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Short communication

Expression of bioactive porcine interferon-gamma by recombinant *Lactococcus lactis*

Prithy Rupa^a, Vicente Monedero^b, Bruce N. Wilkie^{a,*}^aDepartment of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada N1G 2W1^bDepartamento de Biotecnología, Instituto de Agroquímica y Tecnología de Alimentos, CSIC, Burjassot, Spain

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Abstract

Lactococcus lactis is an immunomodulator and candidate live mucosal delivery vehicle for vaccine antigens and for biologically active molecules, including immunoregulatory cytokines such as interferon-gamma (IFN- γ). To provide a tool for investigating downregulation of allergic predisposition of pigs to experimental food allergy, porcine IFN- γ was cloned and expressed as a fusion protein with the *usp45* secretion signal. Immunoblot analysis with polyclonal anti-pIFN- γ -antibody demonstrated that the recombinant porcine IFN- γ (rpIFN- γ) protein was expressed in the *L. lactis* transformants as a secreted product. Activity of rpIFN- γ was confirmed by ability to upregulate class II major histocompatibility complex (MHC) on cells of the porcine monocytic cell line 3D4/31. The *L. lactis* producing biologically active rpIFN- γ will be used to investigate its possible ability to modulate the allergic immune response phenotype of pigs.

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1. Introduction

Interferon-gamma (IFN- γ) plays a major role in immunoregulation (Young and Hardy, 1995). Immune regulatory effects of IFN- γ include upregulation of many genes involved in antigen processing and presentation, including major histocompatibility complex (MHC) class I and II expression and enhancement of macrophage function (Collins et al., 1984; Delves

and Roitt, 2000). It also downregulates type 2-interleukin-4 (IL-4)-induced allergic immune responses (Romagnani, 1996). The porcine IFN- γ gene has been cloned and expressed in *Escherichia coli* (Vandenbroeck et al., 1993), insect Sf9 cell lines (Vandenbroeck et al., 1994) and mammalian cells (Cencic et al., 1999). Lactic acid bacteria (LAB) are natural gastro-intestinal tract commensals in humans and animals which stimulate IL-12 (p70) production, causing a shift from type 2 to type 1 immune response (Sashihara et al., 2006). Live *Lactococcus lactis* fed to animals and human volunteers passed rapidly through the gastrointestinal tract without colonization (Klijn

* Corresponding author. Tel.: +1 519 824 4120x54760; fax: +1 519 824 5930.

E-mail address: bwilkie@uoguelph.ca (B.N. Wilkie).

et al., 1995) yet effectively induced local immune response (Robinson et al., 1997).

L. lactis is a well-characterized food-grade LAB and generally regarded as safe (GRAS), bacterium which is non-pathogenic, non-colonizing and is a candidate vehicle for vaccine and biologically active molecule delivery by mucosal routes (Wells et al., 2004). This bacterium has been engineered to express several bacterial and viral antigens (Hirt et al., 2000) and cytokines (Steidler et al., 1995, 2000; Bermudez-Humaran et al., 2003a,b). The purpose of the present study was to express biologically active rpIFN- γ protein in *L. lactis* as a delivery vehicle to be tested in newborn piglets for ability to alter immune response and to reduce the frequency of food-induced allergic sensitization.

2. Materials and methods

2.1. Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 1.

2.2. Isolation of porcine blood mononuclear cells

Blood was collected from three 45-day-old healthy female Yorkshire pigs (University of Guelph, Arken Research Station) into heparin-coated tubes (BD Mississauga, ON, Canada). Blood was diluted 1:2 with phosphate-buffered saline (PBS; pH 7.2), overlaid with Histopaque 1077 (Sigma, Oakville, ON) and centrifuged at $300 \times g$ for 30 min. Blood mononuclear cells, BMCs, were cultured (5×10^6 cells/ml) for 4 h at 37°C in complete DMEM medium (Invitrogen Canada

Inc., ON, Canada) in the presence of $10 \mu\text{g/ml}$ of *E. coli* lipopolysaccharide (LPS) (Sigma). One microliter containing 1×10^4 cells was used for first-strand cDNA synthesis using the SuperScript III CellsDirect cDNA Synthesis system (Invitrogen, ON, Canada) following the manufacturer's guidelines.

2.3. Cloning and expression of rpIFN- γ

The cDNA was amplified with the following set of primer pairs: 5'-CCATGAGTTATACAACCTATTTC-3' and 5'-TTATTTTGATGCTCTCTCTGGCC-3' and subcloned into TA- pCR 2.1 (Invitrogen) vector and the porcine IFN- γ gene was amplified using platinum *Pfx* polymerase (Invitrogen) by PCR reaction with primer set: 5'-CGCGCCGGCATGAGTTATACAACCTATTTC-3' and 5'-TCACTAGTTTATTTTGATGCTCTCTCTGGC-3', that introduced a new NgoMIV site (underlined). The resulting fragment was cloned into NgoMIV and *SpeI* (made blunt with Klenow enzyme) digested pTINX *L. lactis* vector (Schotte et al., 2000). Porcine IFN- γ was cloned under the transcriptional control of lactococcal P1 constitutive promoter and fused to the *usp45* secretion signal from *L. lactis* and transformed to *L. lactis* MG1363 cells by electroporation. The pTINX vector alone was used as a control.

2.4. Protein expression of rpIFN- γ

L. lactis cells containing pTINX-IFN- γ were cultured at 30°C overnight in GM17 medium (Difco-BD, Sparks, MD, USA) with erythromycin ($5 \mu\text{g/ml}$) (Sigma). Cultures were centrifuged at $5000 \times g$ for 10 min at 4°C . Cell pellet was resuspended in 10 ml of GM9 medium comprising

Table 1
Strains and plasmids used in this study

Strains and plasmids	Relevant phenotype or genotype	Source or reference
<i>E. coli</i> Top10	F ⁻ [<i>lacI</i> ^T Tn10(Tc ^r)] <i>mcrA</i> Δ (<i>mrr-hsdRMS mcrBC</i>) ϕ 80 <i>lacZ</i> Δ M15 <i>lacX74 recA araD139</i> Δ (<i>ara-leu</i>)7697 <i>galU galK rpsL</i> (Str ^r) <i>endA1 nupG</i>	Invitrogen
<i>L. lactis</i> MG1363	Plasmid- and phage-free derivative of NCDO712 strain	Laboratory stock (IATA, Spain)
pCR 2.1	Ap ^r Km ^r : ampicillin and kanamycin resistance; <i>lacZ</i> α <i>oriR</i> from ColE1	Invitrogen
pTINX	Em ^R : erythromycin resistance; <i>L. lactis</i> vector carrying the P1 promoter and <i>usp45</i> secretion signal	Schotte et al. (2000)
pTINX-IFN- γ	pTINX with cloned porcine IFN- γ gene	This work

1 ml of 10× Minimal medium (Sigma); 250 µl of 20% glucose (Sigma); 500 µl of 10% casitone (BD Bioscience); 20 µl of 1 M MgSO₄ (Sigma); 1 µl of 1 M CaCl₂ (Sigma) dissolved in 50 mM carbonate buffer (pH 7.4) containing 5 µg/ml of erythromycin. After 4 h of incubation at 30 °C, cells were centrifuged (5000 × *g* for 10 min). Pellet and supernatant from approximately 1×10^9 cells were precipitated with 1/10 volume of 100% trichloro-acetic acid, electrophoresed on a 12% SDS-polyacrylamide gel and immunoblotted. Rabbit anti-swine IFN-γ (Biosource, Camarillo, CA, USA) and goat anti-rabbit-IgG (whole molecule) conjugated to alkaline-phosphatase (Sigma) were used to probe the filter. The blot was developed using 5-bromo-4-chloro-3-indolyl-1-phosphate (BCIP) and nitro-blue tetrazolium (NBT) substrate (Invitrogen).

2.5. Bioassay for rpIFN-γ using MHC class II staining

Biological activity of rpIFN-γ was determined by its ability to upregulate cell surface MHC class II expression on cells of a porcine myelomonocytic cell line 3D4/31 (Weingartl et al., 2002). Cells were cultured and maintained at 37 °C in RPMI medium (Sigma). The resulting adherent monolayer was dissociated (dissociation solution, Sigma) and free cells were resuspended at 2.5×10^5 cells/ml in RPMI medium. Cells were cultured in triplicate with or without rpIFN-γ as various dilutions of *L. lactis* (pTINX-IFN-γ) cell culture supernatant in 100 µl of complete RPMI or 10 ng of rpIFN-γ (Prospec Technogen Ltd., Israel). Cells were incubated for 24 h prior to evaluation of MHC class II expression by enzyme-linked immunoassay (ELISA). Cells were reacted with porcine MHC II-specific mouse monoclonal antibody MSA-3 (J. Lunney, USDA, MD, USA) and binding was detected with biotin-conjugated goat anti-mouse IgG (heavy and light chain-specific; Kirkegaard and Perry Laboratories, MD, USA) followed by phosphatase-labelled streptavidin (Kirkegaard and Perry Laboratories) and chromogenic substrate (polynitro-phenyl phosphate, pNPP) (Sigma). Optical density was read at 405/630 nm using an ELISA plate reader (EL808, Bio-Tek Instrument Inc., WI, USA).

2.6. Statistics

The differences between control (*L. lactis* MG1363 with pTINX) and transformed (*L. lactis* MG1363 with pTINX-IFN-γ) in regulation of MHC class II expression were examined by Student's *t*-test. Significant difference was taken as $P \leq 0.05$.

3. Results

3.1. Detection of the protein from *L. lactis* cells

Total cell extract and culture supernatant proteins fractionated by SDS-PAGE were probed with anti-IFN-γ antibody. A 20 kDa protein consistent with

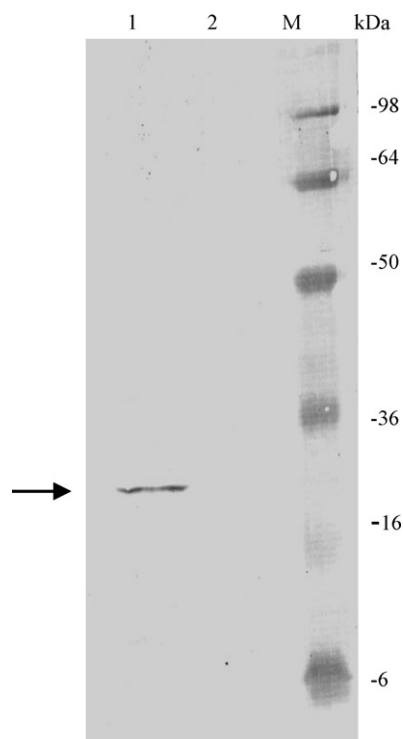


Fig. 1. Immunoblot analysis of rpIFN-γ expression. Immunoblot of *L. lactis* MG1363 culture supernatant samples from bacteria carrying pTINX-IFN-γ (lane 1) and control vector (pTINX) (lane 2). Rabbit anti-swine IFN-γ antibody was used for probing the membrane and detected using enzyme-conjugated goat anti-rabbit IgG (whole molecule). Migration positions of the molecular mass markers (lane M) are given on the right in kilodaltons (kDa). Migration position of rpIFN-γ is indicated by an arrow.

pIFN- γ was detected in culture supernatant of *L. lactis* (pTINX-IFN- γ) (Fig. 1). The immunoreactive band was absent from negative control (*L. lactis* (pTINX)) culture supernatant and present only in trace amounts in bacterial cell pellet (data not shown). Hence, fusion of the pIFN- γ gene to the *usp45* lactococcal secretion signal resulted in secretion of the recombinant protein into the extracellular space. Degradation fragments of pIFN- γ were not detected suggesting that proteolysis did not occur in *L. lactis*, obviating the need for protease inhibitors.

3.2. Recombinant pIFN- γ upregulates MHC class II expression

Cells of a porcine monocyte cell line were used to detect expression of MHC class II molecules by ELISA after treatment with control (pTINX vector) and test (pTINX-IFN- γ)-derived samples. Expression was significantly enhanced with rpIFN- γ -positive culture supernatant at all dilutions and with commercial rpIFN- γ ($P \leq 0.001$) (Fig. 2). Control samples (*L. lactis* containing pTINX alone) induced less but significant ($P \leq 0.05$) change in expression when

compared to the unstimulated cells that had minimum or no expression of MHC class II.

4. Discussion

Biologically active rpIFN- γ was produced in *L. lactis* and upregulated MHC class II expression on porcine monocyte cells suggesting that the modified *L. lactis* might be able to enhance type 1 immune response and reduce susceptibility to allergy in animals/humans. This is apparently the first report of expression of functional porcine IFN- γ , a type 1 cytokine, in a safe, food-grade bacterium, *L. lactis*, that has potential as a delivery vehicle. Preliminary attempts to express rpIFN- γ as a fusion protein in *E. coli* tagged to glutathione-S-transferase, suggested efficient secretion of the cytokine into extra-cellular medium (data not shown). However, *L. lactis*, unlike *E. coli*, does not produce LPS or endotoxins, and as a delivery vehicle, orally administered *L. lactis*, a food-grade GRAS bacterium, is non-pathogenic and has been regularly used to produce fermented milk products. Also, gram-positive bacteria are more potent

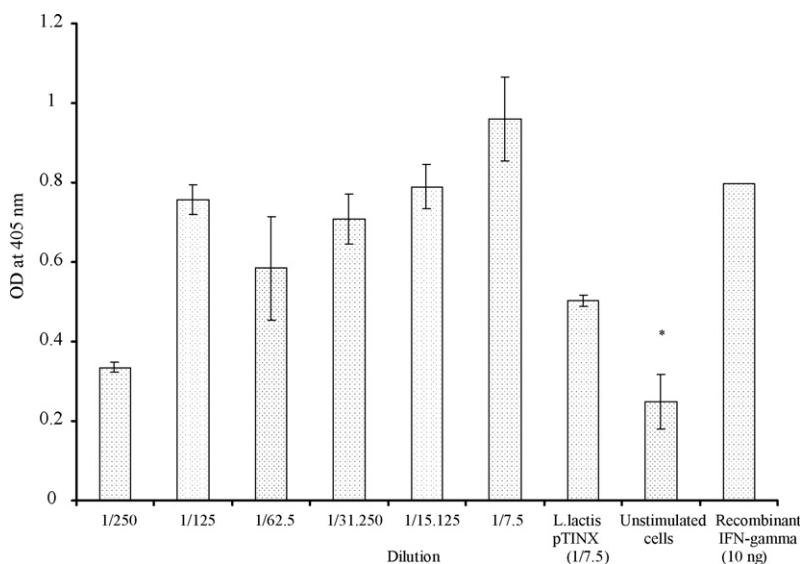


Fig. 2. Detection of MHC class II molecule expression using ELISA. Freshly cultured porcine alveolar macrophage cell line 3D4/31 was analyzed using mouse monoclonal antibodies (MSA-3) against porcine MHC class II after stimulation with various dilutions of *L. lactis* culture supernatants containing rpIFN- γ (1/7.5–1/250 dilutions in RPMI medium). Culture supernatants of *L. lactis* transformed with the pTINX vector (1/7.5 dilution), commercial rpIFN- γ (10 ng) and unstimulated cells were used as controls. Asterisk indicates a significant difference from stimulated cells ($P \leq 0.05$).

inducers of IL-12, a type 1 cytokine like IFN- γ , than gram-negative bacteria (Hessle et al., 2000).

The upregulation of class II MHC expression by rpIFN- γ confirms that the recombinant molecule is biologically active. The present results also confirm the inherent ability of *L. lactis* without expression of rpIFN- γ to upregulate cell surface MHC II expression. The upregulation of MHC class II by *L. lactis* (pT1NX) alone reflects other studies in which probiotic alone enhanced expression of these molecules (Christensen et al., 2002).

L. lactis is a safe vector for delivery of heterologous genes via foodstuffs to the digestive tract (Nouaille et al., 2003). Recently developed constitutive vectors for *L. lactis* have been used to construct recombinant lactococcal vaccines and to confirm in mice that lactococci can deliver biologically active molecules, such as cytokines, to the systemic and mucosal immune systems via mucosal routes (Steidler et al., 1998; Adel-Patient et al., 2005). We have engineered and expressed a functional rpIFN- γ gene in *L. lactis* that would allow delivery to the pig's intestinal mucosa. Although use of this construct is limited by lack of control over quantity and conditional expression of recombinant protein production, containment of live genetically modified *L. lactis* secreting human IL-10 in pigs is possible using an auxotrophic thymidine mutant (Steidler et al., 2003). However, the presently described construct will be used as a heat-killed bacterial vehicle expressing the recombinant porcine IFN- γ protein, for delivery both orally and systemically. Daily intake of heat-killed *Lactobacillus plantarum* augments acquired immunity in healthy human subjects (Hirose et al., 2006) and suppresses antigen-specific IgE in mice (Murosaki et al., 1998).

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References

- Adel-Patient, K., Ah-Leung, S., Creminon, C., Nouaille, S., Chatel, J.M., Langella, P., Wal, J.M., 2005. Oral administration of recombinant *Lactococcus lactis* expressing bovine beta-lactoglobulin partially prevents mice from sensitization. Clin. Exp. Allergy 35, 539–546.
- Bermudez-Humaran, L.G., Langella, P., Commissaire, J., Gilbert, S., Le Loir, Y., L'Haridon, R., Corthier, G., 2003a. Controlled intra- or extracellular production of staphylococcal nuclease and ovine omega interferon in *Lactococcus lactis*. FEMS Microbiol. Lett. 224, 307–313.
- Bermudez-Humaran, L.G., Langella, P., Cortes-Perez, N.G., Gruss, A., Tamez-Guerra, R.S., Oliveira, S.C., Saucedo-Cardenas, O., Montes de Oca-Luna, R., Le Loir, Y., 2003b. Intranasal immunization with recombinant *Lactococcus lactis* secreting murine interleukin-12 enhances antigen-specific Th1 cytokine production. Infect. Immun. 71, 1887–1896.
- Cencic, A., LeFevre, F., Koren, S., La Bonnardiére, C., 1999. Tetracycline-controlled expression of glycosylated porcine interferon-gamma in mammalian cells. Anim. Biotechnol. 10, 63–79.
- Christensen, H.R., Frokiaer, H., Pestka, J.J., 2002. Lactobacilli differentially modulate expression of cytokines and maturation surface markers in murine dendritic cells. J. Immunol. 168, 171–178.
- Collins, T., Korman, A.J., Wake, C.T., Boss, J.M., Kappes, D.J., Fiers, W., Ault, K.A., Gimbrone, M.A., Strominger, J.L., Pober, J.S., 1984. Immune interferon activates multiple class II major histocompatibility complex genes and the associated invariant chain gene in human endothelial cells and dermal fibroblasts. Proc. Natl. Acad. Sci. U.S.A. 81, 4917–4921.
- Delves, P.J., Roitt, I.M., 2000. The immune system. N. Engl. J. Med. 343, 108–117.
- Hessle, C., Andersson, B., Wold, A.E., 2000. Gram-positive bacteria are potent inducers of monocytic interleukin-12 (IL-12) while gram-negative bacteria preferentially stimulate IL-10 production. Infect. Immun. 68, 3581–3586.
- Hirose, Y., Murosaki, S., Yamamoto, Y., Yoshikai, Y., Tsuru, T., 2006. Daily intake of heat-killed *Lactobacillus plantarum* L-137 augments acquired immunity in healthy adults. J. Nutr. 136, 3069–3073.
- Hirt, H., Erlandsen, S.L., Dunny, G.M., 2000. Heterologous inducible expression of *Enterococcus faecalis* pCF10 aggregation substance Asc10 in *Lactococcus lactis* and *Streptococcus gordonii* contributes to cell hydrophobicity and adhesion to fibrin. J. Bacteriol. 182, 2299–2306.
- Klijn, N., Weerkamp, A.H., de Vos, W.M., 1995. Genetic marking of *Lactococcus lactis* shows its survival in the human gastrointestinal tract. Appl. Environ. Microbiol. 61, 2771–2774.
- Murosaki, S., Yamamoto, Y., Ito, K., Inokuchi, T., Kusaka, H., Ikeda, H., Yoshikai, Y., 1998. Heat-killed *Lactobacillus plantarum* L-137 suppresses naturally fed antigen-specific IgE production by stimulation of IL-12 production in mice. J. Allergy Clin. Immunol. 102, 57–64.
- Nouaille, S., Ribeiro, L.A., Miyoshi, A., Pontes, D., Le Loir, Y., Oliveira, S.C., Langella, P., Azevedo, V., 2003. Heterologous

- protein production and delivery systems for *Lactococcus lactis*. Genet. Mol. Res. 2, 102–111.
- Robinson, K., Chamberlain, L.M., Schofield, K.M., Wells, J.M., Page, R.W.F., 1997. Oral vaccination of mice against tetanus with recombinant *Lactococcus lactis*. Nat. Biotechnol. 15, 653–657.
- Romagnani, S., 1996. Development of Th1- or Th2-dominated immune responses: what about the polarizing signals? Int. J. Clin. Res. 26, 83–98.
- Sashihara, T., Sueki, N., Ikegami, S., 2006. An analysis of effectiveness of heat-killed lactic acid bacteria in alleviating allergic diseases. J. Dairy Sci. 89, 2846–2855.
- Schotte, L., Steidler, L., Vandekerckhove, J., Remaut, E., 2000. Secretion of biologically active murine interleukin-10 by *Lactococcus lactis*. Enzyme Microb. Technol. 27, 761–765.
- Steidler, L., Hans, W., Schotte, L., Neirynck, S., Obermeier, F., Falk, W., Fiers, W., Remaut, E., 2000. Treatment of murine colitis by *Lactococcus lactis* secreting interleukin-10. Science 289, 1352–1355.
- Steidler, L., Neirynck, S., Huyghebaert, N., Snoeck, V., Vermeire, A., Goddeeris, B., Cox, E., Remon, J.P., Remaut, E., 2003. Biological containment of genetically modified *Lactococcus lactis* for intestinal delivery of human interleukin 10. Nat. Biotechnol. 21, 785–789.
- Steidler, L., Robinson, K., Chamberlin, L., Schofield, K.M., Remaut, E., Le Page, R.W., Wells, J.M., 1998. Mucosal delivery of murine interleukin-2 (IL-2) and IL-6 by recombinant strains of *Lactococcus lactis* coexpressing antigen and cytokine. Infect. Immun. 66, 3183–3189.
- Steidler, L., Wells, J.M., Raeymaekers, A., Vandekerckhove, J., Fiers, W., Remaut, E., 1995. Secretion of biologically active murine interleukin-2 by *Lactococcus lactis* subsp. *lactis*. Appl. Environ. Microbiol. 61, 1627–1629.
- Vandenbroeck, K., Martens, E., D’Andrea, S., Billiau, A., 1993. Refolding and single-step purification of porcine interferon-gamma from *Escherichia coli* inclusion bodies conditions for reconstitution of dimeric IFN-gamma. Eur. J. Biochem. 215, 481–486.
- Vandenbroeck, K., Willems, L., Billiau, A., Opdenakker, G., Huybrechts, R., 1994. Glycoform heterogeneity of porcine interferon-gamma expressed in Sf9 cells. Lymphokine Cytokine Res. 13, 253–258.
- Weingartl, H.M., Sabara, M., Pasick, J., van Moorlehem, E., Babiuk, L., 2002. Continuous porcine cell lines developed from alveolar macrophages—partial characterization and virus susceptibility. J. Virol. Methods 104, 203–216.
- Wells, J.M., Robinson, K., Chamberlain, L.M., Schofield, K.M., Le Page, R.W.F., 2004. Lactic acid bacteria as vaccine delivery vehicles. Antonie Van Leeuwenhoek 70, 317–330.
- Young, H.A., Hardy, K.J., 1995. Role of interferon-gamma in immune cell regulation. J. Leukoc. Biol. 58, 373–381.