



Cytoplasmic and extracellular expression of pharmaceutical-grade mycobacterial 65-kDa heat shock protein in *Lactococcus lactis*

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ABSTRACT. Lactic acid bacteria (LAB) are an attractive and safe alternative for the expression of heterologous proteins, as they are nonpathogenic and endotoxin-free organisms. *Lactococcus lactis*, the LAB model organism, has been extensively employed in the biotechnology field for large-scale production of heterologous proteins, and its use as a “cell factory” has been widely studied. We have been particularly interested in the use of *L. lactis* for production of heat shock proteins (HSPs), which reportedly play important roles in the initiation of innate and adaptive immune responses. However, this activity has been questioned, as LPS contamination appears to be responsible for most, if not all, immunostimulatory activity of HSPs. In order to study the effect of pure HSPs on the immune system, we constructed recombinant *L. lactis* strains able to produce and properly address the *Mycobacterium leprae* 65-kDa HSP (Hsp65) to the cytoplasm or to the extracellular medium, using a xylose-induced expression system.

Approximately 7 mg/L recombinant Hsp65 was secreted. Degradation products related to lactococcal HtrA activity were not observed, and the *Limulus* amoebocyte lysate assay demonstrated that the amount of LPS in the recombinant Hsp65 preparations was 10-100 times lower than the permitted levels established by the U.S. Food and Drug Administration. These new *L. lactis* strains will allow investigation of the effects of *M. leprae* Hsp65 without the interference of LPS; consequently, they have potential for a variety of biotechnological, medical and therapeutic applications.

Key words: Heat shock proteins; LPS-free Hsp65; *Lactococcus lactis*; *Mycobacterium leprae*

INTRODUCTION

Over the last 30 years, there have been considerable advances in technology for producing recombinant proteins in bacterial expression systems. The use of bacteria to produce recombinant proteins is very attractive because they are genetically well-characterized and because they are easy to culture on inexpensive substrates (Brondyk, 2009). *Escherichia coli* was the first bacterial host used to produce recombinant proteins and remains one of the most widely used bacteria for the expression of heterologous proteins. The advantage of *E. coli* consists in its ability to produce proteins in large quantities and to grow very quickly. However, despite these useful characteristics, it also has some drawbacks (Terpe, 2006; Demain and Vaishnav, 2009). The main problem associated with the use of *E. coli* is the accumulation of endotoxins (referred to as lipopolysaccharides - LPS). LPS are large molecules found in the outer membrane of Gram-negative bacterial cell walls that can bind to many kinds of receptors, resulting in the activation of various types of intracellular signaling pathways and production of proinflammatory cytokines. Consequently, LPS acts as a powerful activator of innate immune responses and is responsible for endotoxic shock (Cardoso et al., 2007; Bryant et al., 2010). Moreover, this bacterium cannot express very large proteins and high expression of recombinant protein often leads to the accumulation of inactive protein aggregates (inclusion bodies) (Brondyk, 2009).

To overcome these limitations, lactic acid bacteria (LAB) have emerged as an attractive and safe alternative, not only due to their "GRAS" (Generally Regarded As Safe) status granted by the U.S. Food and Drug Administration (FDA), but also because they are noninvasive, nonpathogenic, endotoxin-free and do not form inclusion bodies (Wells and Mercenier, 2008). *Lactococcus lactis*, the LAB model, has been widely used for large-scale production of heterologous proteins for the last two decades (Mierau and Kleerebezem, 2005). Its metabolism is relatively simple and well-known, and laboratory strains do not produce the extracytoplasmic protease PrtP. Furthermore, there are several genetic tools available for the production of heterologous proteins in *L. lactis* (Morello et al., 2008). Its genetic accessibility has allowed a variety of new applications to be developed, beyond its traditional use in food fermentation. For example *L. lactis* has been genetically engineered to produce cytokines (Marinho et al., 2010), enzymes (Du et al., 2010), membrane proteins (Frelet-Barrand et al., 2010), and bacterial and viral antigens, expressed either intra- or extracellularly (Van Huynegem et al., 2009).

Therefore, its use as a “cell factory” has been extensively studied.

We have been particularly interested in the use of *L. lactis* as a factory for the production of heat shock proteins (HSPs), which is a group of molecular chaperones that are highly conserved across species. Their expression is induced by a wide variety of stresses (including high temperature, anoxia, and ethanol) (Lindquist and Craig, 1988). Under stress conditions, HSPs promote selective degradation of misfolded proteins and they prevent protein aggregation by facilitating their correct conformation (Rajaiah and Moudgil, 2009). These proteins have also been reported to play important roles in the initiation of innate as well as adaptive immune responses by activating antigen-presenting cells, such as dendritic cells and macrophages (Tsan and Gao, 2009). However, the immunostimulatory activity of HSPs has been questioned. Molecules such as LPS and other endotoxins have the same effect on the immune system (Osterloh et al., 2007). Gao and Tsan (2003) demonstrated that the reported proinflammatory cytokine-inducing effect of 60- and 70-kDa HSP (Hsp60 and Hsp70) is due to contamination with LPS and LPS-associated molecules. These contaminants appear to be responsible for most, if not all, of the reported *in vitro* capacity to induce intracellular signalling, as highly purified HSPs did not alter cell function, such as upregulation and/or downregulation of genes that control cytokine production (Tsan and Gao, 2009).

To be able to study how pure HSPs affect the immune system, we constructed recombinant *L. lactis* strains secreting *Mycobacterium leprae* 65-kDa HSP (Hsp65), using a xylose-inducible expression system (XIES) (Miyoshi et al., 2004). This protein is reported to be an immunodominant antigen eliciting both humoral and cellular responses. Immunization with DNA-hsp65 produced prophylactic and therapeutic effects in a murine model of tuberculosis (Silva, 1999). This protein also induced the reduction of established tumors in mouse models (Victora et al., 2009), and it is protective against experimentally induced arthritis in mice (Santos-Junior et al., 2005). These *L. lactis* strains offer the significant advantage of producing recombinant Hsp65 that is endotoxin-free, which could be used for biotechnological and therapeutic applications.

MATERIAL AND METHODS

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. The *L. lactis* NCD02118 strains were grown in Difco M17 broth, supplemented with 0.5% glucose (GM17) or 1% xylose (XM17), at 30°C, without agitation. When required, 10 µg/mL chloramphenicol was added to the medium. The *E. coli* strains were aerobically grown at 37°C in Luria-Bertani medium containing 100 µg/mL ampicillin or 10 µg/mL chloramphenicol, when needed.

DNA manipulation

The procedures for DNA manipulation were carried out as described by Sambrook et al. (1989), with a few modifications. The plasmids were purified by the alkaline lysis method, and the quality of the products that were obtained, including their concentration and purity, was determined by measuring absorbance at 260 and 280 nm in a spectrophotometer (Bio-Photometer plus Eppendorf). Restriction and modification endonucleases, as well as ligation reactions, were used and run according to recommendations of the supplier (Invitrogen).

Table 1. Bacterial strains and plasmids.

Bacterial strains and plasmids	Relevant characteristics	Source or reference
Bacterial strains		
<i>E. coli</i> TOP10	<i>hsdR</i> , <i>mcrA</i> ; <i>lacZAM15</i> ; <i>endA1</i> ; <i>recA1</i>	Invitrogen
<i>L. lactis</i> NCDO2118	Wild-type <i>L. lactis</i> strain (<i>L. lactis</i> subsp. <i>lactis</i>)	Collection strain
<i>E. coli</i> 83b	<i>E. coli</i> TG1 [(<i>supE</i> , <i>hsd</i> , $\Delta 5$, <i>thi</i> , Δ <i>lac-proAB</i>), F' (<i>traD36proAB-lacZAM15</i>)] harboring the p χ yIT::CYT:: <i>nuc</i> plasmid	Collection strain
<i>E. coli</i> 84c	<i>E. coli</i> TG1 [(<i>supE</i> , <i>hsd</i> , $\Delta 5$, <i>thi</i> , Δ <i>lac-proAB</i>), F' (<i>traD36proAB-lacZAM15</i>)] harboring the p χ yIT::SEC:: <i>nuc</i> plasmid	Collection strain
Plasmids		
pcDNA3- <i>hsp65</i>	Vector carrying <i>M. Leprae hsp65</i> ORF (Neo-Km ^r /ColEI ORI)	Lowrie et al., 1997
pCR [*] -Blunt II- TOPO [*]	Cloning vector (Zeo [*] -Km ^r /pUC ORI)	Invitrogen
pTP- <i>hsp65</i>	pCR [*] -Blunt II-TOPO [*] harboring <i>M. leprae hsp65</i> ORF (cytoplasmic form)	This study
p χ yIT::CYT:: <i>nuc</i>	pCR [*] -Blunt II-TOPO [*] harboring <i>M. leprae hsp65</i> ORF (secreted form)	This study
p χ yIT::SEC:: <i>nuc</i>	pWV01/Cm ^r ; expression vector containing the fusion <i>rhsUsp45::nuc</i> , under the control of P χ yIT	Miyoshi et al., 2004
p χ yIT::CYT- <i>hsp65</i>	pWV01/Cm ^r ; expression vector containing the fusion <i>rhsUsp45::spUsp45::nuc</i> , under the control of P χ yIT	Miyoshi et al., 2004
p χ yIT::SEC- <i>hsp65</i>	pWV01/Cm ^r ; expression vector containing the fusion <i>rhsUsp45::hsp65</i> , under the control of P χ yIT	This study
	pWV01/Cm ^r ; expression vector containing the fusion <i>rhsUsp45::spUsp45::hsp65</i> , under the control of P χ yIT	This study
Collection strains of the Laboratório de Genética Celular e Molecular (LGCM), Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais (UFMG), Brazil.		

Cloning and expression of recombinant *M. leprae* Hsp65 in *E. coli* and *L. lactis*

To construct *L. lactis* strains producing either cytoplasmic (CYT) or secreted (SEC) forms of the *M. leprae* Hsp65 antigen, the *hsp65* ORF was PCR-amplified from the pcDNA3:*hsp65* plasmid (Table 1). The primers that were used, which contained an artificial restriction site at both extremities, were based on the genomic DNA sequence of *M. leprae* TN (GenBank Accession No. M14341). These primers are listed in Table 2. Subsequently, the amplicons corresponding to the CYT and SEC forms of the antigen were cloned into the pCR[®]-Blunt II-TOPO[®] in *E. coli* TOP10 to generate the intermediate plasmids: pTP:*chsp65* and pTP:*shsp65*. These plasmids were later digested with the restriction endonucleases *Nsi*I and *Eco*RI, allowing the release of the ORFs “CYThsp65” and “SEChsp65”. These fragments were purified and cloned into “pXylT:CYT” and “pXylT:SEC” backbones, respectively, which were purified from *Nsi*I/*Eco*RI cut of pXylT:CYT:*nuc* and pXylT:SEC:*nuc*. The final plasmids, pXylT:CYT:*hsp65* and pXylT:SEC:*hsp65* (Figure 1), were first obtained in *E. coli* and then transferred to *L. lactis* NCDO2118 by electroporation. The presence of the expression vectors with the inserts was confirmed by PCR and DNA sequencing.

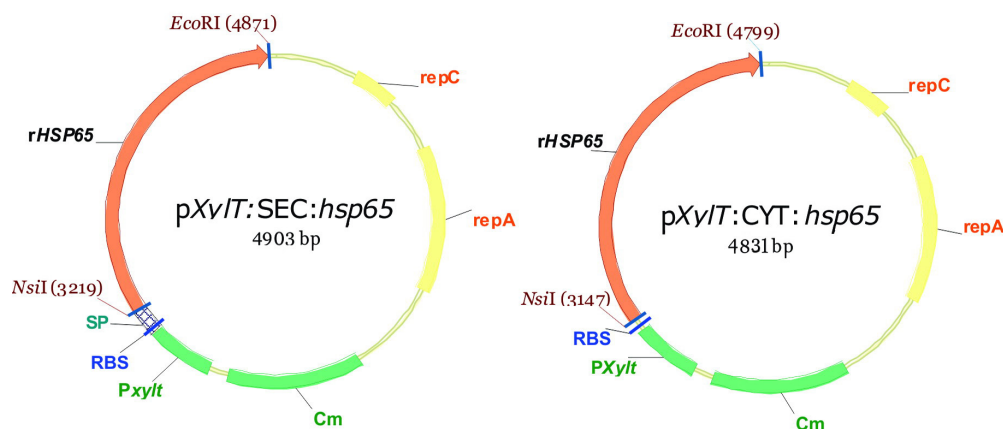


Figure 1. Schematic representation of the xylose-inducible expression vectors for intracellular or extracellular production of rHsp65. For details of plasmid construction, see the text. P_{XylT} = xylose-inducible promoter; RBS_{Usp45} = ribosome binding site of Usp45; rHsp65 = recombinant *Mycobacterium leprae hsp65* coding sequence; Cm = chloramphenicol resistance; SP = signal peptide; repA and repC = replication origin; *Eco*RI and *Nsi*I = restriction enzymes.

Conditions of xylose induction

On the first day, a single colony of wild-type or recombinant *L. lactis* NCDO2118 (harboring pXylT:CYT:*hsp65* or pXylT:SEC:*hsp65*) was grown at 30°C, without agitation, in 5 mL GM17, containing 10 µg/mL chloramphenicol, when needed. On the second day, the overnight culture was diluted 1:10,000 in 10 mL fresh XM17, supplemented with 10 µg/mL chloramphenicol, when required, to induce the expression of the *hsp65* ORF. On the third day, when a 2.0 optical density at 600 nm (OD_{600 nm}) was reached, protein was extracted and analyzed by Western blotting.

Protein extractions

Protein sample preparation from *L. lactis* cultures was performed as previously described (Le Loir et al., 1998), with some modifications. Samples were prepared from 2 mL both induced and noninduced cultures. Next, they were centrifuged for 10 min at 4°C, at 12,000 g. Later, the supernatant and the pellet were treated separately. The supernatant was run through a 0.2- μ m pore-size filter and 100 μ L 100% trichloroacetic acid was then added for protein precipitation. Dithiothreitol (DTT; 10 mM) and 1 mM phenylmethylsulfonyl fluoride (PMSF) were also added to the filtrate. This was then incubated for 1 h on ice and centrifuged (20 min, 12,000 g, 4°C). The pellet was resuspended in 50 mM NaOH. The original cell pellet was resuspended in 100 μ L TES-Lys buffer (25% sucrose, 1 mM EDTA, 50 mM Tris-HCl, pH 8.0, 10 mg/mL lysozyme), 1 mM PMSF and 10 mM DTT. Afterwards, samples were incubated at 37°C for 30 min and 50 μ L 20% SDS was then added to the lysate.

Protein quantification and Western blot analyses

The Bradford method (Bradford, 1976) was used to determine the concentration of total proteins extracted from *L. lactis*. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970). Western blotting analyses were conducted according to Sambrook et al. (1989), using anti-Hsp65 antibodies (1:2500) (Farmacore Biotecnologia Ltda.) and rabbit anti immunoglobulin (IgG), conjugated to alkaline phosphatase (Sigma). The immunodetection blots were scanned and the intensity of the Hsp65 signals was compared to that of the positive control used, which was a known amount of a purified Hsp65 produced in *E. coli* (Farmacore Biotecnologia Ltda.).

Endotoxin analysis

The Limulus amoebocyte lysate (LAL) test was conducted to check the endotoxin-free condition of the wild and recombinant strains of *L. lactis*, as recommended by European and US Pharmacopeias, using the QCL 1000-LAL test kit (Lonza). Briefly, proteins were extracted from *L. lactis* and endotoxin concentrations in the samples were calculated based on a standard curve, which ranged from 0.025 to 50 endotoxin units (EU)/mL. The absorbance in each well was measured at 405 nm.

RESULTS AND DISCUSSION

Construction of recombinant *L. lactis* strains to produce either cytoplasmic or secreted forms of Hsp65 antigen

We produced, for the first time, both cytoplasmic and secreted forms of *M. leprae* Hsp65 in *L. lactis* strain NCDO2118 using XIES. The choice of XIES was based on some advantageous features compared to other expression systems, such as its ability to be switched on or off by simply adding either xylose or glucose, respectively (Miyoshi et al., 2004). It is less expensive and more secure for laboratory use compared to the other available methods.

As described in Material and Methods, two expression vectors were initially constructed, p*XylT*:CYT:*hsp65* (cytoplasmic Hsp65 production) and p*XylT*:SEC:*hsp65* (secreted Hsp65 production). These plasmids are derived from two broad-host-range expression vectors, pCYT:*Nuc* and pSEC:*Nuc* (Bermudez-Humaran et al., 2003). The former contains i) the *PxyIT* promoter (Jamet, 2001), ii) the ribosome-binding site of the lactococcal *usp45* gene (van Asseldonk et al., 1990), and iii) the DNA fragment encoding the mature part of the staphylococcal nuclease, NucB (Le Loir et al., 1994). The latter contains all of this DNA sequence, plus the signal peptide of the *Usp45* coding sequence (*spUsp45*), necessary for the secretion of NucB.

The vector to target the Hsp65 protein to the cytoplasm of *L. lactis* (p*XylT*:CYT:*hsp65*) was obtained as follows: A 1626-bp DNA fragment encoding Hsp65 was PCR-amplified from the plasmid pcDNA3:*hsp65*. Primers were designed using the GenBank nucleotide database of the *M. leprae* TN *hsp65* gene (GenBank accession No. M14341). Artificial restriction sites (*NsiI* and *EcoRI*) and extra bases were introduced into the primers to clone the *hsp65* ORF in-frame into the expression vector p*XylT*:CYT:*nuc* (Table 2). The amplicon generated was treated with the enzymes *NsiI* and *EcoRI* prior to cloning into purified backbone isolated from *NsiI*-*EcoRI*-cut pCR®Blunt II-TOPO® cloning vector. The construction of this intermediate plasmid (pTP:*chsp65*) was important because treatment with restriction enzymes releases inserts with cohesive extremities; this allowed correct cloning into the *L. lactis* plasmid p*XylT*:CYT:*nuc*. The vector pTP:*chsp65* was transformed in *E. coli* TOP10 and then recovered to confirm the presence of *hsp65* by PCR analysis. Later, pTP:*chsp65* was digested with *NsiI* and *EcoRI*, and the released fragment was cloned into the purified backbone isolated from *NsiI*-*EcoRI*-cut p*XylT*:CYT:*nuc*, replacing the DNA sequence encoding NucB. The vector to direct Hsp65 to the extracellular medium, p*XylT*:SEC:*hsp65*, was obtained using the same procedures. First, the *hsp65* ORF was PCR-amplified from pcDNA3:*hsp65*. The oligonucleotides again contained artificial restriction sites for *NsiI* and *EcoRI*, along with extra bases, which were added to adapt the reading frame of the *spUsp45* coding sequence (Table 2). The PCR product was digested with *NsiI* and *EcoRI* and then subcloned into the pCR®Blunt II-TOPO® vector, previously cut with the same restriction enzymes. Subsequently, the intermediate plasmid, pTP:*shsp65*, was introduced into *E. coli* TOP10 by electroporation and then recovered to check for the *hsp65* ORF by PCR analysis. Subsequently, pTP:*shsp65* was cut with *NsiI* and *EcoRI*, and the *shsp65* fragment, containing cohesive extremities, was cloned into purified backbone isolated from *NsiI*-*EcoRI*-cut pSEC:*Nuc*. The final plasmids, p*XylT*:CYT:*hsp65* or p*XylT*:SEC:*hsp65* (Figure 1), were successfully transformed in both *E. coli* TOP10 and *L. lactis* NCDO2118. All constructs were confirmed by PCR and DNA sequencing analysis.

Table 2. Primers used for the polymerase chain reactions.

Primer	Sequence
SCF <i>hsp65</i> (coding strand for the SEC form)	5'-CCATGCATCAGCCAAGACAATTGCGTACG-3'
CTF <i>hsp65</i> (coding strand for the CYT form)	5'-CCATGCATGCCAAGACAATTGCGTACG-3'
SCTR <i>hsp65</i> (complementary strand for the CYT and SEC form)	5'-CCGAATTCAGAAAGTCCATACCACCC-3'

The *NsiI* site is underlined and the *EcoRI* site is underlined and in bold. A (in the first line) was added to adapt the reading frame of *spUsp45*.

***L. lactis* is able to produce and secrete LPS-free *M. leprae* Hsp65**

Western blot experiments of xylose-induced and noninduced cultures were conducted to detect the production of Hsp65 in *L. lactis* carrying the plasmid pXylT:CYT:hsp65 or pXylT:SEC:hsp65, allowing us to characterize the strains. Analysis of induced stationary-phase cell lysates and culture supernatants demonstrated that both *L. lactis* (pXylT:CYT:hsp65) (hereafter called CYT:hsp65) and *L. lactis* (pXylT:SEC:hsp65) (hereafter called SEC:hsp65) were able to produce and properly direct the recombinant protein. No signal of the antigen was detected in the noninduced cultures (Figures 2 and 3). Analysis of protein content showed that the band migrated at the expected position (65 kDa), which is the size of *M. leprae* Hsp65. The immunoblotting assay also revealed that this protein was expressed without signs of degradation, since only one band was detected (Figure 2). Usually, a large number of heterologous proteins produced in wild-type *L. lactis* are degraded by the major extracellular protease, HtrA (Poquet et al., 2000). Absence of degradation by this protease can be explained by the fact that HSPs are among the most highly conserved proteins, which is evident from its conserved DNA and protein sequences (Silva, 1999). This lack of degradation products suggests that *L. lactis* does not recognize this antigen as an exogenous protein. Recombinant Hsp65 (rHsp65) produced by induced *L. lactis* strain CYT:hsp65 was retained in the cytoplasm, as expected, since this strain harbors a vector that lacks the *spUsp45* coding sequence (lanes CYT:hsp65, Figure 2). We also found that induced *L. lactis* strain SEC:hsp65 secreted large amounts of this antigen. We found that approximately 7 mg/L rHsp65 were exported to the extracellular medium. Moreover, the secretion efficiency (ratio of mature protein secreted in the supernatant as a fraction of intracellular content of this protein) was estimated to be 50% (Figure 3). Accumulation of rHsp65 within the cytoplasm can be attributed to intrinsic allosteric properties of HSPs (Sigler et al., 1998). This protein can bind to polypeptides and alter their conformations; consequently, it remains in the cytoplasm and cannot be exported by the *L. lactis* secretion machinery. Few studies have reported the production of recombinant proteins in different cellular locations (i.e., cytoplasm, cell wall or extracellular medium) under the same induction conditions and using the same promoter (Le Loir et al., 2005). We demonstrated that rHsp65 is efficiently produced in both the cytoplasm and secreted forms to the extracellular medium, confirming the ability of XIES to produce and correctly target recombinant proteins.

To determine if this antigen was produced without any traces of LPS, the quantity of endotoxin in bacterially expressed Hsp65 preparations was evaluated using the chromogenic LAL test (Table 3). The samples contained less LPS than the limit set by the FDA, as expected, since *L. lactis* is a Gram-positive bacterium and since this endotoxin occurs as part of the cell structure of Gram-negative bacteria (Cardoso et al., 2007). The FDA currently requires the LAL test to be performed on all bacterial, human and animal products intended to be used for medical proposes. The LPS levels that we found varied from 0.00137-0.01119 ng/ μ g protein, approximately 10-100 times lower than the levels established by the FDA, which stipulates that commercial preparations of recombinant proteins must contain less than 1 EU or 0.1 ng LPS/ μ g protein. According to Salek-Ardakani et al. (2002), it is crucial to eliminate or at least significantly reduce LPS levels because sub-nanogram quantities can alter the phenotype of various cell types, induce complement activation, and cause production of proinflammatory cytokines and reactive oxygen species, both *in vitro* and *in vivo*. These effects can complicate studies of the biological effect of proteins, constituting a serious problem for research on such

proteins. For instance, there have been concerns that the reported cytokine-inducing effects of HSPs may be a result of something other than the HSPs; they could be due to substances bound to HSPs or present as contaminants, such as LPS and lipoproteins (Majde, 1993). Using highly purified HSP preparations, a series of papers have demonstrated that the HSP cytokine functions are in fact a result of contaminating bacterial products (for example lipoproteins, flagellins and mainly LPS) (Tsan and Gao, 2009). Moreover, although several purification protocols have been developed and can reduce the endotoxin content below the threshold level, an absolute guarantee cannot be given. A part of the final product may become accidentally contaminated and fail quality control assays, increasing the cost of the final product. Despite the development of novel methods designed to remove endotoxins from biological samples, more research is needed in this field (Magalhaes et al., 2007). For these reasons, *L. lactis* is an attractive alternative compared to other expression systems, especially *E. coli*, since it is capable of naturally producing LPS-free recombinant proteins.

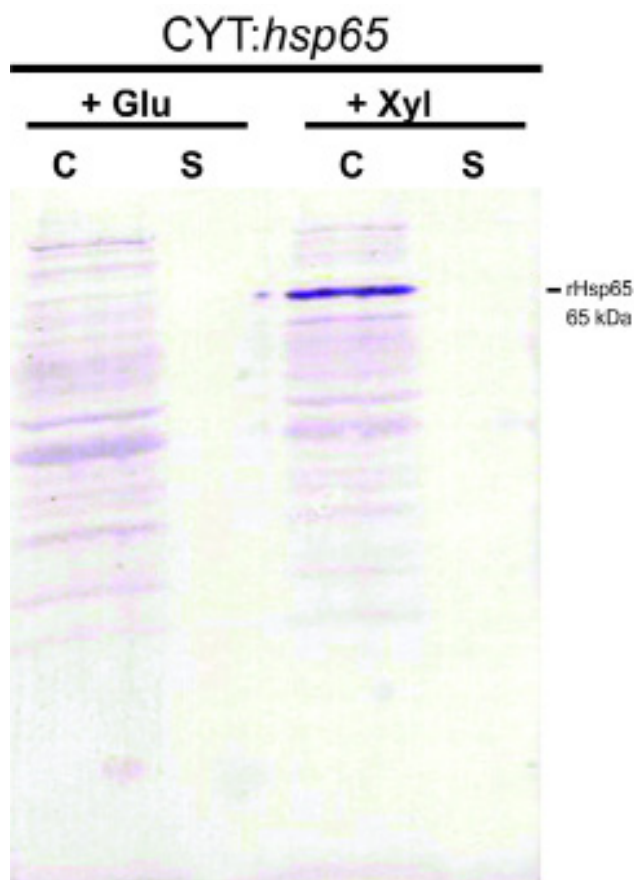


Figure 2. Cytoplasmic rHsp65 production. Protein extracts of noninduced (*Glu* lanes) and xylose-induced (*Xyl* lanes) culture samples of *Lactococcus lactis* NCDO2118 (CYT:hsp65) strain in stationary-phase ($OD_{600} = 2.0$) were prepared from cell (*C* lanes) and supernatant (*S* lanes) fractions and were analyzed by Western blotting using anti-Hsp65 antibodies.

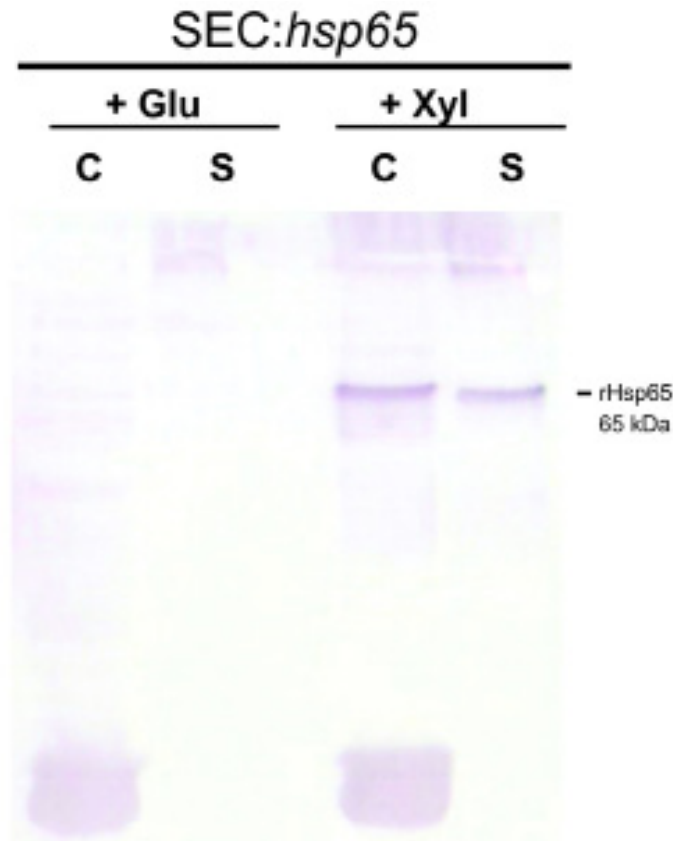


Figure 3. Extracellular rHsp65 production. Protein extracts of noninduced (*Glu lanes*) and xylose-induced (*Xyl lanes*) culture samples of *Lactococcus lactis* NCDO2118 (*SEC:hsp65*) strain in stationary-phase ($OD_{600} = 2.0$) were prepared from cell (*C lanes*) and supernatant (*S lanes*) fractions and were analyzed by Western blotting using anti-Hsp65 antibodies.

Table 3. Primers used for the polymerase chain reactions.

<i>Lactococcus lactis</i> strain	Accepted value in EU/ μ g	Mean EU/ μ g obtained	Final
CYT: <i>hsp65</i> C I	<0.1	0.00881	A
CYT: <i>hsp65</i> C NI	<0.1	0.00501	A
CYT: <i>hsp65</i> S I	<0.1	0.00458	A
CYT: <i>hsp65</i> S NI	<0.1	0.00641	A
SEC: <i>hsp65</i> C I	<0.1	0.00192	A
SEC: <i>hsp65</i> C NI	<0.1	0.00542	A
SEC: <i>hsp65</i> S I	<0.1	0.00137	A
SEC: <i>hsp65</i> S NI	<0.1	0.00205	A
Wild-type strain NCDO2118 C	<0.1	0.01119	A
Wild-type strain NCDO2118 S	<0.1	0.00225	A

A = approved; C = cellular fraction; S = secreted fraction; I = xylose-induced *L. lactis* culture; NI = noninduced *L. lactis* culture; EU/ μ g (endotoxin units/ μ g) = 0.1 ng LPS/ μ g protein; CYT:*hsp65* = *L. lactis* producing the cytoplasmic form of Hsp65; SEC:*hsp65* = *L. lactis* producing the secreted form of Hsp65.

CONCLUSIONS

In conclusion, we engineered recombinant *L. lactis* strains able to produce and target the *M. leprae* Hsp65 to the cytoplasm or to the extracellular medium. Degradation products, related to lactococcal HtrA activity, and LPS contamination were not observed in any fraction from induced *L. lactis* culture samples (CYT:*hsp65* and SEC:*hsp65*). Thus, these strains seem to be able to produce and deliver (cytoplasm or extracellular medium) a stable and LPS-free form of Hsp65. This alternative expression strategy can open up a wide range of pharmaceutical and medical applications for this antigen.

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