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Oral immunization of mice with expressing the rotavirus VP8* protein

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1 **Section: Animal Cell Technology**

2

3 **Oral immunization of mice with *Lactococcus lactis* expressing the rotavirus VP8***

4 **protein**

5

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ABSTRACT

The efficacy of recombinant *Lactococcus lactis* as a delivery vehicle for a rotavirus antigen was evaluated in a mouse model. The rotavirus VP8* protein was expressed intracellularly and extracellularly in *L. lactis* wild type and in an *alr* mutant deficient in alanine racemase activity, necessary for the synthesis of the cell-wall component D-alanine. When the mucosal immune response was evaluated by measuring VP8*-specific IgA antibody in faeces, wild-type *L. lactis* triggered a low IgA synthesis only when the secreting strain was used. In contrast, VP8*-specific IgA was detected in faeces of both groups of mice orally given the *alr* mutant expressing extracellular VP8* and intracellular VP8*, which reached levels similar to that obtained with the wild type secreting strain. However, oral administration of the recombinant strains did not induce serum IgG or IgA responses. *L. lactis* cell-wall mutants may therefore provide certain advantages when low-antigenic proteins are expressed intracellularly. However, the low immune response obtained by using this antigen-bacterial host combination prompts to the use of new strains and vaccination protocols in order to develop acceptable rotavirus immunization levels.

48 **INTRODUCTION**

49 Rotaviruses are enteric viruses and are a main cause of diarrhea in children worldwide. They are responsible of
50 more than 500,000 deaths of children under 5 years each year (Parashar et al. 2009). The VP4 protein from the
51 outside viral layer is the viral hemagglutinin and is digested by trypsin into two polypeptides, the *N*-terminal
52 VP8* (28 kDa) and the *C*-terminal VP5* (60 kDa) proteins. Mucosal immunity against rotavirus infections is
53 based on the production of IgA antibodies at the intestinal mucosa (Kapikian 2001; Ward 2003). In fact,
54 antibodies to VP8* produced *in vivo* can protect against rotavirus produced diarrhoea (Ruggeri and Greenberg
55 1991; Gil et al. 2000), making VP8* a good target for the design of new rotavirus vaccines.

56 *Lactococcus lactis* is a well-characterized lactic acid bacterium which has been the chosen candidate vehicle for
57 the delivery of active molecules by mucosal routes, including numerous bacterial and viral antigens for the
58 development of live vaccines (Wells and Mercenier 2008; Bermudez-Humaran 2009; Rottiers et al. 2009).
59 Earlier work has addressed the effects of expressing antigens at different levels and locations (cytoplasmic,
60 secreted or cell-wall anchored), showing a clear dependence of the amount of expressed antigen in the elicited
61 immune response in animal models when the oral route was employed (Grangette et al. 2002). This led to the
62 evaluation of mutants altered in cell-wall synthesis with the aim of increasing the delivery capacity to the
63 mucosal immune system by promoting the *in vivo* release of antigens. Strains of *L. lactis* and *Lactobacillus*
64 *plantarum* deficient in the synthesis of D-alanine (alanine racemase mutants; *alr*), essential for the synthesis of
65 peptidoglycan, showed enhanced capacity to deliver the tetanus toxin fragment C (TTFC) model antigen to the
66 mucosal immune system in mice (Grangette et al. 2004).

67 The purpose of the present study was to assess the efficacy of *L. lactis* and its *alr* mutant in the mucosal delivery
68 of an antigen different to TTFC and with a potential use as rotavirus vaccine. To this end, wild-type *L. lactis* and
69 its *alr* mutant producing rotavirus VP8* intracellularly and extracellularly were assayed for immunization in a
70 murine model.

71

72

73 **MATERIAL AND METHODS**

74

75 **Bacterial strains and growth conditions**

76 *Lactococcus lactis* MG1363 and *L. lactis* PH3960 (*alr*), an MG1363 derivative deficient in the alanine racemase
77 gene (Hols et al. 1999), were used in this study. Both strains were routinely grown in M17 medium (Difco)
78 supplemented with 0.5 % (w/v) glucose at 30°C in static conditions. For growth of the PH3960 strain the
79 medium was supplemented with 200 µg D-alanine ml⁻¹. *E. coli* DH5α was used as cloning host and *E. coli* M15
80 was used for protein expression. They were grown in LB medium at 37°C under shaking. For plasmid selection,
81 erythromycin was used at 5 µg ml⁻¹ for *L. lactis* and ampicillin was used at 100 µg ml⁻¹ for *E. coli*.

82

83 **Expression and purification of VP8* in *E. coli* and anti-VP8* serum preparation**

84 The VP8* cDNA from the rotavirus simian strain SA11 (P5B[2] G3 genotype) was obtained from plasmid
85 pGALVP8 (Gil et al. 2001) by digestion with BamHI and KpnI and cloned into pQE30 plasmid (Qiagen)
86 digested with the same enzymes. The resulting pQE-VP8 plasmid was used to transform *E. coli* M15 and protein
87 expression was induced with 1 mM IPTG. VP8* was totally present as inclusion bodies and it was dissolved
88 from the pellet by treatment with 8 M urea in 100 mM Tris/HCl pH 7.4 for 2 h at room temperature. The
89 denatured protein was then purified in Ni-NTA columns according to the supplier (Qiagen) including 8 M urea
90 in all the buffers. Urea was removed by sequential dialysis with decreasing urea concentrations followed by a
91 final dialysis in 50 mM Tris/HCl pH 7.4, 100 mM NaCl, 1mM EDTA and the protein was stored at -80°C. Anti-
92 VP8* serum was obtained by intramuscular injection of three doses of 100 µg of the purified protein to rabbits
93 at the animal facilities of the Instituto de Biomedicina of Valencia.

94

95 **Construction of plasmid vectors expressing VP8***

96 The VP8* cDNA was obtained by treating plasmid pGALVP8 (Gil et al. 2001) with *Xba*I/Klenow followed by
97 digestion with *Bam*HI and cloned into the pT1NX vector (Schotte et al. 2000) digested with *Spe*I/Klenow and
98 *Bam*HI, giving pRo157. In this construct the VP8* cDNA was fused to the sequence coding the Usp45
99 lactococcal secretion signal and expressed from the constitutive P1 lactococcal promoter (Waterfield et al.
100 1995). A vector for intracellular expression was constructed based in pT1NX. The P1 promoter region and the
101 ribosome-binding site of *usp45* were obtained from pT1NX by PCR with oligonucleotides 5'-
102 AATAGAATTCGATTAAGTCATCT-3' and 5'-TGAGGATCCGTCGCCGCCATGCGTATATCTCCTTT-

103 3' (restriction sites introduced for cloning are underlined). The PCR fragment was digested with *EcoRI* and
104 *BamHI* and used to replace the *EcoRI-BamHI* fragment from pT1NX which contained the promoter and *usp45*
105 secretion signal. The resulting plasmid was used for cloning the VP8* cDNA as a *XbaI/Klenow* and *BamHI*
106 fragment, giving pRo298. All plasmid constructions were performed in *L. lactis*, which was transformed by
107 electroporation in a GenePulser apparatus (BioRad).

108

109 **VP8* expression in *Lactococcus* and inocula preparation**

110 The day before immunizations, 5 ml GM17 were inoculated with 10 µl frozen bacterial stock and were
111 incubated for 16 h at 30 °C. The cultures were washed in GM9 medium [1X minimal M9 salts, 0.5 % (w/v)
112 glucose, 0.5 % (w/v) Casitone, 20 mM MgSO₄, 2 mM CaCl₂, 50 mM carbonate buffer pH 7.4] and diluted 1/10
113 in the same medium and further incubated for 4 h at 37°C. For the *L. lactis alr* strain a suboptimal concentration
114 of D-alanine (25 µg ml⁻¹) was added to allow bacterial growth. For inocula preparation, bacteria were recovered
115 by centrifugation washed three times with GM9 and resuspended to 10⁹ c.f.u. in 100 µl of GM9 containing 10
116 µg the B subunit of cholera toxin (CTB) as adjuvant. CTB was purified using plasmid pAE-ctxB as described
117 (Areas et al. 2002). For western blot analysis, cells corresponding to 10⁹ cfu were washed and resuspended in 50
118 µl of 50 mM Tris/HCl pH 7.5 containing 0.5 M sucrose and 50 mg lysozyme ml⁻¹. After 1 h at 37°C, cells were
119 washed with 1 ml 50 mM Tris/HCl pH 7.5 and resuspended in 50 µl Laemmli loading buffer and boiled for 5
120 min. One ml growth medium supernatants were treated with trichloroacetic acid at 10 % (w/v), incubated on ice
121 for 1 h and proteins were precipitated at 12,000 x g 10 min. Cell lysates and supernatant precipitated proteins
122 corresponding to 5 x 10⁸ cfu were separated in 10 % SDS-PAGE gels and expression of VP8* was analyzed by
123 western blot with rabbit anti-VP8* serum and the ECL system (GE Healthcare).

124

125 **Mice inoculation and sample collection**

126 Each immunization group consisted of 5 female BALB/c mice, 6 to 10 weeks old. Each individual was analyzed
127 by ELISA to confirm the absence of anti-rotavirus antibodies prior to the immunization schedule. The
128 inoculation was performed intragastrically using a mice intragastric probe (Harvard Apparatus) to ensure equal
129 administration (10⁹ c.f.u. in 100 µl) to each mouse. The immunization consisted of three inoculation cycles of
130 three doses in three consecutive days spaced by three weeks intervals. Serum samples and faeces were collected
131 10 days after each immunization cycle. Blood samples were obtained from the tail vein and sera were obtained
132 by centrifugation at 3000 x g and stored at -32°C. Stool samples were collected and dissolved to at 100 mg ml⁻¹

133 in PBS containing the Mini EDTA-free Protease inhibitor cocktail (Roche) and frozen at -32°C until use. Prior
134 to be used the faecal suspensions were homogenized and centrifuged at 5000 x g for 10 min.

135

136 **Viral challenge**

137 After the last blood and faecal sampling, mice were inoculated by the intragastric route with 50 µl suspension of
138 rotavirus SA11 strain (6×10^7 pfu ml⁻¹). Faeces were recovered from individual mice for seven days and blood
139 samples were taken at 15 days post-infection. Samples were tested by ELISA for rotavirus antigens and
140 rotavirus-specific antibodies as described (Garcia-Diaz et al. 2004)

141

142 **ELISA assays**

143 Polystyrene plates were coated with 1 µg purified (6XHis)-VP8* protein ml⁻¹ in 0.1 M carbonate/bicarbonate
144 buffer pH 9.6 incubated at 37 °C for 2 h and stored at 4°C until use. The serum samples were serially diluted in
145 PBS with 0.5 % Tween 20 (v/v) (PBS-T) plus 1 % BSA (w/v) as blocking agent. 100 µl of undiluted faecal
146 suspensions were used. After washing with PBS-T, a 1:2000 dilution of anti-mouse IgG- or anti-mouse IgA-
147 HRP-conjugate (Sigma) in PBS-T containing 1 % BSA (w/v) was added as secondary antibody. The
148 colorimetric reaction was developed with *o*-phenylenediamine and H₂O₂ for 10 min and the reactions stopped
149 with 50 µl 3 M H₂SO₄. The final absorbance was read at 492 nm. The statistical significance was calculated
150 using the Mann-Whitney U-test with SPSS 17.0 software (SPSS, Inc., Chicago, IL)

151

152 **RESULTS**

153

154 **VP8* expression in *L. lactis***

155 Two vectors were constructed for intracellular and extracellular expression of VP8* from the rotavirus strain
156 SA11 in *L. lactis* (Fig. 1a). The extracellular construct (pRo157) was based on the widely used lactococcal
157 Usp45 secretion signal. As can be seen in Figure 1B, this signal efficiently promoted the secretion of VP8*, with
158 low amounts of VP8* detected in the cellular fractions. Secreted VP8* was partially degraded into smaller
159 peptide products. When the intracellular vector (pRo298) was used, most of VP8* was detected in the cellular
160 fraction, with a small portion present in the supernatant probably as a result of cell lysis (Fig. 1b). Expression
161 was assayed in *L. lactis* wild type and an *alr* deficient mutant which was totally dependent on the addition of D-
162 alanine for growth. However, to ensure differences in cell-wall characteristics between both strains, a

163 suboptimal D-alanine concentration was used in the expression and inoculation experiments (Grangette et al.
164 2004). Compared to the wild type, the presence of the *alr* mutation did not affect VP8* expression in either the
165 extracellular or intracellular constructs.

166

167 **Oral immunization of mice with *L. lactis* expressing VP8* and viral challenge**

168 An inoculation schedule was designed consisting in three consecutive daily oral doses of 10^9 bacterial cells
169 repeated three times at three weeks intervals. Serum samples were analyzed by ELISA for VP8*-specific IgG
170 and IgA. After the three immunization cycles no antibody response was obtained in sera with either of the
171 strains. However, as seen in Figure 2, administration of *L. lactis* expressing VP8* led to a significant increase in
172 the VP8*-specific IgA response in faeces, indicating that the recombinant strains only triggered a local immune
173 response. However, strains producing secreted VP8* resulted in IgA levels that were already significant after the
174 second inoculation cycle for the wild type ($P=0.01$) and after the third inoculation cycle for the *alr* mutant
175 ($P=0.004$). When the intracellular constructs were used, the wild type induced specific IgA production only after
176 the third inoculation cycle which did not reach statistical significance ($P=0.134$). On the contrary, a significant
177 IgA production was already measured with the *alr* mutant after the second and third cycle ($P=0.04$ and 0.01 ,
178 respectively). This indicated that the low immunogenicity of the wild type was increased by the *alr* mutation,
179 which resulted in IgA levels similar to that obtained with the strains producing secreted VP8*.

180 Mice treated with recombinant *L. lactis* were orally challenged with rotavirus strain SA11. Although this simian
181 strain is able to infect and induce diarrhea in mice pups, its limited infection capacity in adult mice resulted in
182 background levels of rotavirus antigens in faeces with no differences between groups (data not shown). ELISA
183 analysis of sera from infected mice showed that some groups of mice were primed. This was reflected by
184 significantly ($P=0.013$) higher anti-rotavirus IgG levels in mice treated with wild-type *L. lactis* expressing
185 extracellular VP8*. The same effect was observed for the *alr* mutant expressing intracellular VP8*, although
186 differences were not significant ($P=0.052$) (Fig. 3).

187

188 **DISCUSSION**

189

190 Vaccination against rotavirus is currently regarded as the most efficient preventive measure against rotavirus
191 severe diarrhoea (Parashar et al. 2003). The present work aimed to investigate if *L. lactis* was a suitable vector
192 to confer mucosal immunity against a rotavirus antigen that has previously shown to elicit a rotavirus

193 neutralizing response and to prove if cell-wall mutants of this bacterium behaved as better antigen delivery
194 vehicles. We showed that orally administered recombinant *L. lactis* to mice was able to deliver the VP8* antigen
195 to the intestinal mucosa. VP8* displayed a limited immunogenicity, as reflected by the need of a repeated
196 inoculation scheme and the use of CTB as adjuvant to trigger specific IgA production. When CTB was omitted
197 in the inoculations, no immune responses were observed (data not shown). Additionally, the low immune
198 response against VP8* can be the result of other factors. The P1 promoter has been successfully employed for
199 the delivery of recombinant proteins to mice intestinal mucosa by *L. lactis* (Steidler et al. 2000; Robinson et al.
200 2004; Vandembroucke et al. 2004). Nevertheless, the actual strength of this promoter under the conditions of the
201 different segments of mice gastrointestinal tract is not known and the possibility exists that VP8* in situ
202 expression was a limiting factor.

203 When immunized mice were challenged with rotavirus SA11, a priming effect was only observed in the groups
204 that showed the higher local immune response. However, the use of the simian SA11 strain makes difficult to
205 assess the efficacy of protection and further experiments using murine VP8* antigens and virus strains are
206 required to address this point.

207 *L. lactis* producing VP8* elicited only a local response, with no production of serum specific antibodies. These
208 results are in contrast with very recent data reported by Qiao et al., who showed that *Lactobacillus casei*
209 expressing porcine VP8* produced an IgG humoral response when given orally to mice (Qiao et al. 2009). This
210 suggests that immune responses to antigens expressed by lactic acid bacteria depend not only on the nature of
211 the antigen but also in the intrinsic adjuvant characteristics of the host strain and questions the suitability of *L.*
212 *lactis* as a host for rotavirus VP8* delivery. However, in agreement to the results reported here, a VP8*-LTB
213 (heat-labile toxin B subunit from *E. coli*) fusion was required to increase adjuvancy and obtain a strong immune
214 response in mice (Qiao et al. 2009).

215 When wild-type *L. lactis* producing VP8* was used, an IgA response was only observed with the extracellular
216 expression vector. The amount of secreted VP8* in culture medium was higher than that observed in the
217 intracellular constructs. This, together with the fact that soluble antigens are generally less immunogenic,
218 suggested that the lack of immune response elicited by the wild type with the intracellular construct was
219 possibly due to the limited amounts of VP8* produced. We tried to circumvent this by following a similar
220 strategy to that reported for the delivery of the TTFC model antigen by *L. lactis* and *L. plantarum* (Grangette et
221 al. 2004). The use of an *alr* mutant expressing intracellular VP8* only resulted in a slight increase of specific
222 IgA in faeces compared to the wild type expressing VP8* at the same location. Nevertheless, in comparison to

223 the wild-type strain producing secreted VP8*, the use of the *alr* strain did not result in a stronger immune
224 response. The introduction of this mutation causes *L. lactis* cells to lyse upon D-alanine depletion (Steen et al.
225 2005). It is thus conceivable that the release of intracellular VP8* during the gastrointestinal passage of
226 recombinant *L. lactis alr* could be the reason for the improved delivery capacity of intracellularly-expressed
227 VP8* obtained with this mutant. Following this reasoning, no clear differences could be observed between the
228 wild type and the *alr* mutant when the VP8* antigen was produced extracellularly. However, as previously
229 suggested (Grangette et al. 2004), other reasons, such as a different interaction with the host immune cells and
230 the activation of antigen-presenting cells, cannot be ruled out.

231

232 **In summary**, *L. lactis* expressing VP8* induces a low local response in mice when given orally. This response
233 could not be enhanced by the use of *L. lactis* cell-wall mutants. This implies that new lactic acid bacteria strains
234 with better adjuvant/delivery capacity, in combination with appropriated immunization protocols and viral
235 strains, must be tested in order to develop an efficient rotavirus vaccine based on VP8*.

236

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243

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305

306

307

308 **FIGURE LEGENDS**

309

310 **Fig 1**

311 VP8* expression in *L. lactis*. **a** Schematic representation of the plasmid constructs for intracellular and
312 extracellular production of VP8*. P1, lactococcal constitutive promoter; SD, Shine-Dalgarno sequence from
313 *usp45*; Usp45; secretion signal from the *L. lactis* Usp45 protein. **b** Western blot analysis of VP8* production.
314 Cell extracts and supernatants corresponding to 5×10^8 cfu were loaded onto each lane and VP8* was detected
315 with a rabbit antiserum. wt is wild-type *L. lactis* and *alr* is the *alr* mutant strain. C is an intracellular extract of
316 wild-type *L. lactis* transformed with the pT1NX vector. MW indicates the position of molecular weight markers
317 in kDa. The asterisks point to degradation products of extracellular VP8*. Usp45::VP8* is non-secreted
318 unprocessed VP8*

319

320 **Fig 2**

321 Mice IgA antibody response after oral inoculation with *L. lactis* producing VP8*. The ELISA reads of VP8*-
322 specific IgA in faeces of mice inoculated with *L. lactis* expressing VP8* are shown. (□) first cycle of
323 inoculation; (■) second cycle of inoculation; (■) third cycle of inoculation. wt is wild-type *L. lactis* and *alr* is
324 the *alr* mutant strain. The control is wild-type *L. lactis* transformed with the pT1NX vector.

325

326 **Fig 3**

327 Serum anti-SA11 IgG response in mice treated with recombinant *L. lactis* and challenged with rotavirus SA11
328 strain. Serum samples were tested at 1:100 dilution for rotavirus-specific IgG by ELISA in rotavirus SA11
329 antigen-coated wells.

Figures 1, 2 and 3

[Click here to download line figure: VP8 figuresII.ppt](#)

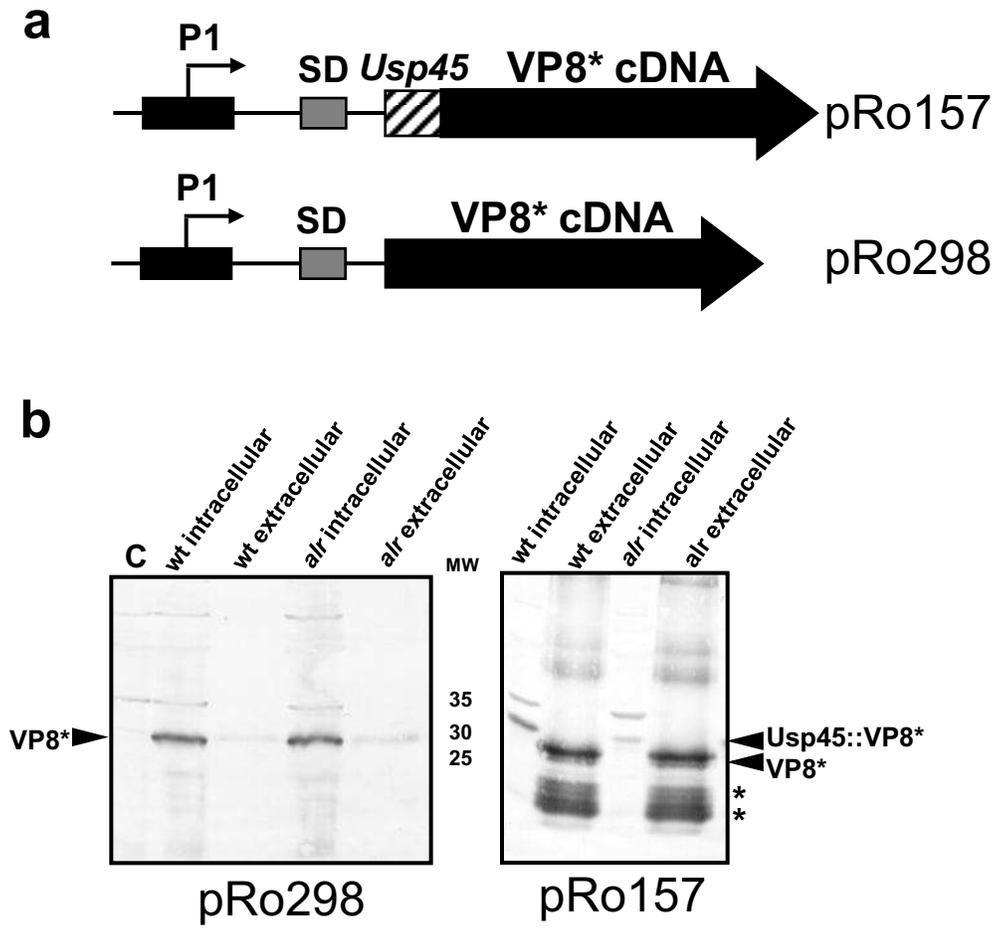


Fig 1

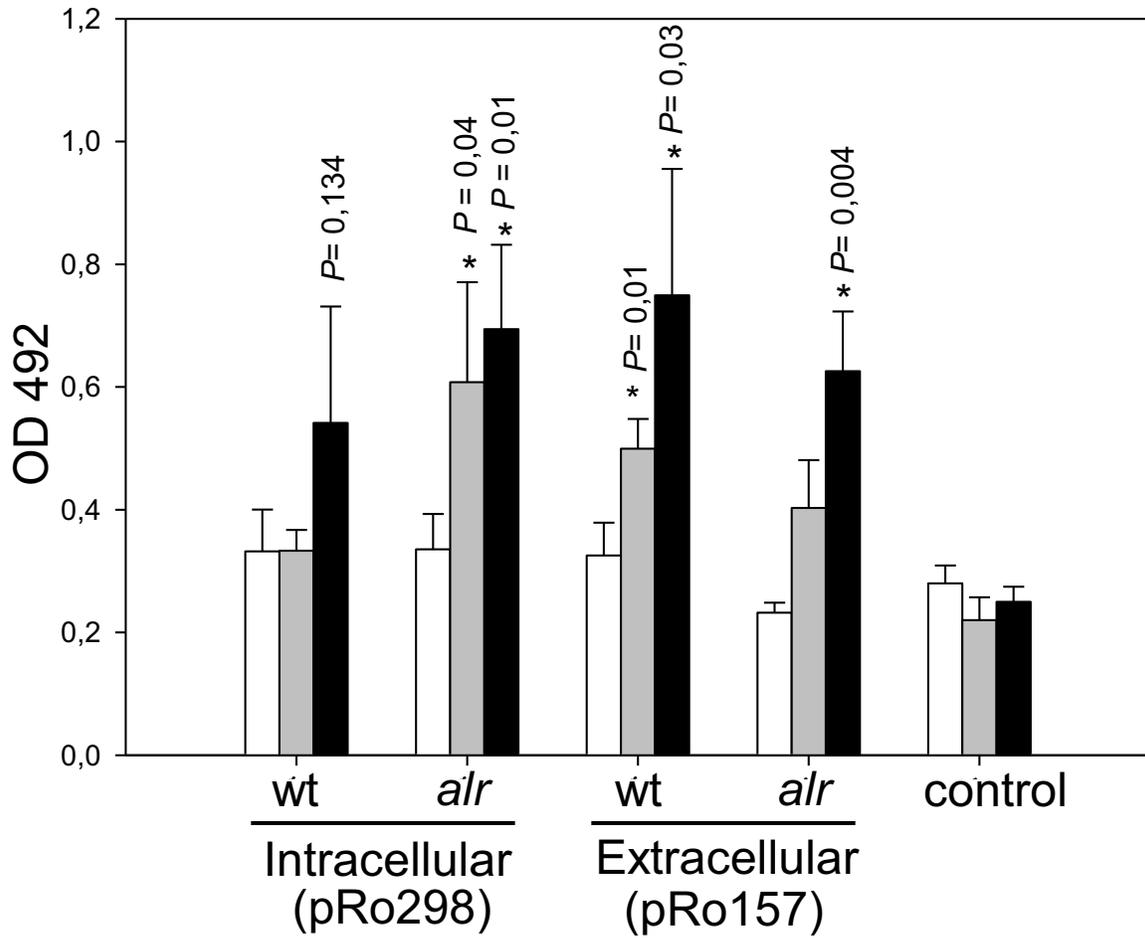


Fig 2

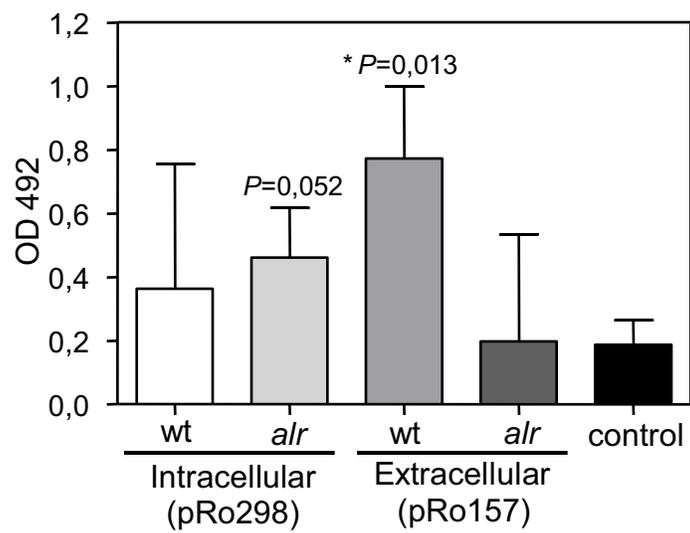


Fig 3