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ORIGINAL RESEARCH PAPER

A novel binary expression vector for production of human IL-10 in *Escherichia coli* and *Bifidobacterium longum*

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Abstract A novel expression vector (pLR) driven by *hup* promoter and *Bifidobacterium* β-galactosidase signal peptide was constructed. The pLR vector was used for the expression of the optimized human IL-10 synthetic gene in *Escherichia coli* and *Bifidobacterium longum*. In both microorganisms, rhIL-10 was in a soluble form in total extract cells. The recombinant hIL-10 was partially processed in *E. coli*, whereas in *Bifidobacterium* all rhIL-10 was found in the mature form.

Keywords Cytokine · Delivery systems · Protein expression · Synthetic gene · Sec pathway

Introduction

Delivery systems based on non-pathogenic bacteria have several advantages over DNA vaccines: transduction of the therapeutic gene into the human cell genome is not necessary and target gene expression can be stopped at any time by administration of suitable antibiotics. Moreover, a bacterial delivery system is not dependent on the presence of the tumor-specific antigens and could be applied to all patients that present tumor hypoxic/necrosis regions.

Bifidobacterium spp. are Gram-positive anaerobic bacteria and an predominant members of intestinal microflora that play important role in health promoting properties (Guarner and Malagelada 2003). Their economic importance is beyond doubt; these microorganisms are widely used in the fields of food science, medicine and industry. Using non-pathogenic microorganisms as delivery systems for cancer gene therapy (Yazawa et al. 2001) have increased since have been demonstrated that *Bifidobacterium* can selectively colonize and grow on the hypoxic region of solid tumors after intravenous injection (Kimura et al. 1998). *Bifidobacterium* has also been evaluated within the context of enzyme prodrug therapy (Fujimori et al. 2002; Theys et al. 2003; Fu et al. 2005; Yi et al. 2005), these data indicates the potential of *Bifidobacterium* as a tumor-specific gene delivery system for cancer treatment; however, no commercial expression vectors are available for the transformation of these microorganisms.

In this work, we describe the design of a novel binary expression system, named pLR, for *E. coli* and *Bifidobacterium*. pLR is driven by the *Bifidobacterium hup* promoter and terminator sequences. The functionality of the system was tested in both microorganisms by the expression of the human interleukin-10 (hIL-10) synthetic gene. The hIL-10 has been proposed as therapeutic treatment for autoimmune mediated illness

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such as inflammatory bowel disease (IBD) (deWaal and Moore 1998).

Materials and methods

Bacterial strains and media

E. coli TOP 10 was used for general cloning purposes and was grown at 37°C in Luria-Bertani broth containing at 100 μg ampicillin ml^{-1}. *Bifidobacterium longum* ATCC 15707 was routinely grown in MRS broth (Difco) supplemented with 0.05% (w/v) L-cysteine at 37°C under anaerobic conditions. Electrocompetent *Bifidobacterium* cells were obtained following the methodology of Argnani et al. (1996). Transformant cells were selected in MRS media containing 10 μg chloramphenicol ml^{-1}. The production of recombinant human interleukin-10 (rhIL-10) was made in MRS medium buffered at pH 8 using filter sterilized 50 mM carbonate solution (Schotte et al. 2000).

Construction of expression vector

The promoter and terminator sequences from *hup* gene were obtained from the *Bifidobacterium longum* NCC2705 genome database (GenBank AEO14295). The β-galactosidase signal peptide sequence (BIF3) from *B. bifidum* (GeneBank Access No. AJ224435) was fused in frame to hIL-10 gene. The construction denominated as ''expression cassette'' consisted of *hup* promoter, BIF3 signal peptide, hIL-10 optimized gene and *hup* terminator sequences, was synthesized and ligated into a shuttle vector pCR4 to yield pCR4-796 (Entelechon). The expression cassette was excised from pCR4-796 by *Eco*RI restriction and ligated into pDG7 *Eco*RI restricted plasmid (Fig. 1). The resulting plasmid of 8.0 kb was named as pLR (Mexican Patent Pending).

Expression of human Interleukine-10

E. coli TOP10 strain transformed with pLR was inoculated in LB medium containing 100 μg ampicillin ml^{-1} and incubated overnight at 37°C. Cells were separated by centrifugation, the pellet was resuspended and sonicated in TNT buffer (50 mM Tris/HCl pH 8, 300 mM NaCl, 0.1% Triton X-100) and centrifuged at 1500*g* for 10 min. Proteins from the supernatant were precipitated with five volumes of methanol and subsequently dissolved in Laemmli sample buffer (Schotte et al. 2000).

Transformed *B. longum* was grown for 48 h in MRS broth with chloramphenicol, and fresh broth was inoculated with this culture. After 3 h of incubation at 37°C, the cells were centrifuged at 1500*g* for 10 min and pellets were resuspended in MRS medium buffered with carbonate solution, and incubated for another 6 h. Cells and culture supernatants were separated by centrifugation. *Bifidobacterium* cell pellets were resuspended in TNT buffer and disrupted with glass beads. Cell debris was separated by centrifugation and supernatants were treated as described above.

Western blot and ELISA analysis

Protein fractions were separated by SDS-PAGE and electroblotted onto nitrocellulose membrane (GE Healthcare) for Western blot analysis. Recombinant hIL-10 (rhIL-10) was detected by immunoblotting and commercial rhIL-10 (Prepro Tech) was used as standard. The polyclonal rabbit anti-human IL-10 (Prepro Tech) was used as the primary antibody diluted to 1:1000. The goat anti-rabbit IgG (H+L) coupled to alkaline phosphatase (Bio-Rad) diluted to 1:2000 was used as a secondary antibody. Enzymatic activity was revealed with NBT/BCIP substrate (Invitrogen). Quantification of rhIL-10 was assayed by ELISA kit (eBiosciences).

Results and discussion

Expression vector construction

Protein expression using the plasmid pLR is driven by the constitutive *hup* promoter (Takeuchi et al. 2002). The pLR vector contains the *B. bifidum* β-galactosidase BIF3 signal peptide (Moller et al. 2001) was fused in frame to the codon optimized hIL-10 synthetic gene. The synthetic hIL-10 gene was designed guided by the preferred codons to be expressed in *E. coli* and *Bifidobacterium longum*. The resultant optimized gene had 81% identity with respect to the wild-type gene (Fig. 1). A summary of codon preference in *E. coli* and *Bifidobacterium longum* and codon usage in the wild type and the optimized synthetic hIL-10 gene is shown in Table 1, where the most significant changes were for

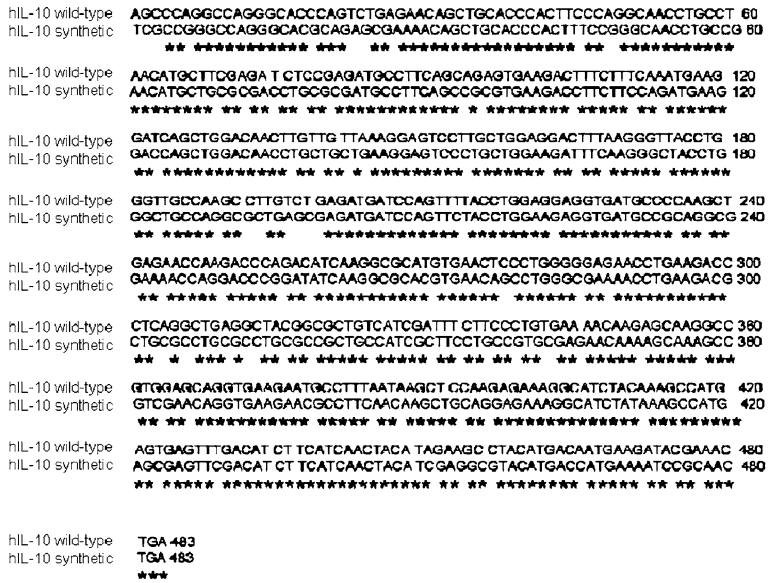

Fig. 1 Alignment of nucleotide sequences of wild-type and the optimized synthetic hIl-10 gene

arginine, leucine, proline and serine codons. The restriction sites, *Sal*I and *Nco*I (upstream signal peptide), *Eco*47III between the signal peptide and hIL-10 gene, and *Sma*I and *Sph*I (downstream the gene), were included to facilitate the subcloning of other target genes in pLR (Fig. 2). Clones where the expression cassette was ligated in the same direction as the *bla* gene and clones in contrary sense were named pLR1 and pLR2, respectively (Fig. 2). *E. coli* was transformed with each pLR constructions. After *Hin*dIII–*Sma*I double restriction, one fragment of 720 bp was obtained in pLR1 clones (Fig. 3, lane 2), and as expected no fragment were observed with pLR2 clones (Fig. 3, lane 3).

Expression of rhIL-10

Functionality of pLR1 and pLR2 was tested in *E. coli*. The amount of rhIL-10 attained with pLR1 was 4.2 times more than pLR2, interestingly the

Table 1 Summary of codon preference in *E. coli* and *B. longum* and codon usage in the wild-type and the optimized synthetic hIL-10 gene

Amino acid	Native gene	Synthetic gene	Codon usage (%)	
			E. coli	*B. longum*
Ala	GCC, GCG,	GCG, GCC, GCU	33.8, 25.6, 15.3	26.8, 59.8, 12.4
Arg	CGC, CGA, AGA, AGG, CGG	CGC	22.0	28.5
Asn	AAC, AAU	AAC	21.7	25.2
Cys	UGU, UGC	UGC	6.4	7.7
Gln	CAA, CAG	CAG	28.9	26.7
Gly	GGU, GGG, GGC	GGC	29.8	51.5
Ile	AUA, AUC	AUC	25.2	37.8
Leu	CUA, CUC, CUG, CUU, UUA, UUG	CUG	52.9	42.3
Pro	CCA, CCU, CCC	CCG	23.3	26.6
Ser	AGU, UCU, UCC, AGC	AGC, UCC, UCG	16.1, 8.6, 8.9	12.4, 24.8, 13.3
Thr	ACA, ACC, ACU	ACG, ACC	14.4, 23.5	15.4, 36.9
Tyr	UAU	UAC, UAU	12.2, 16.6	18.5, 8.2
Val	GUC, GUG	GUG	26.3	39.5

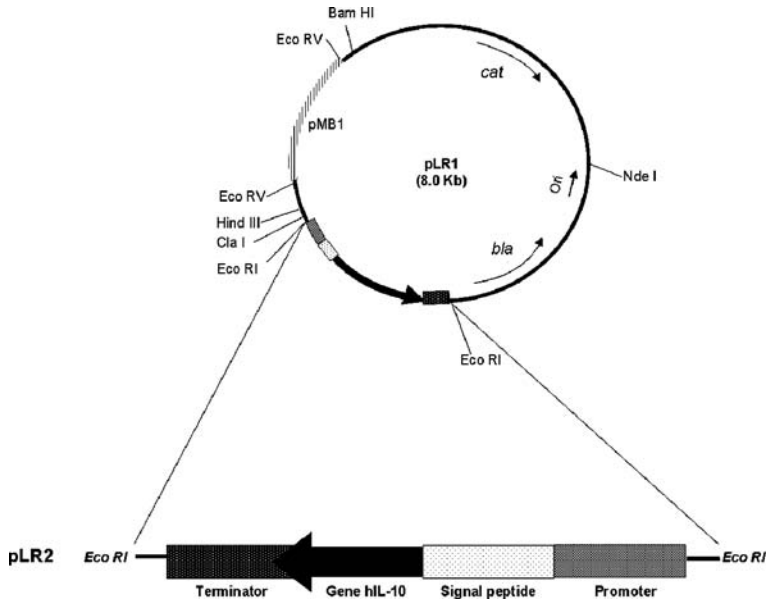

Fig. 2 Esquematic representation of pLR plasmid. The HU promoter and terminator, the signal peptide BIF3 was fused to the synthetic hIL-10 gene. When the expression cassette was ligated in the sense of *bla* gene was named as pLR1, contrary to gene *bla* was named as pLR2 (zoomed image). pMB1 represents the replicon for *Bifidobacterium*; Ori, *E. coli* origin; *bla*, Ampicillin resistance; *cat*, Chloramphenicol resistance

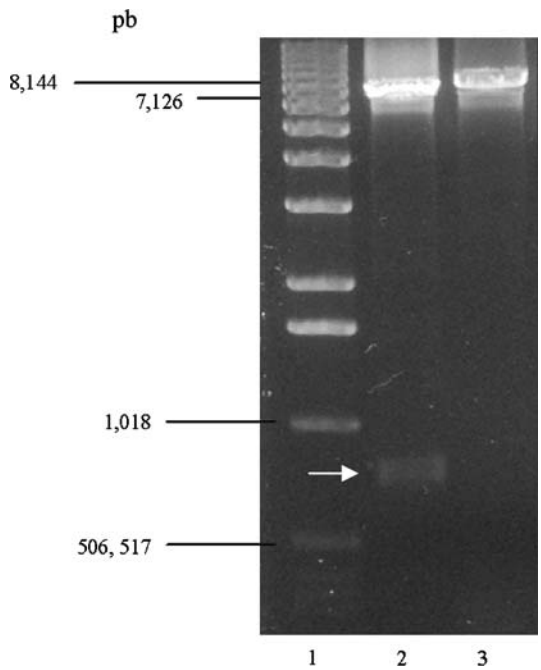

Fig. 3 Restriction assay of pLR1 and pLR2. Lane 1, 1 kb DNA ladder; Lane 2, *Hin*dIII–*Sma*I restriction of clone 1 shows an 800 pb fragment (white arrow); Lane 3, *Hin*dIII–*Sma*I restriction of clone 2, no fragments were liberated

recombinant protein was detected in higher amounts in the culture medium than inside of the cells (Table 2). Western blot analysis from cell pellets

Table 2 Expression and secretion of hIL-10 in *Escherichia coli*

E. coli TOP10 transformed with	pg rhIL-10 (pg ml^{-1})		
	Cell extracts	Culture broth	Total
pLR1	140	220	360
pLR2	17	68	86

showed two forms of rhIL-10 in *E. coli*, the 18.6 kDa band represents the mature form, while the higher molecular weight band corresponds to the non-processed protein (Fig. 4A).

In the case of *B. longum*, only transformants with pLR2 were obtained. It has been claimed that the presence of a thick (multi-layered) cell wall generally forms a barrier for the uptake of exogenous DNA molecules; this gives the low efficiency of *Bifidobacterium* transformation (Argnani et al. 1996). As shown on Fig. 4B, all protein expressed for *B. longum* was correctly processed; only the 18.6 kDa protein was observed.

The pLR plasmid was useful to express rhIL-10 in both *Escherichia coli* and *Bifidobacterium longum*. This is the first report showing that promoters and signal peptides from *Bifidobacterium* are recognized for *E. coli*. Comparative analysis from *E. coli* and *Bifidobacterium* promoters showed that -35, -10 box

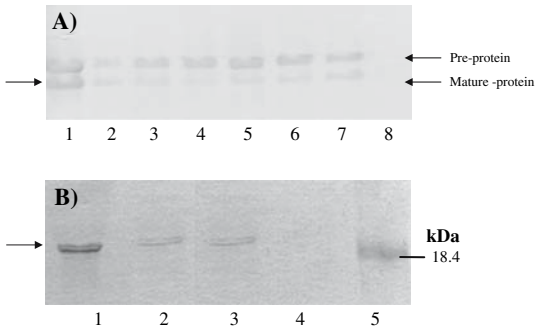

Fig. 4 Immunoblot detection for rhIL-10 in (**A**) *E. coli* and (**B**) *Bifidobacterium longum*. (**A**) Lane 1, rhIL-10 standard; Lanes 2–7, rhIL-10 expressed from different *E. coli* clones transformed with pLR1; lane 8, control expression, *E. coli* clone with pLR without hIL-10 gene. (**B**) Lane 1, rhIl-10 standard; lane 2 and 3 rhIL-10; lane 4, *Bifidobacterium* clone with pLR without hIL-10 gene; lane 5, MW markers

and ribosome binding sites (RBS) shares a high degree of homology (Takeuchi et al. 2002), this homology could explains the functionality of *Bifidobacterium hup* promoter in *E. coli*. Since the concentration of mature rhIL-10 in *B. longum* was low (22 pg ml^{-1}), the optimization of expression conditions is further recommended for larger scale production. However, high amounts of IL-10 could have secondary effects; the IL-10 produced for *B. longum* could be enough for an in situ delivery. Recombinant proteins in *B. longum* can bind to the outside of the cell wall (Rhim et al. 2006) and this could be the reason why IL-10 was not detected in the culture medium. This will be important in the design of more functional system for in situ pro-drug directed therapy. Further studies for immunolocalization should be carried out. Several others proteins such as β-galactosidase, lipase, amylase, among others therapeutic proteins, could be achieved with pLR plasmid for in vivo delivery.

The alternative approach with more potential to specifically target cancer cells are based on the use of non-toxic bacteria as *Bifidobacterium*. Further improvements of pLR systems could be achieved by incorporating stronger inducible promoters.

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References

Argnani A, Leer RJ, van Luijk N, Pouwels PH (1996) A convenient and reproducible method to genetically transform bacteria of the genus *Bifidobacterium*. Microbiology 142:109–114

de Waal MR, Moore KW (1998) Interleukin-10. In: Thomson A (ed) The cytokine handbook, 3rd edn. San Diego, CA, Academic Press, pp 333–364

Fu GF, Li X, Hou YY, Fan YR, Liu WH, Xu GX (2005) *Bifidobacterium longum* as an oral delivery system of endostatine for gene therapy on solid liver cancer. Cancer Gene Ther 12:133–140

Fujimori M, Amano J, Taniguchi S (2002) The genus *Bifidobacterium* for cancer gene therapy. Curr Opin Drug Discov Devel 5:200–203

Guarner F, Malagelada JR (2003) Gut flora in health and disease. Lancet 361:512–519

Kimura NT, Taniguchi S, Aoki K, Baba T (1998) Selective localization and growth of *Bifidobacterium bifidum* in mouse tumors following intravenous administration. Cancer Res 40:2061–2068

Moller PL, Jorgensen F, Hansen OC, Madsen SM, Stougaard P (2001) Intra and extracellular β-galactosidases from Bifidobacterium bifidum and B. infantis: Molecular cloning, heterologous expression, and comparative characterization. Appl Environ Microbiol 67:2276–2283

Rhim SL, Park MS, Ji GE (2006) Expression and secretion of *Bifidobacterium adolescentis* amylase by *Bifidobacterium longum*. Biotechnol Lett 28:163–168

Schotte L, Steidler L, Vandekerckhove J, Remaut E (2000) Secretion of biologically active murine interleukin-10 by *Lactococcus lactis*. Enzyme Microbiol Technol 27:761–765

Takeuchi A, Matsumura H, Kano Y (2002) Cloning and expression in *Escherichia coli* of a gene, *hup*, encoding the histone-like protein HU of *Bifidobacterium longum*. Biosci Biotech Biochem 66:598–603

Theys J, Barbé S, Landuyt S, Nuyts L, Mellaert V, Wouters B, Anné J, Lambin P (2003) Tumor specific gene delivery using genetically engineered bacteria. Curr Gene Ther 3:207–221

Yazawa K, Fujimori M, Nakamura T, Sasaki T, Amano J, Kano Y, Taniguchi S (2001) *Bifidobacterium longum* as a delivery system for gene therapy of chemically induced rat mammary tumors. Breast Cancer Res Treat 66:165–170

Yi C, Huang Y, Guo ZY, Wang S (2005) Antitumor effect of cytosine deaminase/5-flurocytosine suicide gene therapy system mediated by *Bifidobacterium infantis* on melanoma. Acta Pharmacol Sin 26:629–634