5) and resuspended in 2 ml of 10 mM Tris-HCl (pH 7.5). Cell extracts were prepared with a French-Press (Bioritech) and immediately mixed with an antiprotease cocktail (Complete; Boehringer Mannheim). Cell debris was removed by centrifugation for 5 min at 1,300 × *g* at 4°C in a microcentrifuge. When relevant, the protein concentration was determined by the Bradford method (2) using the Bio-Rad protein assay kit. The soluble proteins were separated by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (Mini Protean II; Bio-Rad) and blotted (semidried system; OWL) onto nitrocellulose membranes (Optitran BA-S85; Schleicher & Schuell). Polyclonal rabbit anti-TTFC antibodies (provided by E. Sablon, Innogenetics N.V., Ghent, Belgium) and goat anti-rabbit–alkaline phosphatase antibodies (Promega) were used as primary and secondary antibodies, at dilutions of 1/1,000 and 1/7,000, respectively. Detection was performed by addition of 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate (Boehringer Manneim). Semiquantification of the TTFC bands after scanning was performed by using GelCompar (Applied Maths, Kortrijk, Belgium). Intensity (pixels per band area) of the strongest band was considered 100% and used as a reference to estimate percentages of intensity in the other lanes.

Mouse immunization experiments. Bacterial inocula were prepared as follows. Fresh medium was inoculated 1/50 (vol/vol) with an overnight culture and incubated at 37°C. Induction was achieved by adding nisin at a concentration of 0, 0.5, 2, or 25 ng/ml after 1 h of growth. Cultures were harvested by centrifugation (3,000 × *g*, 4°C) after 5 h of incubation at 37°C. The collected cells were washed twice with sterile phosphate-buffered saline (pH 7.2) (GibcoBRL) and resuspended in phosphate-buffered saline to a final concentration of 10^{10} CFU/ml. Groups (four female mice/group) of 8-week-old C57BL6 mice were immunized subcutaneously on days 0, 15, and 37 with 10^9 CFU (100 μl) of *L. plantarum* NCIMB8826 Int-1(pMEC46) that produced increasing amounts of TTFC after nisin induction. Control mice were inoculated with *L. plantarum* NCIMB8826 Int-1 (nonexpressor strain). Serum samples were taken before immunization (preimmune serum) and 7 days after primer and booster injections. A last sample was collected 70 days after the last boost, to evaluate the persistence of the immune response.

ELISA. The anti-TTFC immunoglobulin G (IgG) response was analyzed by enzyme-linked immunosorbent assay (ELISA) with the end point titer method

(KC4 software; Bio-Tek Instruments). The microtiter plates (Immulon 3; Dynatech) were coated with 200 ng of TTFC (Boehringer Manneim). Sequential dilutions of serum samples were distributed in the wells, and IgG detection was performed with biotinylated goat anti-mouse IgG (Southern Biotechnology Associates). Streptavidin-horseradish peroxidase complex (Amersham) was added, and plates were read with an ELX800GUV machine (Bio-Tek Instruments) at 490 nm after developing with *o*-phenylenediamine (Sigma) and hydrogen peroxide. The end point titer was calculated as the dilution of serum producing an optical density of three times the background level. The results were evaluated by the Mann-Whitney U test, and differences were considered significant when P values were <0.05.

RESULTS

Implementation of the NICE system in *L. plantarum* NCIMB8826. The transferable NICE system consists of an expression vector and a regulatory plasmid (20) which are derived from two compatible broad-host-range replicons (36). The expression plasmid (pSH71 derivative) carries the *E. coli gusA* reporter gene transcriptionally or translationally fused (ATG fusion) to the *nisA* promoter (pNZ8008 or pNZ8032, respectively) (Table 1). The regulatory *nisRK* genes are carried on different pAMβ1-derived replicons, leading to increasing NisRK dosage in the following order: pNZ9530, pNZ9531, pNZ9520, and pNZ9521 (Table 1). To assess the NICE system in *L. plantarum* NCIMB8826, the following combinations of the two plasmids were introduced into this host: pNZ8008 associated with pNZ9530, pNZ9531, pNZ9520 or pNZ9521 and pNZ8032 associated with pNZ9530. The resulting double transformants were first cultured at 37°C in the presence of erythromycin (7.5 μg/ml) and (chloramphenicol) (10 μg/ml). Under these conditions, the strains containing pNZ9520 or pNZ9521 were severely disabled, while those carrying lower *nisRK* dosage (pNZ9530 and pNZ9531) appeared to be viable but unstable upon propagation. However, their stability could be improved by growth at 30°C in the presence of erythromycin (5 μg/ml) and chloramphenicol (5 μg/ml). Viable double transformants (pNZ8008 or pNZ8032 combined with pNZ9530 or pNZ9531) grew approximately half as fast as the plasmid-free strain, while recombinant *L. plantarum* containing a single plasmid behaved like the parent strain.

The progressive induction of the *nisA* promoter by increasing doses of nisin was followed by measuring the β-glucuronidase activity in the different double-transformant cultures. At the maximal dose of nisin (100 ng/ml), the most stable plasmid combination was pNZ9530 associated with pNZ8008. The corresponding recombinant strain showed no basal activity in the absence of the inducer (0.002 ± 0.002 activity units [AU]). As in the case of the pNZ9530- and pNZ8032-containing strain, β-glucuronidase activity was found to be induced by nisin in a dose-dependent manner (data not shown). However, the maximal β-glucuronidase activity remained low and variable from one culture to another in NCIMB8826(pNZ9530) and NCIMB8826(pNZ8008) (0.028 ± 0.018 AU). When *nisRK* genes were transcribed at a slightly higher level (NCIMB8826-pNZ9531 and -pNZ8008), the strains exhibited a higher β-glucuronidase activity after nisin induction (0.073 AU) but also a basal activity (0.025 ± 0.009 AU). In view of these results, the dual-plasmid NICE system seemed poorly suited to *L. plantarum* NCIMB8826.

Since high expression levels of the nisin regulatory genes might be detrimental to *L. plantarum*, the *nisRK* genes were integrated with their own promoter into the genome of this host. Site-specific integration of *nisRK* in *L. plantarum* NCIMB8826 was achieved by applying the system that was based on the integration property of the mv4 temperate bacteriophage and originally developed for *L. plantarum* LP80 (12). Chromosomal integration of mv4 is mediated by site-specific recombination between the homologous attachment sites *attP* and *attB*, carried by the phage and the bacterial genome, respectively. *attB* is located at the 3′ end of the $tRNA^{Ser}$ locus, and integration into this site, catalyzed by a phage-encoded integrase (*int*), is nondisruptive. The promoterless mv4 *int-attP* cassette carried by pMC1 (12) was introduced into pNZ9500 (Table 1), which contains *nisRK* under the control of the *nisR* promoter. The *int-attP* sequence was inserted into pNZ9500 downstream of the erythromycin resistance marker, in order to allow read-through transcription from this gene, which lacks a functional transcription terminator. The resulting integrative plasmid, pMEC10 (Table 1), is derived from pUC19 and is thus unable to replicate in *L. plantarum*. Transformation of *L. plantarum* NCIMB8826 with pMEC10 and selection for erythromycin- and lincomycin-resistant colonies yielded putative integrants that appeared after 3 days of incubation at 37°C. Analysis of these clones by PCR and Southern blotting confirmed integration of pMEC10 at the $tRNA^{Ser}$ locus (data not shown). One integrant presenting the correct structure, *L. plantarum* NCIMB8826 Int-1, was kept for further studies. The plasmid-containing integrant grew on 10 μg of chloramphenicol/ml as fast as the corresponding plasmid-free strain or the wild-type NCIMB8826 strain on nonselective medium. The segregational and structural stability of pNZ8008 and pNZ8032 were then evaluated in the NCIMB8826 Int-1 background. After 100 generations in nonselective medium, 70 colonies out of 70 retained the Cm^r phenotype. Analysis of the plasmid content of 6 randomly picked colonies showed no structural rearrangements.

Optimization of the nisin induction conditions in *L. plantarum* NCIMB8826 Int-1. The optimal growth phase for nisin induction was the first parameter studied. β-Glucuronidase activity was determined in NCIMB8826 Int-1(pNZ8008) cultures incubated at 30 or 37°C after inoculation at 1/20 (vol/vol) or 1/50 (vol/vol) with an overnight culture. Nisin was added at 50 ng/ml every 30 min during 3 h after the start of the culture, and β-glucuronidase activity was measured 2 h 30 min after induction. As shown in Fig. 1A, maximal levels were reached when the inducer was added early in the exponential phase, i.e., 1 h or 1 h 30 min after bacterial inoculum, depending on the inoculation conditions (1/50 inoculum, 37°C; 1/20 inoculum, 37°C; or 1/20 inoculum, 30°C). The higher β-glucuronidase activity was obtained with cultures grown at 37°C with 1/50 inoculum. These conditions were kept for further experiments, and nisin was added after 1 h of growth.

Next, we determined the optimal production levels as a function of induction time. β-Glucuronidase activity was measured at several time points after the addition of nisin and was found to quickly rise after induction, the maximum level being reached 4 h 30 min to 5 h after induction (Fig. 1B).

These conditions (addition of nisin 1 h after inoculation at 1/50 [vol/vol] with an overnight culture and incubation at 37°C for 5 h) were finally applied to characterize the induction of the *nisA* promoter by different nisin concentrations. Both transcriptional (pNZ8008) and translational (pNZ8032) fusions of P*nisA-gusA* were analyzed. Repeated β-glucuronidase assays were performed with six individual clones of each recombinant strain to evaluate the reproducibility of induction and stability of the expression system. Cultures were induced with nisin concentrations ranging from 0 to 50 ng/ml. With both plasmids, β-glucuronidase activity was found to be nisin dose dependent, essentially in the range of 0 to 5 ng of nisin/ml, and remained very low in uninduced cultures (Fig. 1C). The maximum level was reached with 25 ng of nisin/ml and was three times higher for strains containing pNZ8032 (2.9 ± 0.4 AU) than for those containing pNZ8008 (1.0 ± 0.1 AU), which was comparable to

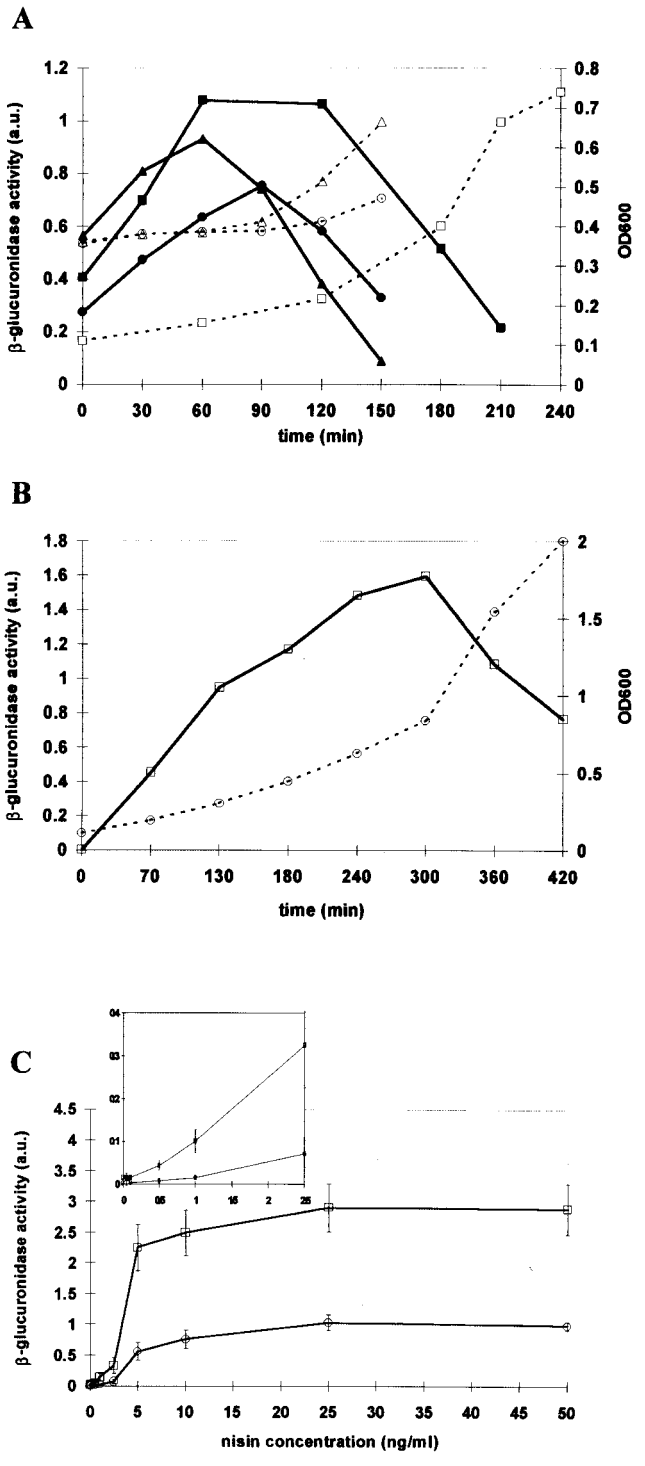

FIG. 1. β-Glucuronidase activity (AU and optical density at 600 nm [OD_{600}]) of *L. plantarum* NCIMB8826 Int-1(pNZ8008) cell extracts. (A) β-Glucuronidase activity (solid lines) determined during growth (dotted lines) in different conditions (squares, 1/50 inoculum and incubation at 37°C; triangles, 1/20 inoculum and incubation at 30°C; and circles, 1/20 inoculum and incubation at 37°C) after induction with 50 ng of nisin/ml at different times after inoculation. β-Glucuronidase activity was measured after 2 h 30 min of contact with nisin. (B) β-Glucuronidase activity (solid line) after different periods of contact with 50 ng of nisin/ml. Optical density (dotted line) was measured at 600 nm (OD_{600}). (C) Dose-response curves of *gusA* expression in *L. plantarum* NCIMB8826 Int-1 harboring pNZ8008 (circles) or pNZ8032 (squares) induced with increasing concentrations of nisin (0 to 50 ng/ml). Error bars represent standard deviations. The inset shows β-glucuronidase activity for nisin concentrations ranging from 0 to 2.5 ng/ml.

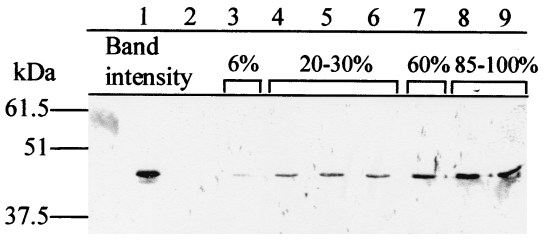

FIG. 2. Dose-dependent nisin induction of TTFC expression in *L. plantarum* NCIMB8826 Int-1(pMEC46). Western blotting was performed on 2 μg of soluble proteins (lanes 2 to 9) from total cell extracts. Lane 1, purified recombinant TTFC; lane 2, *L. plantarum* NCIMB8826 Int-1 (negative control); lanes 3 to 9, NCIMB8826 Int-1(pMEC46) uninduced and induced with 0.5, 1, 1.5, 2, 5, or 20 ng of nisin/ml, respectively. Sizes as deduced from molecular mass markers are shown at the left. Relative band intensities in lanes 3 to 9 are shown at the top.

the results obtained in *L. lactis* (6). All clones exhibited reproducible enzymatic activities (Fig. 1C).

Controlled production of TTFC by the integrant NCIMB8826 Int-1. We next investigated whether the nisin-inducible system would allow us to produce increasing amounts of a model antigen (TTFC). A plasmid carrying the TTFC gene translationally fused to the *nisA* promoter (pMEC46) (Table 1) was constructed and introduced into NCIMB8826 Int-1. Cultures were induced with increasing concentrations of nisin in the conditions defined for β-glucuronidase production, and the corresponding cell extracts (soluble proteins) were analyzed by Western blotting. As shown in Fig. 2, TTFC was produced in a nisin dose-dependent manner. However, a low production level was detected in uninduced cultures (lane 3), probably due to the high sensitivity of the anti-TTFC antibodies used, which allowed detection even of a few nanograms of the antigen. By scanning, it was estimated that TTFC constituted about 10% of the total soluble proteins in NCIMB8826 Int-1(pMEC46), which is notably higher than the results obtained with the strong constitutive promoters that we have studied so far (Chagnaud et al., unpublished results).

Immunization of mice by the subcutaneous route. NCIMB8826 Int-1(pMEC46) was grown in the presence of 0, 0.5, 2, or 25 ng of nisin/ml as described above, and 10^9 CFU of each culture was administered by the subcutaneous route to C57BL6 mice. The serum anti-TTFC IgG response of the mice was analyzed by ELISA at different time points. NCIMB8826 Int-1 containing no plasmid was used as the negative control. As shown in Fig. 3, only the mice that received recombinant lactobacilli induced by 25 ng of nisin/ml showed a statistically significant ($P < 0.05$) immune response after priming. In contrast, all mice, including those immunized with the noninduced culture of NCIMB8826 Int-1(pMEC46), produced anti-TTFC serum IgG ($P < 0.05$) after the first and second boosts. Notably, the titers reached after the first boost reflected the amount of TTFC produced by the bacterial vector (Fig. 2 and 3). A 1- to 2-log increase in the anti-TTFC IgG titers was observed for mice immunized with recombinant *L. plantarum* strains grown in the presence of high doses of the inducer (2 and 25 ng/ml; mean titer, 55,100) compared to those which were given strains induced with low amounts of nisin (0.5 ng/ml; mean titer, 7,278). Uninduced cultures led to a mean antibody titer of 378. No difference was seen after the first boost between the two groups of mice immunized with the two highest doses of antigen. The induced immune response persisted for up to 70 days after the last booster injection, regardless of the dose of antigen delivered at priming.

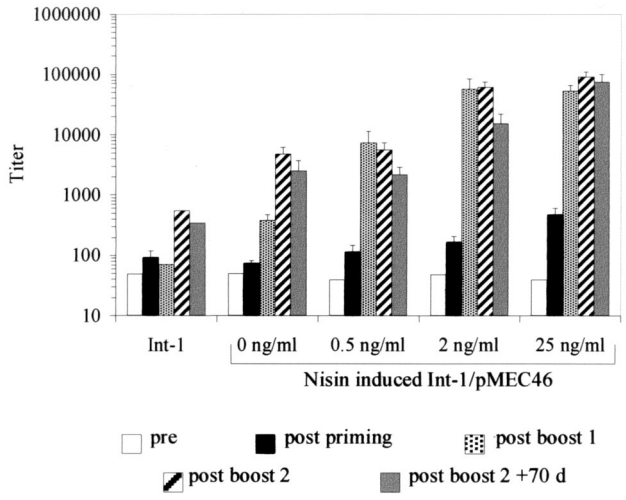

FIG. 3. Anti-TTFC IgG titers of individual sera of mice inoculated subcutaneously with *L. plantarum* NCIMB8826 Int-1 (negative control) (Int-1) or NCIMB8826 Int-1(pMEC46) induced by increasing nisin concentrations. Data represent the ELISA mean end-point titers for each group in the preimmune sera (pre), the sera obtained 7 days after each inoculation (postpriming, post-boost 1, and post-boost 2), and the sera obtained 70 days after the second boost (post boost 2 + 70 d). Error bars represent standard errors of the means.

DISCUSSION

In this paper, we describe the implementation of the NICE system in *L. plantarum* NCIMB8826, which necessitated integrating the *nisRK* regulatory genes into the chromosome of this strain. In contrast with the use of the dual-plasmid NICE system, the *nisRK* integration has led to genetic stabilization of the recombinant strain and to reproducible and dose-dependent nisin induction of the *nisA* promoter.

Lactobacilli constitute a very broad genus including over 60 species that may be quite distant from each other from a phylogenetic point of view (35). As a consequence of this diversity, the efficacy of transcription, translation, or secretion signals may vary greatly from one *Lactobacillus* species to another (17, 27, 30). This is well illustrated with *L. plantarum*, in which the dual-plasmid NICE system appeared unstable and poorly regulated, even when it could be successfully transferred to other lactobacilli (20). However, some common features could be observed between *L. plantarum* and other LAB. Firstly, the expression levels of the *nisRK* regulatory elements in the final expression host are very important (this work and references 13 and 20). In NCIMB8826, these expression levels have to remain very low in order to retain both the inducibility to nisin and no basal activity. This is the case with the double transformant carrying pNZ9530 and with NCIMB8826 Int-1, which contains a single chromosomal copy of *nisRK*, thus mimicking the optimal *L. lactis* system (*pepN*::*nisRK* integrants [6]). Secondly, cultures and induction conditions have to be optimized for each host. For NCIMB8826 Int-1, it is necessary to add higher doses of the inducer (20 to 25 ng/ml) (this work) than those used for *L. lactis* (1 to 5 ng/ml) (6). However, these concentrations remain notably lower than those described for other gram-positive bacteria (13). Also, *L. plantarum* is more receptive to nisin when its addition occurs very early in the exponential growth phase (this work), while the best induction time for *L. lactis* is located in the middle of the exponential growth (7). Moreover, the optimal contact time with the inducer, leading to maximal protein production, can vary greatly depending on the host (this work and reference 20).

Adaptation of the NICE system in *L. plantarum* also allowed it to reach very high β-glucuronidase activity levels, especially in strains harboring pNZ8032 (2.9 AU versus 0.02 AU with the dual-plasmid system), in which initiation of translation is optimized. Induction factors, as defined by the ratio of the β-glucuronidase activity (observed after maximal induction) to the basal activity (no inducer), are equal to 500 or 200 with NCIMB8826 Int-1 containing pNZ8008 or pNZ8032, respectively. This is only two- to threefold lower than in *L. lactis* (7). When fully induced, the *nisA* promoter appears to be more efficient than strong endogenous *L. plantarum* promoters (unpublished observations), leading to an estimated intracellular production of about 10% in the case of TTFC. These levels are close to those obtained with the best recombinant *L. lactis* strains producing TTFC (39). Contrary to *L. lactis*, very few inducible expression systems are available for lactobacilli. Thus, the adaptation of the NICE system in *L. plantarum* provides a powerful, well regulatable, and stable expression tool for this bacterium.

Because of the potential health applications of certain *Lactobacillus* strains, it is of utmost importance to develop controlled gene expression systems which would allow dose-response curves to be established for the biological effect under study. In the second part of this work, we used live recombinant *L. plantarum* to deliver increasing levels of the model antigen TTFC by subcutaneous administration to mice and we showed a correlation between the amount of antigen delivered and the specific serum IgG response induced after priming and the first boost. This is one of the first successful attempts to establish dose-response curves with bacterial live vectors, based on the delivery of incremental amounts of protein by equivalent numbers of live cells. It will allow us to evaluate the contribution of protein and bacterial doses in the induction of a biological effect. This point may be particularly relevant in the case of LAB, as some *Lactobacillus* strains are reported to exhibit adjuvant properties (26, 31). Thus, we have taken advantage of the main property of the NICE system, i.e., controlled expression level by nisin concentration, while it is usually used at the fully induced stage for overproduction of heterologous proteins. The optimized NICE system was successfully applied to tag *L. plantarum* with green fluorescent protein and to follow the fate of these fluorescent lactobacilli in vivo or in cell cultures (16), providing a powerful tool to study the health-promoting effects of lactobacilli in humans or animals. In vivo induction of the NICE system by adding nisin in food, as shown by Drouault et al. (11), could be considered.

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