Effects of orally applied Fes p1-displaying *L. plantarum* WCFS1 on Fes p1 induced allergy in mice

Rajna Minic[a], Marija Gavrovic-Jankulovic[c], Vladimir Petrusic[a], Irena Zivkovic, [a], Vincent G.H. Eijsink [b], Ljiljana Dimitrijevic[a] , Geir Mathiesen[b]

Affiliations:

[a]Institute of Virology, Vaccines and Sera, Torlak, Belgrade, Serbia,

[b]Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Aas, Norway

[c]Department of Biochemistry, Faculty of Chemistry, University of Belgrade, Serbia

Corresponding author:

Rajna Minic, Institute of Virology, Vaccines and Sera, Torlak, Vojvode stepe 458, Belgrade, Serbia, tel: +381648852431, fax: +381113975501, e-mail: minicrajna@gmail.com

Abstract

Group I grass pollen allergens are major contributors to grass pollen-related seasonal allergic rhinitis, and as such a primary target for allergen specific immunotherapy. In this study the potential therapeutic role of oral application of *Lactobacillus plantarum* WCFS1, directing cell wall attachment of the recombinant Fes p 1 allergen, from *Festuca pratensis* was tested in a mouse model of Fes p 1 allergy. For surface expression of Fes p 1 allergen in *L. plantarum* WCFS1 pSIP system with inducible expression was used. Balb/c mice were sensitized with Fes p 1 protein in alum and subsequently received live recombinant *L. plantarum* orally. Antibody levels (IgE, total IgG, IgG1, IgG2a, and IgA) were determined by ELISA. Differential eosinophil count in peripheral blood was performed. Reduced peripheral blood eosinophilia and increased serum IgG2A levels was detected in both groups which received live *L. plantarum* orally. Specific serum IgA levels were increased only in mice treated with the recombinant bacteria. Oral application of *L. plantarum* WCFS1 has a beneficial therapeutic effect in a mouse model of Fes p 1 allergy. Cell surface expression of Fes p 1 allergen potentiates this phenomenon in an allergen specific way.

Keywords:

Allergy, Oral therapy, recombinant *L. plantarum* WCFS1, *Festuca pratensis*

1. Introduction

Type I allergy is one of the leading disorders of the immune system affecting about 25% of the population worldwide. Subcutaneous allergen specific immunotherapy has been used for almost a century and numerous studies have shown the benefits of this strategy (Bousquet et al., 1998). However, the efficacy of this strategy varies and it is associated with a risk of anaphylaxis and lengthy therapeutic regimes. Mucosal tissues contain antigen-presenting cells with a tolerogenic phenotype and their immunostimulation is less likely to give rise to anaphylactic shock (Calderon et al., 2012). Therefore, it is of interest to target mucosal tissues when developing strategies to combat allergy.

In recent years probiotic lactic acid bacteria have received massive attention because of their potential beneficial effects on human health (Isolauri et al., 2002). There is increasing

experimental evidence for claims that lactic acid bacteria exert beneficial effects on gut health (Kalliomaki et al., 2003; Sazawal et al., 2006) and that some of these effects relate to favorable immunomodulating properties, such as tolerance induction (van Baarlen et al., 2009). To date, several clinical trials have shown that probiotic strains may reduce allergy symptoms (Kalliomäki et al., 2010; Snel et al., 2011). In order to achieve immunological memory towards the specific allergen it is of interest to use the relevant allergen together with lactobacilli, to prevent the possibility of future sensitization to the same allergen.

L. plantarum WCFS1 is one of the best characterized lactic acid bacteria both in terms of genome analysis, behavior in the gastrointestinal tract and immunomodulatory properties (Kleerebezem et al., 2003; Marco et al., 2009; van Hemert et al., 2010). Considering the tolerance-inducing immunomodulatory effects of *L. plantarum* (van Baarlen et al., 2009), it is of interest to explore the possibility to use allergen expressing lactobacilli as delivery vehicles of allergy vaccines or in immunotherapy (Kruisselbrink et al., 2001; Daniel et al., 2006; Rigaux et al., 2009). Indeed, it has been shown that intranasal prophylactic application of allergen-expressing *L. plantarum* in different mouse allergy models reduces allergic sensitization by shifting the immune response to a non-allergic Th1 immune response as evidenced by production of specific IgG2a antibodies and increased secretion of IFN-γ (Repa et al., 2003; Daniel et al., 2006; Rigaux et al., 2009; Schwarzer et al., 2011). It has also been shown that therapeutic oral administration of *L. acidophilus* ATCC 4356 expressing the Der p5 allergen results in drastically decreased allergen-specific serum IgE levels and lowered eosinophilia in bronchoalveolar lavage samples (Charng et al., 2006).

Based on these promising previous results, the goal of the present study was to explore the possibility to use an engineered *L. plantarum* with a surface-displayed respiratory allergen Fes p1 in immunotherapy for pollen allergy. Covalent surface-anchoring of the antigen has the advantage that it minimizes the risk of the antigen entering the blood stream. Fes p1, a group I allergen from *Festuca pratensis* (Poeae grasses) was chosen because of its global distribution across cool, temperate areas (Borrill et al., 1976). Furthermore, sensitization to Fes p1 usually implies sensitization to several grass species due to group I grass pollen allergens being highly conserved among these species (Laffer et al., 1994). One closely related allergen is Phl p1 from *Phleum pratense*, which was recently found to be the initiator of IgE sensitization in 75% of children with grass pollen-related seasonal allergic rhinitis in Germany (Hatzler et al., 2012).

Group I allergens are usually glycoproteins, with a carbohydrate content comprising up to 5 % of the molecular mass (Petersen et al., 1995).

To engineer a suitable recombinant *L. plantarum* strain, we used recently developed genetic tools (Sørvig et al., 2003; Sørvig et al., 2005; Mathiesen et al., 2009; Fredriksen, et al., 2010) for expression and cell-wall anchoring of heterologous proteins in lactobacilli. Thus, a *L. plantarum* strain was generated in which Fes p1 is covalently anchored to the bacterial cell wall and displayed on the bacterial surface, as documented by immunofluorescence microscopy. This effect of this engineered strain on Fes p1-induced allergy was studied in a mouse model.

2. Methods

2.1. Bacterial strains and culture conditions

E. coli Top 10 cells (Life Technologies Ltd Paisley, UK) were grown in BHI broth (Oxoid Ltd, Basingstoke, UK) at 37 °C with shaking. *Lactobacillus plantarum* WCSF1 (Kleerebezem et al., 2003) was propagated in MRS (Oxoid) medium at 37 °C without agitation. For plates, the medium was solidified with 1.5% agar (w/v). The antibiotic concentrations were 200 μg ml^{-1} and 10 μg ml^{-1} erythromycin for *E. coli* and *L. plantarum*, respectively.

2.2. Plasmid construction and transformation

All vectors used in the present study are derivatives of the modular pSIP vectors developed for inducible expression in *Lactobacillus* (Sørvig, et al., 2003; Sørvig et al., 2005) and further developed for secretion and anchoring of target proteins (Mathiesen et al., 2008; Mathiesen et al., 2009). The gene for Fes p1, (GenBank: CCD28290) with codon usage optimized for *L. plantarum* and inserted in pUC57 was purchased from GenScript (Piscataway, NJ, USA). The sequence was N-terminally truncated, and started with amino acid 43. The coding sequence was PCR amplified with the primers: Forward, 5'-TCAGAAGAGGATCTGGATGCTAAAAGTACATGGT-3, and Reverse 5'-AAA*ACGCGT*CTTTGCAGCATAACTCGT-3' (MluI site italics), both from Operon Biotechnologies GmbH (Cologne, Germany). A Myc-tag (N-EQKLISEEDL-C) was fused N-terminally to the coding sequence by reamplification of the PCR product with an extended forward primer 5'-AAA*GTCGAC*GAACAAAAACTCATCTCAGAAGAGGATCTG-3' (SalI site italics). The PCR product was digested with SalI and MluI and ligated into SalI/MluI

digested pLp_0373sOFAcwa2 (Fredriksen et al., 2010), yielding pLpFesp1CWA. In the constructed plasmid, Fes p1 containing an N-terminal Myc-tag is N-terminally fused to the *L. plantarum* signal peptide of *lp_0373* (Mathiesen et al., 2008), and C-terminally fused to the 194 amino acid long LPxTG anchor sequence of the *lp_2578* gene (Fredriksen et al., 2012). *E. coli* Top 10 cells made competent by calcium chloride were used for subcloning, and the final plasmid, pLpFesp1CWA, was transformed to electrocompetent *L. plantarum* WCFS1 (Aukrust et al., 1995). The expression of Fes p1 in *L. plantarum* WCFS1 was checked by Western blot analysis using an Anti-Myc antibody (Life Technologies).

1.3. Indirect immunofluorescence microscopy of Fes p1 expressing *L. plantarum*

Surface display of Fes p1 was analyzed by indirect immunofluorescence microscopy of *L. plantarum*. For Fes p1 expression the bacteria were induced at optical density (610 nm) of 0.25 by the addition of the inducing peptide (IP-673) (CASLO Laboratory ApS, Denmark) at 10 ng ml^{-1} final concentration, essentially as described previously (Halbmayr, Mathiesen et al. 2008). Two hours post induction, the bacteria were harvested by centrifugation at 3 000 x g for 5 min, and washed once with PBS containing 2% bovine serum albumin (PBS/2% BSA). Approximately 2 x 10^7 of harvested bacteria were resuspended in 0.5 ml PBS/2% BSA and incubated with 5 µg Anti-Myc antibody, for 15 min at 4 °C. After incubation the cells were washed twice with 1 ml 2% B-PBS, followed by incubation in 0.1 ml PBS/2% BSA containing 5 µg Anti-Mouse IgG (Fc specific)–FITC antibody produced in goat (Sigma Aldrich, St. Louis, Missouri, USA). After incubation for 15 min at 4 °C, the bacteria were collected by centrifugation at 3000 x g for 2 min and washed twice in PBS. The signal was visualized using a Leica SP5 confocal scanning laser microscope using a 488-nm argon laser (FITC photomultiplier tube [PMT]) and a bright field (BF) PMT for transmitted light (Leica Microsystems, GmbH, Wetzlar, Germany). *L. plantarum* harboring a pSIP plasmid without the *Fes p 1* gene (named pEV) (Fredriksen et al., 2012) was used as a negative control.

2.4. Purification of Fes p 1

Festuca pratensis pollen was obtained from the Institute of Virology, Vaccines and Sera, Torlak, Serbia. The pollen were lysed by incubation of 1 g of pollen in 10 ml 20 mM acetate buffer (pH 4.5) over night at 4°C, after which debris was removed by centrifugation at 10 000 x g for 10

minutes. The supernatant was passed through a 5 cm x 0.7 cm CM-cellulose column (Sigma–Aldrich, St. Louis, Missouri, USA), equilibrated with 20 mM acetate buffer (pH 4.5). Stepwise elution was performed with 100 mM, 200 mM, 500 mM, and 1000 mM NaCl in 20 mM acetate buffer (pH 4.5) and the protein was eluted from the column in the 1 M NaCl fraction. Protein concentrations were determined by measuring absorbance at 280 nm, using the theoretical extinction coefficient for Fes p1.

2.5. Experimental protocol for animal tests

The therapeutic potential of the engineered *L. plantarum* strains was tested *in vivo* using a mouse model of Fes p1 induced allergy. Four week old female Balb/c mice were purchased from Military Medicine Academy (VMA, Belgrade, Serbia), divided into groups of six animals, kept in individual ventilated cages, and fed with standard food and water *ad libitum* in a pathogen-free part of the animal house. Humidity, temperature and light/dark cycles were maintained at 55%±5%, 21±2°C and 12/12 hours, respectively. All experiments were approved by the Ethics Committee for the welfare of experimental animals, at the Institute of Virology, Vaccines and Sera in Torlak, Serbia, and conformed to Serbian laws and European regulations on animal welfare (Approval No. 011-00-00510/2011-05/4). At day 0 and 7 the mice were sensitized by intra peritoneal (i. p.) injection with 7 µg Fes p1 allergen in 100 µl PBS mixed with an equal amount of aluminum hydroxide (alum) suspension (Fig. 1). At days 18, 19, 20, 25, 26, 27, 55, 56, 57, 60, 61 and 62 (Fig. 1) the mice were given 1 x 10^9 recombinant *L. plantarum* in 20 µl fresh MRS medium without antibiotics by orall gavage. The bacteria were grown, induced and harvested as described for immunofluorescence microscopy, above. Untreated mice were subjected to the same experimental protocol, but were given sterile MRS medium. At day 68 all mice were euthanized by cervical dislocation and samples of peripheral blood were collected, PBMC were immediately isolated, and serum samples were collected and stored at -80°C.

2.6. Specific antibody detection

MaxiSorp plates (Nunc A/S, Denmark) were coated with 0.5 µg purified Fes p 1 allergen in 50 µl 0.1 M sodium carbonate buffer (pH 9.6) overnight at 4°C. After blocking with PBS/2% BSA (1h, 37°C), the wells were incubated with 50 µl diluted mice sera (100 fold dilution for IgG, IgG1, IgG2, IgG3 and IgA and 6 fold dilution for IgE) for 1h at 37°C. Dilutions were made in

PBS containing 1% BSA. Biotinylated anti-mouse antibodies (Biolegend, San Diego, CA) were used for specific antibody class/subclass determination. Streptavidin peroxidase (Sigma–Aldrich, St. Louis, Missouri, USA) was used for detection with ortho-phenylendiamin as the substrate.

2.7. Eosinophil counts

To count eosinophils among leukocytes, peripheral blood smears from each mouse were prepared and after fixation in methanol for 10 min the leukocytes were stained with May-Grünwald Giemsa. Eosinophils were counted in a double blind fashion, using a light microscope at 400-fold magnification, with the total count being 300 leukocytes per slide.

2.8. Statistical analysis

Statistical analysis of antibody levels measurement was done with use of One-Way ANOVA test; Tukey test for means comparison. Statistical analysis of eosinophil number was done with use of Student's t-Test for independent samples. Results are presented as mean values ± standard error of the mean.

3. Results

3.1. Protein expression analysis and protein purification

The Fes p1 allergen was cloned with an N-terminal Myc tag into a previously developed pSIP vector (Fredriksen et al., 2010) where it becomes linked to an N-terminal signal sequence for secretion and a C-terminal cell wall anchor for sortase-mediated covalent attachment of the allergen to the surface of *L. plantarum* WCFS1. Western blot analysis of cell lysates harvested two hours after induction clearly showed that *L. plantarum* harboring pLpFesp1CWA produces Fes p1 (Fig. 2A). The localization of the allergen was analyzed using indirect immunofluorescence microscopy, which confirmed that Fes p1 was displayed at the surface of the recombinant *L. plantarum* cells (Fig. 2B).

3.2. Specific antibody production

Peripheral blood samples were taken at day 68 and used for analysis of Fes p1-specific immunoglobulins using purified Fes p1 (the purity was estimated to be in around 90 % [Fig. 2C]) in the ELISA. As expected, IgE levels increased in all groups injected with purified Fes p 1

allergen, with slightly lower values in pLp_pEV group (Fig. 3A). This shows that the allergy induction protocol was successful in terms of IgE production.

In order to see whether oral administration of *L. plantarum* resulted in changes in allergen specific levels of protective IgG and IgA antibodies the levels of these immunoglobulin classes was determined. All immunized groups showed elevated levels of Fes p1-specific total IgG, with no significant difference between the treated and the non-treated mice (Fig. 3B). ELISA showed that the majority of IgG was of the IgG1 subclass; also with respect to IgG1 levels there were no statistically significant difference between the immunized groups (Fig. 3C). The level of subclass IgG2a was also analyzed, and the production was increased in both groups administered with bacteria (Fig. 3D).

Fes 1p-specific IgA levels are of particular interest because production of these secreted antibodies is indicative of a mucosal immune response, i.e. of the type of response one hopes to achieve when using antigen-displaying lactobacilli. The measurements showed that IgA production was significantly higher in mice treated with *L. plantarum* harboring pLpFesp1CWA compared to any of the other groups (Fig. 3E).

3.3. Peripheral eosinophil counts

Increased eosinophil levels (i.e. higher than 5 % of the total number of leukocytes) in peripheral blood are generally considered as evidence of an increased IL-5 production (Fulkerson and Rothenberg 2013), which is indicative of a Th2 type of response. Fig. 4 shows eosinophil levels in the four different mice groups of this study. In the positive control group (c+) the eosinophil level was higher than 5% of the total leukocytes, confirming that these mice have developed eosinophilia as part of the allergic reaction towards the Fes p 1 protein. Treatment with *L. plantarum* cells with or without the allergen resulted in reduced numbers of eosinophils in the peripheral blood. Similar level of esoinophils as in the negative control mice was found in both mouse groups which received the bacteria.

4. Discussion

In the present study a major group I allergen from *F. pratensis* pollen, Fes p1, was successfully expressed and displayed at the surface of *L. plantarum* WCFS1 (Fig. 2). Surface-display of heterologous proteins in lactobacilli is still not too common and the results obtained with Fes p1 are encouraging. The idea behind the construction of the Fes p1-displaying strain was that surface-exposure will facilitate the interaction with the mucosal immune system. In

addition, covalent attachment to the bacterial cell wall prevents entering the blood stream by the allergen, which in principle could cause anaphylactic shock.

Oral application of both pEV and pLpFesp1CWA harboring lactobacilli resulted in amelioration of several allergy parameters: reduction of peripheral blood eosinophilia and increase in production of anti Fes p 1 IgG2A production antibodies all being indicative of a reduced Th2 response. These effects were further increased in mice treated with the Fes p1 producing *Lactobacillus* which showing accumulation of serum Fes p 1 specific IgA. Previous studies have shown that *L. plantarum* acts in a Th1 adjuvant manner (Repa et al., 2003; Rigaux et al., 2009), we found similar effects in Th1-mediated responses of *L. plantarum* bearing recombinant allergen.

Importantly, we detected significant increase in allergen specific IgA levels in mice treated with *L. plantarum* carrying the surface-exposed allergen (pLpFesp1CWA). Serum levels of allergen-specific IgA are indicative of secreted IgA levels, which again are indicative of a mucosal immune response (Pabst 2012). Interestingly, a previous study on the use *L. plantarum* engineered to intracellularly produce the major birch pollen allergen Bet v1 also showed allergen-specific secretory IgA responses after treatment with the allergen-producing strain only (Daniel et al., 2006).

Eosinophilia is an important effect of eosinophil-associated disorders, such as asthma and allergy (Fulkerson and Rothenberg 2013). Oral application of *L. plantarum/*pEV and *L. plantarum/*pLpFesp1CWA led to a clear reduction of eosinophilia in the peripheral blood of the experimental mice (Fig. 4), thus providing clear evidence of the beneficial effect of the bacterial treatment. As eosinophilia is a consequence of increases in IL-5 production, a marker of a Th2 response, a decrease in eosinophila shows amelioration of the pathological Th2 response.

In conclusion, our data show that the use of recombinant *L. plantarum* WCFS1 as a delivery vehicle for surface-displayed, covalently anchored antigens represents a promising strategy for antigen delivery in general and allergy treatment. Several parameters indicate that in the current experimental setting, oral application of *L. plantarum* cells alone, even without the specific antigen, has a beneficial therapeutic effect, which is not unexpected considering the results of previous studies (Kalliomäki et al., 2010; Snel et al., 2011). However, the data showed that surface display of the Fes p1 antigen potentiates this phenomenon as evidenced by an

increase in specific IgA production. As therapeutic oral administration of lactic acid bacteria is cost effective and safe, and since humans consume probiotic lactobacilli as part of their normal nutrition, it would be interesting to carry out human trials with the type of *Lactobacillus* strains engineered in this study.

Author contributions

Conceived and designed the experiments: RM MGJ VGHE GM LD. Performed the experiments: RM IZ GM. Analyzed the data RM VP LD. Contributed reagents/materials/analysis tools: MGJ GM VGHE LD. Wrote the paper: RM GM VE.

Conflict of interest

The authors have declared no conflict of interest.

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Figure legends

Figure 1. Overview of the experimental design. On days 0 and 7 Balb/c mice received intraperitoneal injections of 7 µg Fes p1 allergen in alum. Oral administration of lactobacilli was performed on 12 days, divided into four periods: 18-20, 25-27, 55-57, and 60-62. At day 68 the mice were sacrificed and blood samples were taken for analysis.

Figure 2. Expression, cell wall anchoring and purification of Fes p1. A) Western blot analysis of the whole cell lysates. Lane 1, *L. plantarum* WCFS1 harboring pLpFesp1CWA; lane 2, *L. plantarum* WCFS1 harboring pEV; lane mm, molecular marker (kDa) (Life Technologies). The predicted mass of the N- and C-terminally tagged Fes p1 protein is 53 kDa. B) Immuno-fluorescence microscopy using mouse Anti-Myc antibody and FITC conjugated anti mouse IgG. 1, *L. plantarum* WCFS1 harboring pLpFesp1CWA; 2, *L. plantarum* WCFS1 harboring pEV. C) Comassie-Briliant blue stained 12 % SDS-PAGE gel showing purified Fes p1 allergen. mm, molecular marker (kDa); lanes 1-4,- step elution of the CM-cellulose column with 100mM NaCl, 250mM NaCl, 500mM NaCl and 1M NaCl, respectively. The predicted mass of the non-glycosilated Fes p1 protein is 28kDa.

Figure 3. Analysis of Fes p1 specific immunoglobulins in mice sera sampled at day 68. A) Fes p1 specific IgE; B) Fes p1 specific total IgG; C) Fes p1 specific IgG1; D) Fes p1 specific IgG2A; E) Fes p1 specific IgA. C+ - mice i.p. injected with Fes p 1 at day 0 and 7; pLp_pEV, Fes p1 immunized mice treated by oral application of *L. plantarum* WCFS1 carrying pEV (control vector); pLp_Fesp1CWA, Fes p1 immunized mice treated by oral application of *L. plantarum* carrying pLpFesp1CWA. * $p<0.05$; ** $p<0.005$; ***$p<0.0005$.

Figure 4. Peripheral blood eosinophil counts, expressed as percentage of eosinophils in the total number of leukocytes. C+ - mice i.p. injected with Fes p 1 at day 0 and 7; pLp_pEV, Fes p1 immunized mice treated by oral application of *L. plantarum* WCFS1 carrying pEV (control vector); pLp_Fesp1CWA, Fes p1 immunized mice treated by oral application of *L. plantarum* carrying pLpFesp1CWA. * $p<0.05$

Figure 1

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Figure 2

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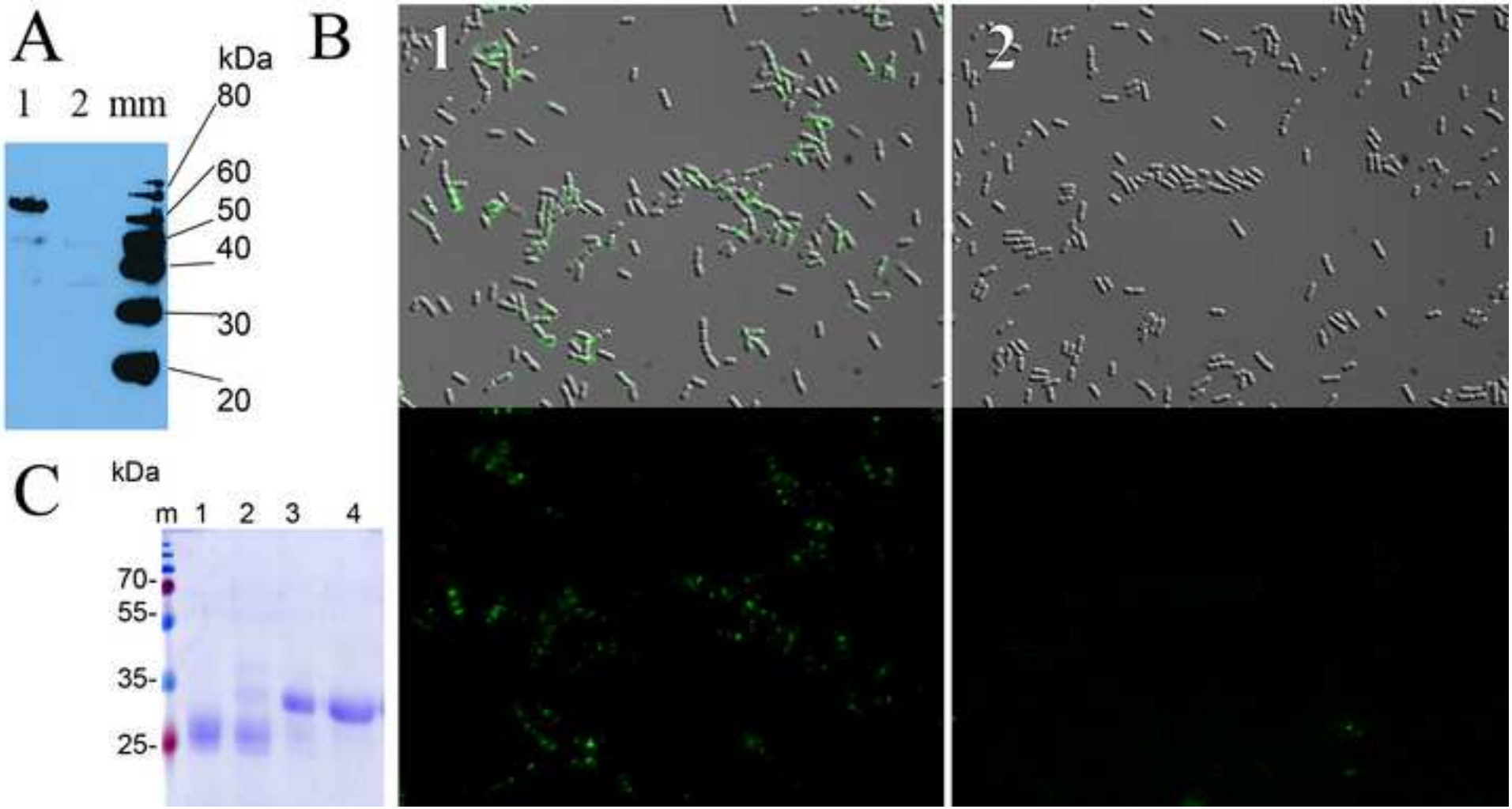

Figure 3

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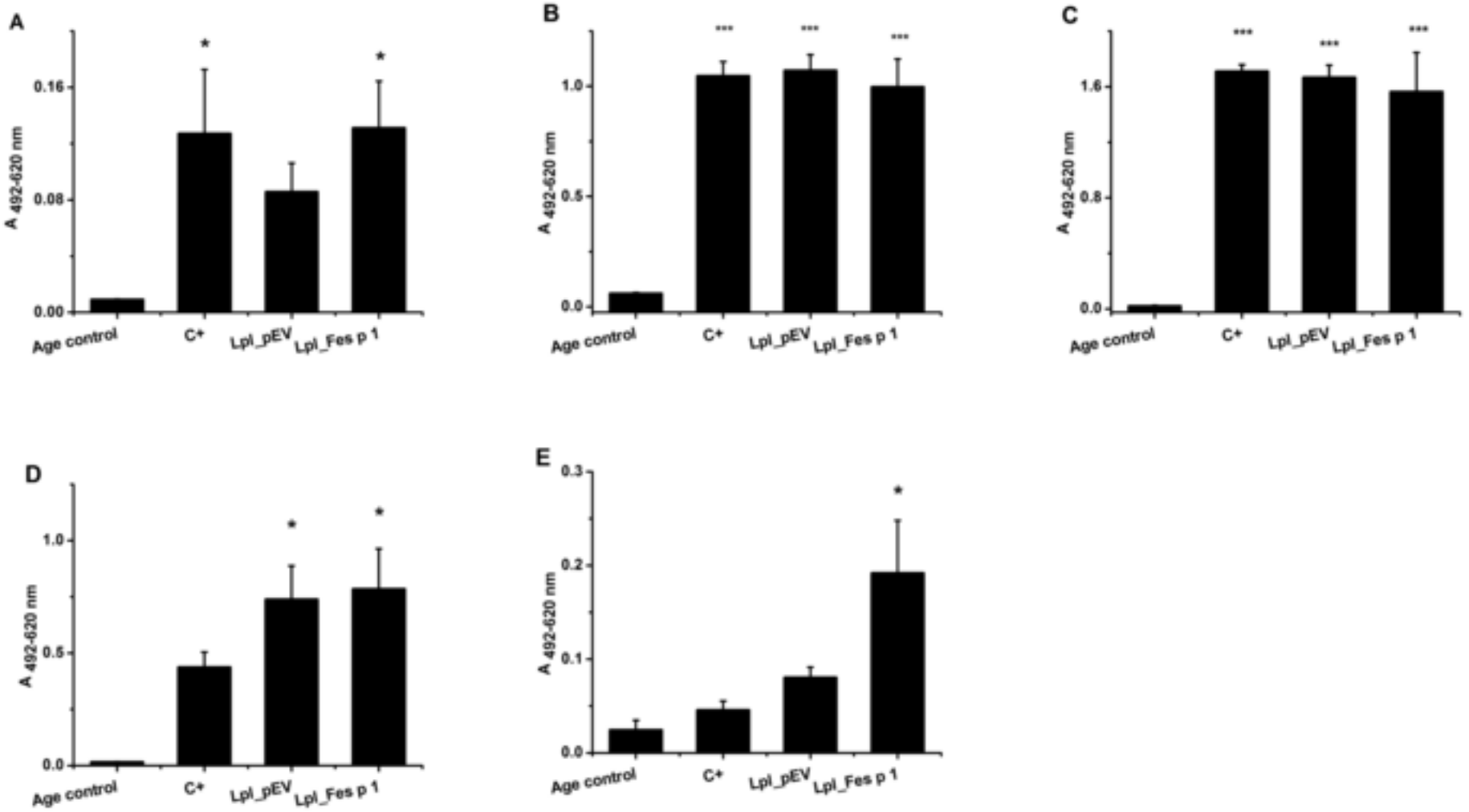

Figure 4

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