Lactococcus lactis as a vehicle for the heterologous expression of fungal ribotoxin variants with reduced IgE-binding affinity

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Abstract

Fungal ribotoxins are a family of extracellular ribonucleases which inhibit protein biosynthesis by inactivating the ribosomes. This inactivation results in the induction of cell death by apoptosis. Ribotoxins show antitumoral properties based on their ability to cross the membrane of some transformed cells. Unfortunately, they also show an unspecific cytotoxicity which has greatly impaired their potential clinical uses. α-Sarcin, produced by *Aspergillus giganteus*, is the best-characterized ribotoxin. Asp f 1, another ribotoxin produced by *A. fumigatus,* is indeed one of its major allergens. In this work, the *Lactococcus lactis* MG1363 strain has been engineered to produce and secrete not only wild-type Asp f 1 and α-sarcin but also three different mutants with reduced cytotoxicity and/or IgE-binding affinity. The proteins were secreted in native and active form when the extracellular medium employed was buffered at pH values around 8.0. Strains producing the wild-type natural α-sarcin were proved to be innocuous when administered intragastrically to mice for a period of 14 days. Overall, the results presented are discussed in terms of its potential application as a vehicle of oral delivery of hypoallergenic variants as well as a starting point to approach the design of strategies to accomplish the safe delivery of these proteins as antitumoral agents.
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1. Introduction

Aspergillus species are responsible for several human lung pathologies, including different allergic inhalant diseases (Kurup et al., 2002), allergic bronchopulmonary aspergillosis (ABPA) being the most severe form among them. *Aspergillus fumigatus* is usually the mold involved in most of those diseases, ABPA included, because this fungus, with small spores, optimally grows at 37 °C, a temperature that is prohibitive for most of the other environmental ubiquitous fungi. Thus, it can colonize the respiratory tract of the host leading to the onset of pathological events (Banerjee and Kurup, 2003).

Ribotoxins are a group of secreted fungal ribonucleases, best represented by α-sarcin (Lacadena et al., 2007), whose toxicity comes from their ability to reach the cytosol via endocytosis without establishing any receptor interaction (Olmo et al., 2001). They inhibit protein biosynthesis by inactivating the ribosomes (Schindler and Davies, 1977; Kao et al., 2001) which results in induction of cell death by apoptosis (Olmo et al., 2001). This ribosome inactivation is achieved by cleaving a unique phosphodiester bond at the so-called sarcin–ricin loop (SRL) of the largest rRNA (Endo and Wool, 1982; Correll et al., 1999). Ribotoxins were discovered during a screening program of the Michigan Department of Health searching for antibiotics and antitumor agents (Olson et al., 1965). Unfortunately, further studies revealed an unspecific cytotoxicity of these proteins, which limited their potential clinical uses (Roga et al., 1971).

All ribotoxins show a high degree of sequence identity with most of their differences appearing on exposed regions, such as their NH$_2$-terminal β-hairpin (α-sarcin residues 1–26), a domain

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which can be considered as two consecutive minor β-sheets connected by a hinge region. The second of them (residues 7–22) juts out as a solvent-exposed protuberance and is one of the protein regions with highest conformational flexibility (Pérez-Cañadillas et al., 2000, 2002). Deletion of this β-sheet results in a $\Delta(7–22)$ mutant that shows no significant conformational differences except for the deleted region (García-Mayoral et al., 2004) but has lost its ability to specifically recognize the ribosome and is much less cytotoxic (García-Ortega et al., 2002).

Asp f 1, the ribotoxin produced by *A. fumigatus*, is also a major and one of its best-characterized allergens (Moser et al., 1992). There is a significant prevalence of Asp f 1-specific IgE antibodies in sera from patients sensitized to *Aspergillus*. Particularly in ABPA, the detection of these antibodies seems to be a promising approach for its otherwise difficult diagnosis (Kao et al., 2001; Greenberger, 2002; Banerjee and Kurup, 2003; García-Ortega et al., 2005). Asp f 1 differs from α-sarcin in only 19 (87% sequence identity) out of 150 residues. Five of these differences are precisely located at the NH_2-terminal β-hairpin. Structural and immunogenic studies of Asp f 1, α-sarcin, and two variants where this β-hairpin had been deleted [Asp f 1 $\Delta(7–22)$ and α-sarcin $\Delta(7–22)$] (García-Ortega et al., 2005) showed that the deleted portion is involved in at least one allergenic epitope (García-Mayoral et al., 2004; García-Ortega et al., 2005). In spite of their decreased IgE reactivity, the prevalence of the two deleted proteins among the sera of patients remained essentially unaffected while they still retained most of the IgG epitopes (García-Ortega et al., 2005). It was then concluded that these ribotoxins' variants might be suitable for use in immunomodulating therapies and diagnosis of *Aspergillus* hypersensitivity.

The digestive tract is inhabited by commensal flora, whose correct settlement has been shown to be very important for human health. In fact, some pathological states can be improved just by means of administration of certain live bacteria, the so-called probiotics (Hooper and Gordon, 2001; Schiffrin and Blum, 2001). In the last years, the setting-up of genetic engineering techniques has made possible the genetic modification of commensal bacteria in order to obtain "biodrugs", i.e., strains of bacteria capable of *in vivo* producing drugs, antimicrobial agents, or vaccines (Hooper and Gordon, 2001; Blanquet et al., 2001). One of the advantages of these "biodrugs" is the specific delivery of the therapeutic agent to their target.

Lactococcus lactis is a non-pathogenic, non-invasive, no colonizing Gram-positive bacterium, mainly used to produce fermented foods. This lactic acid bacterium holds "Generally Regarded As Safe" (GRAS) status and hence is a suitable candidate to be used as one of those "biodrugs". For example, it has been proven useful in producing IL-10 for the treatment of inflammatory bowel disease in mice (Steidler et al., 2000). In this work, the extracellular production by *L. lactis* of the above α-sarcin and Asp f 1 deletion variants is presented. Both wild-type proteins were also included in the study, as well as a properly folded, but catalytically inactive α-sarcin H137Q mutant, as a control (Lacadena et al., 1995; García-Ortega et al., 2002, 2005). The possibility of using this strategy as a potential immunomodulating therapeutic approach is discussed.

2. Materials and methods

2.1. Materials

All reagents were molecular biology grade. Restriction endonucleases and DNA modifying and synthesizing enzymes were purchased from Roche (Indianapolis, IN), New England Biolabs (Beverly, MA), or Promega (Madison, WI). Oligonucleotides were purchased from Sigma-Genosys (Cambridge, UK). PerkinElmer (Wellesley, MA) GeneAmpPCRSystem 2400 thermal cycler was used for the polymerase chain reaction (PCR)-based amplifications. DNA sequencing was performed at the facility of the Universidad Complutense (Madrid, Spain).

2.2. Media

L. lactis strain MG1363 (Table 1) was routinely grown at 30 °C in M17 (Difco) containing 0.5% (w/v) glucose (GM17) under static conditions, as described (Gil et al., 2001), adding erythromycin (5 µg/ml) when needed. In order to prepare electrocompetent cells, they were grown in GM17 medium but supplemented with 23.8 mg/ml L-threonine, and 1 mM $MgSO_4$ (GM17GT). Protein production was initially assayed in a medium (GM9) containing 0.042 M Na_2HPO_4, 0.02 M KH_2PO_4, 0.01 M NH_4Cl, 8.5 mM NaCl, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 5 g/l peptone, and 5 µg/ml erythromycin. Final production of the proteins was accomplished in 0.2 M potassium phosphate buffered GM9 (PM) adjusted at different pH values between 6.0 and 8.5.

2.3. Cloning procedures

Cloning procedures and DNA manipulations were carried out according to standard methods (Lacadena et al., 1994; Maassen, 1999; Schotte et al., 2000; Sambrook and Russell, 2001). Suitable deoxyoligonucleotides were used as primers for PCR amplification, using a series of plasmids constructed before as templates (Lacadena et al., 1994, 1995; García-Ortega et al., 2002, 2005), containing the cDNA corresponding to the five proteins studied: Asp f 1, α-sarcin, Asp f 1 $\Delta(7–22)$, and α-sarcins $\Delta(7–22)$ and H137Q. These amplified DNA fragments were flanked by NgoMIV and BamHI restriction sites that were used to clone the proteins into the corresponding cloning sites of the plasmid pT1NX, in frame with the secretion signal leader of the lactococcal *usp*45 gene placed under the control of the constitutive promoter P1 (Steidler et al., 1995, 2000). The vectors thus obtained (Table 1) were used to electroporate electrocompetent MG1363 *L. lactis* cells.

2.4. L. lactis electroporation

In order to prepare electrocompetent MG1363 *L. lactis* cells, the corresponding strain was grown overnight at 30 °C in 5 ml of GM17GT medium. Next day, this culture was diluted with fresh medium to a final volume of 25 ml and an OD_{600} of 0.1 and further incubated until reaching a value of 0.32. Cells were then harvested by centrifugation at $7500 \times g$ for 15 min. The cel-

Table 1
Strain and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristics	Reference
L. lactis MG1363	Plasmid-free derivative of NCDO712	Laboratory stock
pT1NX	*L. lactis* vector; erythromycin resistance; P1 promoter and *usp*45 secretion signal	Schotte et al. (2000)
pT1αsarcin	pT1NX with cloned α-*sarcin* cDNA	This work
pT1αsarcinH137Q	pT1NX with cloned α-*sarcin* H137Q variant	This work
pT1αsarcin7–22	pT1NX with cloned α-*sarcin* Δ(7–22) deletion variant	This work
pT1aspf	pT1NX with cloned *aspf1* cDNA	This work
pT1aspf 7–22	pT1NX with cloned *aspf1* Δ(7–22) deletion variant	This work

lular pellet was maintained in ice and washed twice with 2 ml of distilled water, once with 1 ml of 50 mM EDTA, and once with 1 ml of 0.3 M sucrose. Finally, washed cells were gently resuspended in 0.2 ml of 0.3 M sucrose and immediately used for electroporation. DNA ligation mixtures, or plasmids, used for electroporation were previously phenolized and ethanol precipitated in the presence of 50 μg/ml glycogen and redissolved in 10 μl of water. Electroporation was performed in a BioRad Gene Pulser apparatus at 2500 V, 200 Ω, and 25 μF, using 0.2 cm cuvettes and 2–3 μl (1 μg of DNA) of either the ligation mixtures or plasmids. Electroporated cells were quickly resuspended in 5 ml of GM17 containing 1% (w/v) sucrose and 1 mM MgSO$_4$ and incubated for 2 h at 30 °C. Selection of the proper colonies was then made for erythromycin (5 μg/ml) resistance on GM17 agar plates containing 1% (w/v) sucrose and 1 mM MgSO$_4$, after incubation at 30 °C.

2.5. Plasmids purification and analysis

Plasmids were purified essentially as described before (O'Sullivan and Klaenhammer, 1993; Maassen, 1999) by a method that combines enzymatic breakdown of the cell wall and purification of the plasmid by commercially available DNA-binding columns. With this purpose, the cells corresponding to 10 ml of a 30 °C GM17 erythromycin (5 μg/ml) containing overnight culture were pelleted, resuspended in 200 μl of 10 mM HCl–Tris, pH 8.0, supplemented with 20% (w/v) sucrose, 10 mM EDTA, 50 mM NaCl, and 15 mg/ml lysozyme, and further incubated for 15 min at 37 °C. This solution was then thoroughly but gently mixed with 200 μl of 0.2 M NaOH, containing 1% (w/v) SDS to induce the cell lysis. Cell lysates were subjected to fractionation using the standard procedure and reagents from GenElute^{TM} Plasmid Miniprep Kit (Sigma–Aldrich). Isolated plasmids were analyzed by electrophoretic restriction maps and DNA sequencing.

2.6. Protein production

Individual *L. lactis* colonies harboring each one of the five pT1NX-derived plasmids (Table 1) containing the different ribotoxins' cDNA were cultured overnight at 30 °C in GM17 supplemented with 5 μg/ml erythromycin. Next day, 500 μl of these saturated cultures were used to inoculate 50 ml of the same medium and further grown at 30 °C to an OD$_{600}$ of 0.3. These cultures were centrifuged at 5000 × *g* for 10 min at room temper-

ature and the supernatant was discarded. The cellular pellet was gently resuspended in 50 ml of either GM9 or buffered PM media and incubated for five additional hours at 30 °C. These cultures were then processed as described in the following section.

2.7. Protein analysis and detection

L. lactis MG1363 grown for 5 h in GM9 or buffered PM media were centrifuged at 15,000 × *g* for 15 min at room temperature and the supernatants were then 20-fold concentrated using Microcon YM-10 centrifugal filter devices, for detecting the proteins by Western blot, or 250-fold using Centiprep YM-10 centrifugal filter devices as well, for detecting them by staining with Coomassie blue or zymogram. These samples were analyzed on 0.1% (w/v) SDS-PAGE performed in 15% (w/v) polyacrylamide gels (Laemmli, 1970). Gels containing the proteins thus fractionated were stained with Coomassie brilliant blue R-250 or blotted onto Immobilon (Millipore) membranes by semidry blotting for 1 h at 0.48 mA/cm^2. Immunodetection on these membranes was achieved as described (Lacadena et al., 1994; Villalba et al., 1994; García-Ortega et al., 2005) employing rabbit polyclonal antibodies raised against wild-type α-sarcin (Lacadena et al., 1994) or Asp f 1 (García-Ortega et al., 2005) and a secondary antibody horseradish peroxidase-labeled goat anti-rabbit IgG. The peroxidase reaction was developed using the ECL Western blotting reagent (Amersham Pharmacia Biotech) as described (Villalba et al., 1994; Barral et al., 2004). The ribonucleolytic activity of the proteins was detected by using a zymogram assay against poly(A). This assay was performed at pH 7.0 in 15% (w/v) polyacrylamide gels containing 0.1% (w/v) SDS and 0.3 mg/ml of the homopolynucleotide. In these zymograms, proteins exhibiting ribonuclease activity appear as colorless bands after appropriate distaining (Lacadena et al., 1999; Kao et al., 2001; García-Ortega et al., 2002).

2.8. L. lactis oral administration to mice

Female, 6 weeks old, BALB/c mice were obtained from Harlan Interfauna Ibérica S.A. (Barcelona, Spain). Animals were maintained at the Animal Care Services of the Faculty of Biology (UCM, Madrid, Spain), according to the local guidelines for animal care. The studies performed were approved by the Animal Experimentation Ethics Committee of the Complutense University.

Mice ($n = 6$) were inoculated daily via intragastric gauge with 2×10^7 *L. lactis* cells suspended in 200 µl of bicarbonate solution during 14 consecutive days. Cells containing the α-sarcin producing pT1α-sarcin plasmid or the pT1NX vector were used. Simultaneously a control group of six mice remained untreated. Two weeks after last inoculation the animals were sacrificed and gut histology was analyzed for injury. Intestine samples from all animals were fixed with formalin, routinely processed and paraffin-embedded. Sections of 3 µm were stained with haematoxylin and eosin (H&E) technique.

These gut sections were examined under light microscope by the mice pathologist blinded to the protocol design.

For optimal α-sarcin production, the saturated cultures employed had been grown for 16 h in GM17 containing 5 µg/ml erythromycin and then were diluted 25-fold in buffered PM medium and further grown for three more hours at 30 °C. Then, the OD_{600} of these cultures was measured and they were diluted with the same medium in order to have the required 2×10^7 bacteria in 200 µl.

3. Results

3.1. α-Sarcin production

α-Sarcin is the best-characterized ribotoxin so far (Lacadena et al., 2007). Thus, this protein was first used to optimize the extracellular ribotoxin's production system in *L. lactis*. To this end, the α-sarcin cDNA was cloned into pT1NX plasmid fused to the usp45 lactococcal secretion signal. The initial production experiments, performed on GM9 medium, revealed the presence of an immunoreactive band in the extracellular culture medium of *L. lactis* carrying pT1αsarcin plasmid (Fig. 1). However, this band was of much higher electrophoretic mobility than wild-type α-sarcin. This suggested that *L. lactis* was able to secrete α-sarcin but also that proteolytic degradation of the recombinant protein was taking place. Considering that the final pH value of the cultures was around 4.5, in agreement with the results described by others employing similar systems (Israelsen et al., 1995; Gil et al., 2001), and that it has been reported the presence of acidic proteases within the extracellular medium of *L. lactis*, it seemed clear that the protein was being degraded. A very similar observation has been reported before for the heterologous expression of IL-10 using an almost identical system (Steidler et al., 2000). Therefore, the use of pH-buffered media was considered. Consequently, cells were grown in PM at different pH values. As can be seen in Fig. 2, production of native wild-type α-sarcin was maximal when pH values were around 8.0. Consequently, further production experiments of ribotoxins were performed in PM buffered at pH 8.0.

3.2. Toxicity of the α-sarcin producing strain on mice

Histological analysis of the three groups of mice studied (non-treated or treated with *L. lactis* carrying the cloning vector pT1NX or the expression plasmid pT1αsarcin) revealed the absence of significant differences among all of them (Fig. 3), suggesting that oral administration of ribotoxins' producing
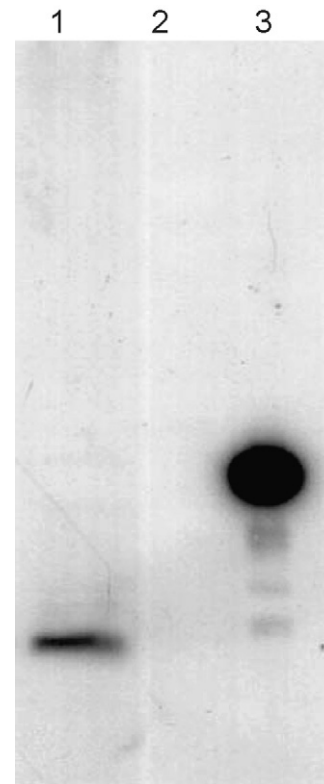

Fig. 1. Electrophoretic separation on 0.1% SDS-15% PAGE followed by Western immunoblot detection of α-sarcin in the extracellular media of overnight cultures of *L. lactis* MG1363 cells carrying the pT1αsarcin expression vector (1) or the pT1NX cloning vector (2). As a positive control, 50 ng of wild-type natural fungal α-sarcin were also loaded in lane (3).

L. lactis strains had not deleterious effects on mice intestinal tracts.

3.3. Production of the other allergenic variants

Once the extracellular production of wild-type α-sarcin was optimized, and its lack of toxicity was assessed, four other ribotoxins' versions were also cloned and produced in *L. lactis* under identical conditions (Table 1). One of them was the wild-type major *A. fumigatus* allergen Asp f 1 (García-Ortega
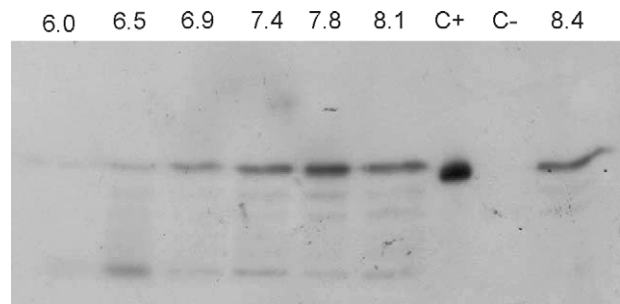

Fig. 2. Electrophoretic separation on 0.1% SDS-15% PAGE followed by Western immunoblot detection of α-sarcin in the extracellular media of buffered cultures of *L. lactis* [pT1αsarcin]. Numbers indicate the final pH values of the culture. Positive (50 ng of wild-type natural fungal α-sarcin, C+) and negative (no protein, C−) controls were also loaded.
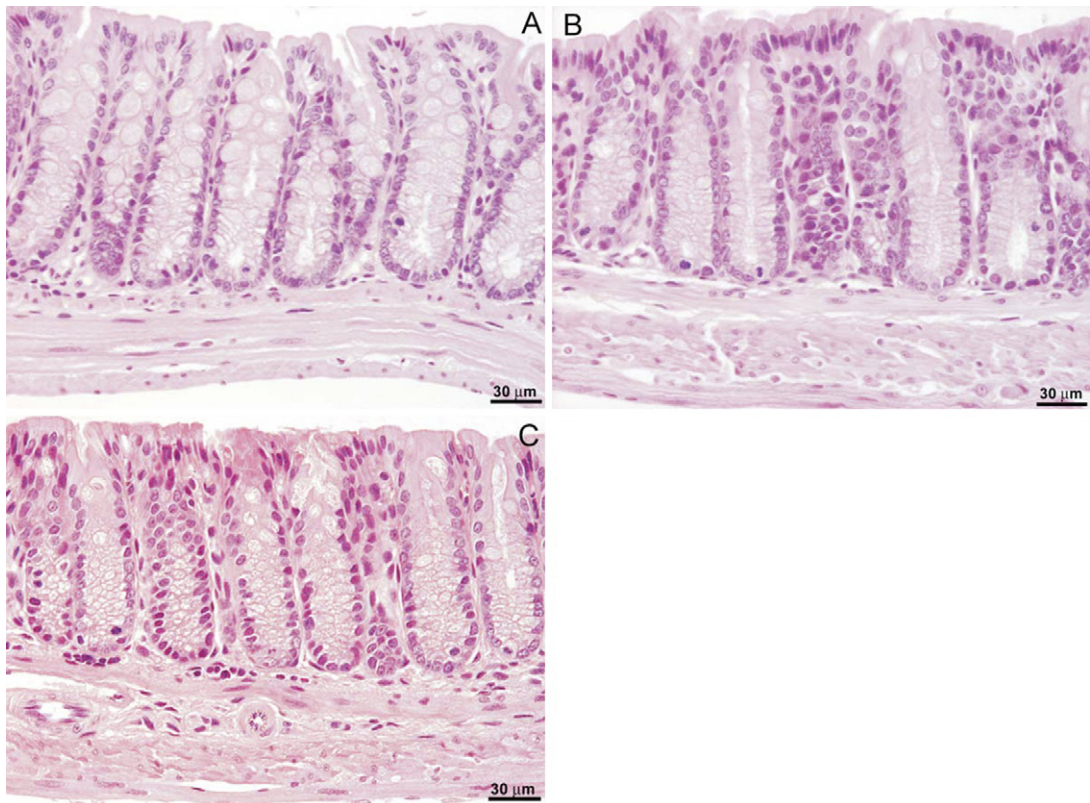
Fig. 3. H&E stains of adult mouse small intestine sections. Samples from not treated mice (A) and from mice treated with *L. lactis* strains producing (B), or not (C) α-sarcin. Note the absence of pathological signs of inflammatory response in the muscularis, submucosa, and mucosa layers in all samples. It is also observed how the epithelium lining the intestine (surface epithelium) and the glands show no discrepancies among the three different groups of animals studied. Equivalent results were obtained with sections of different areas of the large intestine (data not shown).

et al., 2005). Two other ones were the corresponding Δ(7–22) deleted variants of Asp f 1 and α-sarcin, two proteins with highly reduced IgE-reactivity and cytotoxicity (García-Ortega et al., 2002, 2005; García-Mayoral et al., 2004). The fourth one (H137Q) was a mutant version of α-sarcin where only the catalytically essential His-137 has been mutated, being substituted by Gln (Lacadena et al., 1995). This mutation renders a fully inactive and non-cytotoxic protein that however retains the wild-type native conformation (Lacadena et al., 1995, 1999). Protein production was scarcely detected in Coomassie blue stained gels (data not shown), especially for the α-sarcin variants, but it was apparent in all cases when revealed by means of Western immunoblotting and ribonucleolytic activity against poly(A). Thus, immunoreactive bands corresponding to the expected electrophoretic mobilities were observed for all the five proteins studied (Fig. 4A). Indeed, except for the catalytically inactive H137Q mutant, all the other bands corresponded to ribonucle-olytically active proteins and, as proven before (García-Ortega et al., 2002, 2005), this non-specific activity was even higher for the deleted variants (Fig. 4B).

4. Discussion

There are growing evidences that regular consumption of foods containing probiotic bacteria may enhance the immune response and positively affect the indigenous microflora

(Schiffrin and Blum, 2001). In addition, Gram-positive bacteria not only do not have an outer cell membrane and do not produce endotoxins but rather their peptidoglycan layer appears to exhibit natural immuno-adjuvanticity (Pouwels et al., 1996).
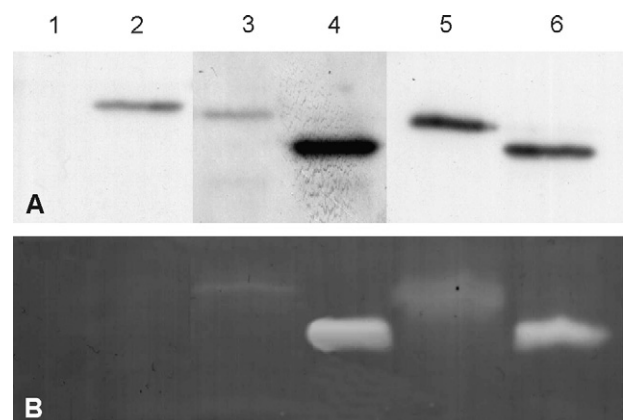

Fig. 4. (A) Electrophoretic separation on 0.1% SDS-15% PAGE followed by Western immunoblot detection and (B) zymogram against poly(A) of concentrated culture supernatants of *L. lactis* carrying pT1αsarcinH137Q (lane 2), pT1αsarcin (lane 3), pT1αsarcin7–22 (lane 4), pT1aspf (lane 5), pT1aspf 7–22 (lane 6). Lane 1 corresponds to *L. lactis* cells carrying the cloning vector pT1NX. Samples were not reduced before loading onto the gels. Immunoblot membrane corresponding to lanes 1–4 in part (A) were stained using a rabbit polyclonal serum raised against wild-type α-sarcin, whereas an anti-Asp f 1 serum was used for the area corresponding to lines 5 and 6.

This explains why oral vaccination using GRAS Gram-positive bacteria is being developed as a promising approach to the onset of different pathologies, allergies included (Pouwels et al., 1996; Robinson et al., 1997; Maassen et al., 1999; Kirjavainen et al., 1999).

L. lactis fed to animals and human volunteers passes rapidly through the gastrointestinal tract without colonization (Gruzza et al., 1994; Klijn et al., 1995). However, genetically modified versions of this microorganism are still effective in delivering antigens to the mucosal immune system and capable of inducing a local immune response (Robinson et al., 1997; Maassen et al., 1999; Adel-Patient et al., 2005; Perez et al., 2005). This seems to happen because *L. lactis* lacks the ability to multiply *in vivo* but it can readily be sampled by dendritic cells. A process that seems to be involved in the development of efficient immune responses (Xin et al., 2003), including the selective induction of IgA (Macpherson and Uhr, 2004). In addition, antigens within *Lactococci* are protected against direct contact with gastric acid and proteolytic enzymes. For example, oral administration of recombinant *L. lactis* expressing bovine β-lactoglobulin, a major cow's milk allergen, was shown to induce a specific Th1 response down-regulating a further Th2 one and thus preventing mice from sensitization (Adel-Patient et al., 2005).

Finally, only a few proteins are naturally secreted in *L. lactis*, but these bacteria can be engineered for secreting heterologous proteins to the extracellular medium, unlike most of Gram-negative bacteria in which the majority of secreted proteins are located in the periplasmic space (Nouaille et al., 2003). Altogether, all these facts make this organism optimally interesting for producing hypoallergenic proteins in order to use it as a vaccination vehicle.

Following this idea, extracellular production of five different fungal ribotoxin variants was optimized in *L. lactis* MG1363. One of these variants was the wild-type ribotoxin Asp f 1, naturally produced by the human fungal pathogen *A. fumigatus* and one of its major allergens. Three other ones are natural and artificial variants of this protein displaying a diminished reactivity against human IgE from allergic patients (García-Ortega et al., 2005). Indeed, both deleted versions [Asp f 1 and α-sarcin Δ(7–22)] show a highly reduced cytotoxicity due to the fact that they lack the ability to inactivate ribosomes specifically (García-Ortega et al., 2005). However, the four proteins secreted preserved their native conformation since all of them retained their ribonucleolytic activity against poly(A) (Fig. 4). The fifth protein produced was the α-sarcin mutant H137Q, a variant which is completely devoid of cytotoxicity and ribonucleolytic activity (Fig. 4) (Lacadena et al., 1995, 1999; Olmo et al., 2001). Additionally, neither the bacterial strain alone nor the transformed producing natural wild-type α-sarcin did induce any deleterious effect on mice intestinal tract when intragastrically administered (Fig. 3), indicating that even the highly toxic wild-type ribotoxins could be safely delivered using *L. lactis* as a vehicle for oral administration.

It is well known how gastric pH values are rather low due the presence of high HCl concentrations. However, it has been also demonstrated how a significant fraction of *L. lactis* inocula can survive and be metabolically active in all the other compartments of the intestinal tract (Klijn et al., 1995; Corthier et al., 1998; Drouault et al., 1999) where pH values are much milder, approaching neutrality in many instances (Blanquet et al., 2001). Certainly, taking into account the results shown in Fig. 2, the possibility that the observed lack of toxicity might be due just to the instability of the protein produced cannot be ruled out. Nevertheless, the overall picture is far more complex. First, it is difficult to predict the *in vivo* suitability of these approaches since many different factors seem to affect cell viability and integrity within the digestive tract compartments (Drouault et al., 1999), including the presence of the many other microorganisms composing the intestinal flora. Second, native wild-type ribotoxins are rather acid pH and proteases resistant (Martínez del Pozo et al., 1988). Third, as mentioned above, genetically engineered *L. lactis* using an identical plasmid to produce IL-10 were still therapeutically active in mice (Steidler et al., 2000) besides the fact that the *in vitro* production of this cytokine was also unstable at low pH values (Schotte et al., 2000). Finally, even if the proteins were being degraded they might be still useful as immunomodulating agents given that the resulting fragments are immunologically active (Fig. 2). Thus, in summary, it seems clear that the system described needs further evaluation in deeper detail at the *in vivo* level, especially in terms of direct detection of the activity and presence of the proteins within the mice gastrointestinal tract. However, at least within the idea of designing new immunomodulating therapeutical approaches, it seems safe to conclude that the system herein described combines the minimal features to be considered as optimally suitable for its potential application as a vehicle of oral delivery of hypoallergenic variants of Asp f 1. Finally, it has not escaped to our attention that given the antitumoral properties of ribotoxins (Olson et al., 1965; Olmo et al., 2001), this system may also constitute a starting point to approach the design of strategies to accomplish the safe delivery of these proteins as antitumoral agents against different gastrointestinal tumors.

Acknowledgements

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