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1 **INFLUENCE OF ENVIRONMENTAL CONDITIONS ON BIOFILM**
2 **FORMATION BY *HAFNIA ALVEI* STRAINS.**

3
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26 **Abstract**

27

28 *Hafnia alvei* is considered an opportunistic pathogen of animal and humans, affecting a
29 wide range of homeothermic and poikilothermic hosts with different body temperatures.
30 In this work, *H. alvei* strains isolated from different sources were studied with regard to
31 their capacity to form biofilms under different environmental conditions. Strain, growth
32 phase, temperature and culture media dependent changes of biofilm formation were
33 semiquantitatively monitored using a microtiter plate method. Our study shows that all
34 strains used could form biofilms *in vitro*, and that biofilm formation increases
35 dramatically during growth at 25°C but not at 37°C, and decreases at both temperatures
36 in presence of glucose. At 16°C only one strain isolated from a lizard was able to form a
37 dense biofilm showing that the ability to form biofilms in this species is regulated by
38 environmental factors and is also strain specific.

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50 **Keywords:** *Hafnia alvei*, biofilms, environmental regulation.

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52 1. Introduction

53 *Hafnia alvei* is a Gram negative, rod-shaped, facultative anaerobic bacterium of the
54 family *Enterobacteriaceae* that has been isolated from various mammals, fish and birds
55 (Gelev et al., 1990; Sharma et al., 1991; Rodríguez et al., 1998). The organism has also
56 been detected in food and in food production units (Gamage et al., 1998; Tornadijo et
57 al., 1993; García-Fontán et al., 2007; Morales et al., 2004; González-Rodríguez et al.,
58 2001). In humans this species causes a wide variety of infections, including pneumonia,
59 bacteremia, and extraintestinal infections (Klapholz et al., 1994; Ramos and Damaso,
60 2000; Casanova-Román et al., 2004). Its thermic ecological niche varies from cold, the
61 temperatures found in the preservation food chains and fish aquatic environments (4-
62 20°C) to the body temperature of humans and birds (37°C-42°C). However, the
63 properties determining its adaptation capacity to these different temperatures are still
64 unknown. Previously, we described an outbreak in commercial laying hens in Spain
65 (Real et al., 1997) and we were able to reproduce the same disease presentation in hens
66 experimentally infected by either the intraperitoneal or oral route. A similar outbreak
67 recently reported in Italy and challenges with the strain isolated was reproduced the
68 disease in healthy pullets (Proietti et al., 2004). *H. alvei* has some characteristics closely
69 related to the genus *Escherichia*, *Salmonella* and *Shigella* (Janda and Abbott, 2006).
70 Septicemic lesions produced by *Hafnia* spp. in several animal species resemble those
71 produced by *Salmonella* spp. (Kelly, 1993; Ridell, 1987; Real et al., 1997).
72 Furthermore, some reports show that *H. alvei* may be misidentified for *Salmonellae* or
73 other pathogens (Wang et al., 2006). Although some virulence factors have been
74 identified in *H. alvei*, little is known about the factors that contribute to its pathogenesis
75 within its host. According to Hall-Stoodley and co-workers (Hall-Stoodley L et al.,
76 2004), biofilms can be defined in a narrower sense as "a structured community of

77 bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or
78 living surface". Since cell surface components play a role on adherence and biofilm
79 formation, we speculate that adherence properties in *H. alvei* may contribute to its
80 pathogenicity. The goal of the present study was to analyse the capacity of *H. alvei*
81 strains to form biofilms during the growth under different environmental conditions.
82 Identifying these parameters should contribute to a better understanding of the
83 correlation between adherence capabilities *in vitro* and *in vivo*, and the pathogenicity of
84 this pathogen.

85

86 **2. Materials and Methods**

87 *2.1. Bacterial strains*

88 Eight *Hafnia alvei* strains were used. Strains 187-95, C-34 and 19-68 are animal
89 isolates, and strains 7-67, 24-65, 1967-82, X-1 are human clinical isolates. These strains
90 were identified with the MicroScan WalkAway system (Rodriguez et al., 1999) and
91 with the standard reference procedures (Smibert and Krieg, 1981). Results were
92 confirmed by use of the API 20E diagnostic system (Biomerieux, Spain) and lysis by
93 phage ATCC 1672 (Guinée and Valkenburg, 1968). The strains were routinely cultured
94 on Trypticase soy agar (TSA; Cultimed) at 25°C for 24 h and stored at -80°C with 15%
95 glycerol. Test for the *eae* gene in *H. alvei* strains were all negative as described
96 previously (Padilla et al, 2005).

97

98 *2.2 Culture conditions and biofilm formation*

99 Tryptic soy broth (TSB), Luria-Bertani (LB), LB diluted ½ (LB ½), LB supplemented
100 with 0.25% and 0.5% of glucose, and brain heart infusion broth (BHIB) were employed
101 in all experiments.

102 Estimation of biofilm formation was performed by the method of O'Toole and Kolter
103 (1998) with some modifications. *Hafnia alvei* strains were grown in TSA for 36 h at 25
104 °C, and a suspension from two to three colonies was prepared in PBS (OD₆₀₀ of 0.05).
105 Twenty microlitres of *H. alvei* suspension was placed in each well containing 100µl of
106 culture medium. The microplates were incubated for 24h at 16°C, 25°C and 37°C. After
107 discarding the medium in the microtiter plate wells by inversion of the plate and tapping
108 on absorbent paper, wells were rinsed three times with distilled water (each 200µl/well)
109 and the remaining adherent bacteria were stained for 12 min with 140µl/well of crystal
110 violet (0.7% [wt/vol] solution). Excess stain was removed by washings (x3) with
111 distilled water (each 170µl/well). Crystal violet was extracted by an ethanol-acetone
112 solution (80:20 v/v) and the plates were incubated at room temperature in an orbital
113 shaker for 1 min at 400 rpm (thermomixer comfort, Eppendorf) to release the dye into
114 solution. Then, a sample of 100µl was transferred to another 96-well flat bottom plate
115 and the amount of dye (proportional to the density of adherent cells) was quantified at
116 620 nm using a microplate reader. For each experiment, correction for background
117 staining was made by subtracting the value for crystal violet bound to uninoculated
118 controls. The biofilm assay was performed two times, with quadruplicates in each assay.
119 Influences of the temperature and culture media on biofilm formation were statistically
120 analyzed by using one way ANOVA and Tukey's test. Statistical significance was set at
121 two-tailed $p < 0.001$.

122

123 *2.3 Motility assays*

124 Bacterial cells grown for 24h on TSA plates served as inoculum for all motility
125 experiments. (1) Swimming. Tryptone swim plates (1% tryptone, 0.5% NaCl, and 0.3%
126 Agar) dried overnight at room temperature were point inoculated with bacteria using

127 sterile toothpicks, incubated for 24h at 16, 25 and 37°C, and flagellar motility assessed
128 by the size of the circular zone. (2) Swarming. Tryptone swarm plates (1% tryptone,
129 0.5% NaCl, and 0.6% Agar) dried overnight at room temperature were inoculated using
130 sterile toothpicks to the bottom of the petri dish; the sizes of the swarming zones were
131 observed macroscopically (3). Twitching. Cells were stab inoculated in the swarming
132 plates and after incubation, the polystyrene surface was examined by removing the agar,
133 washing unattached cells with tap water, and staining the attached cells with crystal
134 violet (0.7% [wt/vol] solution).

135

136 *2.4 Test for surface hydrophobicity*

137 The bacterial surface hydrophobicity was tested by the two-phase partition method
138 described by Rosenberg et al. (1980). Xylene was used as the hydrocarbon phase.
139 Bacteria grown in TSB were washed and suspended in phosphate-buffered saline. The
140 concentration of the bacteria was adjusted to an OD₆₀₀ of 0.1. One milliliter of the
141 suspension and 0.6 ml of xylene were mixed vigorously in a 2 ml tube with a vortex
142 mixer for 30 s. After allowing 30 min for the separation of the aqueous and xylene
143 phases, the aqueous phase was carefully removed and its OD₆₀₀ was recorded. The
144 hydrophobicity was expressed as the percent reduction of the optical density of the
145 aqueous phase after being mixed with xylene. All experiments were performed in
146 triplicate and numerical data are presented as means with error bars representing
147 standard deviations. The data were statistically analyzed by using one way ANOVA and
148 Tukey's test. Statistical significance was set at two-tailed $p < 0.001$.

149

150

151

152 **Results**

153

154 All *H. alvei* strains tested were able to form biofilms in the polystyrene surface of the
155 microtiter plates. Homogeneity seems to be the most common characteristic at 37°C in
156 the animal isolates without notable differences between strains in each culture medium.
157 The amount of biofilm formed by animal strains grown in diluted LB (LB ½), in LB 1X,
158 or in BHIB medium was higher than that of cells grown in LB supplemented with
159 glucose (0.25 or 0.5%) at 37°C (Figure 1a), but at 25°C, only strain 19-68 were able to
160 maintain this difference (Figure 1b). At 25°C, there was a clear difference between the
161 lizard and trout isolates with respect to the laying hen isolate (Fig. 1b). Surprisingly, at
162 16°C, strain 19-68 was able to form a dense biofilm in comparison with the other animal
163 or clinical strains (Fig. 1c). In this point, 25°C was the most favourable temperature to
164 form biofilms for all strains ($p < 0.001$).

165 In the group of human isolates, strain X-1 and 4256-83 seem to form more dense
166 biofilms in diluted, undiluted LB, and in TSB (strain X-1) than in the other media at the
167 higher temperatures (37 and 25°C, $p < 0.001$) (Fig. 2a,b). Again, glucose added to the LB
168 medium seems to reduce the capacity to form biofilms for most of the human strains.

169 To investigate whether motility was involved in biofilm formation, swimming and
170 swarming motility were analyzed at different temperatures in all *H. alvei* strains used in
171 this study. After 24h, only strain 1967-82 failed to swim when compared with other
172 animal or human strains (Fig. 3); strain C-34 showed reduced motility at all
173 temperatures in contrast with other strains (Fig. 3). This reduced motility seems not to
174 affect the capacity the strain to form a dense biofilm under the temperature and culture
175 media tested. Strain 1967-82 is also able to form biofilms despite being nonmotile.

176 All strains were unable to swarm at any temperature (not shown) indicating that
177 presence of lateral flagella is not a prerequisite -or that this type of motility had not high
178 contribution for biofilm formation in *H. alvei*.

179 The three animal isolates used in this study (C-34, 187-95, and 19-68) lack any type of
180 fimbriae whereas four of five human isolates (7-67, 1967-82, 24-65, and 4256-83) have
181 type I and III fimbriae as we reported previously (Padilla et al., 2005). The strain X-1
182 isolated from human enteritidis lacks also these structures. This human strain shows
183 high biofilm formation at 37°C in comparison with the other human isolates in three
184 different culture media (Fig. 2a).

185 The surface hydrophobicity of the *H. alvei* strains are shown in Figure 4. The strains
186 187-95 and C-34 (isolated from animals) have a more hydrophilic surface character
187 ($P < 0.001$), whereas no significant differences were found in the surface hydrophobicity
188 among the human isolates.

189

190 **Discussion**

191 Our results in this study suggest that higher nutrient conditions such as TSB and BHIB
192 favors *H. alvei* biofilm formation. In lower nutrient conditions (LB1/2 or in LB1X
193 without glucose), the addition of 0.25% of glucose seems to affect the capacity to form
194 biofilms in most of strains tested at 25°C and 37°C. Further increase in glucose
195 concentration (from 0.25% to 0.5%) did not significantly alter the number of adherent
196 bacteria. Several authors have suggested that attachment and biofilm formation is
197 modulated by glucose present in the culture media (Dewanti and Wong, 1995; Pillai et
198 al., 2004; Reisner et al., 2006; Stanley and Lazazzera, 2004; Domka et al., 2006). In the
199 present study, the addition of glucose to LB medium affects biofilm formation in 75%
200 of the strains tested (6/8) at 37°C and 25°C, but did not affect biofilm formation at 16°C.

201 Swimming motility is important in the initial approach and attachment to live or inert
202 surfaces. Swarming motility depends on the increased flagellar expression induced on
203 surfaces and it is involved in colonization of surfaces and bacterial cell differentiation.
204 Swarming has been well characterized in species of *Proteus*, *Vibrio*, and *Aeromonas*
205 (Kirov, 2003) but little is known about this phenomenon in *H. alvei* where there are
206 motile and non-motile strains (Brenner, 1981). Although an extensive search has been
207 conducted to discover adherence factors in several pathogenic bacteria, little is known
208 about the identities of fimbrial proteins (proteinaceous surface structures). In our
209 study, we did not find a clear relationship between motility, fimbriae and adherence.
210 Similarly, Janda and co-workers (Janda et al., 2002) reported that two *H. alvei* strains
211 are unable to adhere to Hep-2 cells in a recognisable pattern at 37°C, but assays in other
212 human or animal cell lines or at different temperatures are not described in the
213 literature.

214 The hydrophobicity of many enteric pathogens can be correlated with their capacity to
215 attach to human epithelial cells and subsequently cause disease (Absolom, 1988) and
216 this surface characteristic is often cited when interpreting bacterial adhesiveness
217 because it has been observed that adhesiveness increases or decreases depending on the
218 surface hydrophobicity (Van Loosdrecht et al., 1987). There was a clear difference
219 between the laying hen and trout isolates with respect to human isolates, and it is
220 possible that further *in vitro* or *in vivo* experiments show whether different bacterial
221 surface properties (like hydrophobicity) between these strains may demonstrate
222 different virulence properties.

223 It appears that isolates from different sources (animal, human, or environmental) may
224 have differing abilities to form biofilms at different temperatures. Strain 19-68 that
225 exhibited exceptional biofilm formation at low temperature, and was motile, fimbriae

226 negative (as the other animal isolates), would utilize additional, potentially novel factors
227 for elevated biofilm production. A better understanding of the adherence properties in
228 this and other *H. alvei* strains *in vivo* and *in vitro* will provide great insights into the
229 diseases caused by this bacterium.

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378 **Figures.**

379 **Figure 1. Biofilm formation by animal *H. alvei* isolates in polystyrene plates.**

380 Biofilm formation on polystyrene surface after 24 h was assessed by crystal violet
381 staining. Each bar indicates the mean values with standard deviations. The biofilm assay
382 was performed two times, with quadruplicates in each assay.

383

384 **Figure 2. Biofilm formation by human *H. alvei* isolates in polystyrene plates.**

385 Biofilm formation on polystyrene surface after 24 h was assessed by crystal violet
386 staining. Each bar indicates the mean values with standard deviations. The biofilm assay
387 was performed two times, with quadruplicates in each assay.

388

389 **Figure 3. Motility agar phenotype of *Hafnia alvei* strains.**

390 Swimming zones were observed 24h after inoculating 0.3% LB agar plates and
391 incubation at different temperatures.

392

393 **Figure 4. Surface hydrophobicity of *H. alvei* strains.**

394 Surface hydrophobicity of *H. alvei* determined using the bacterial adherence to
395 hydrocarbon–xylene test. Error bars represent the standard deviations of the mean of
396 three independent experiments.

397

398

Fig. 1.

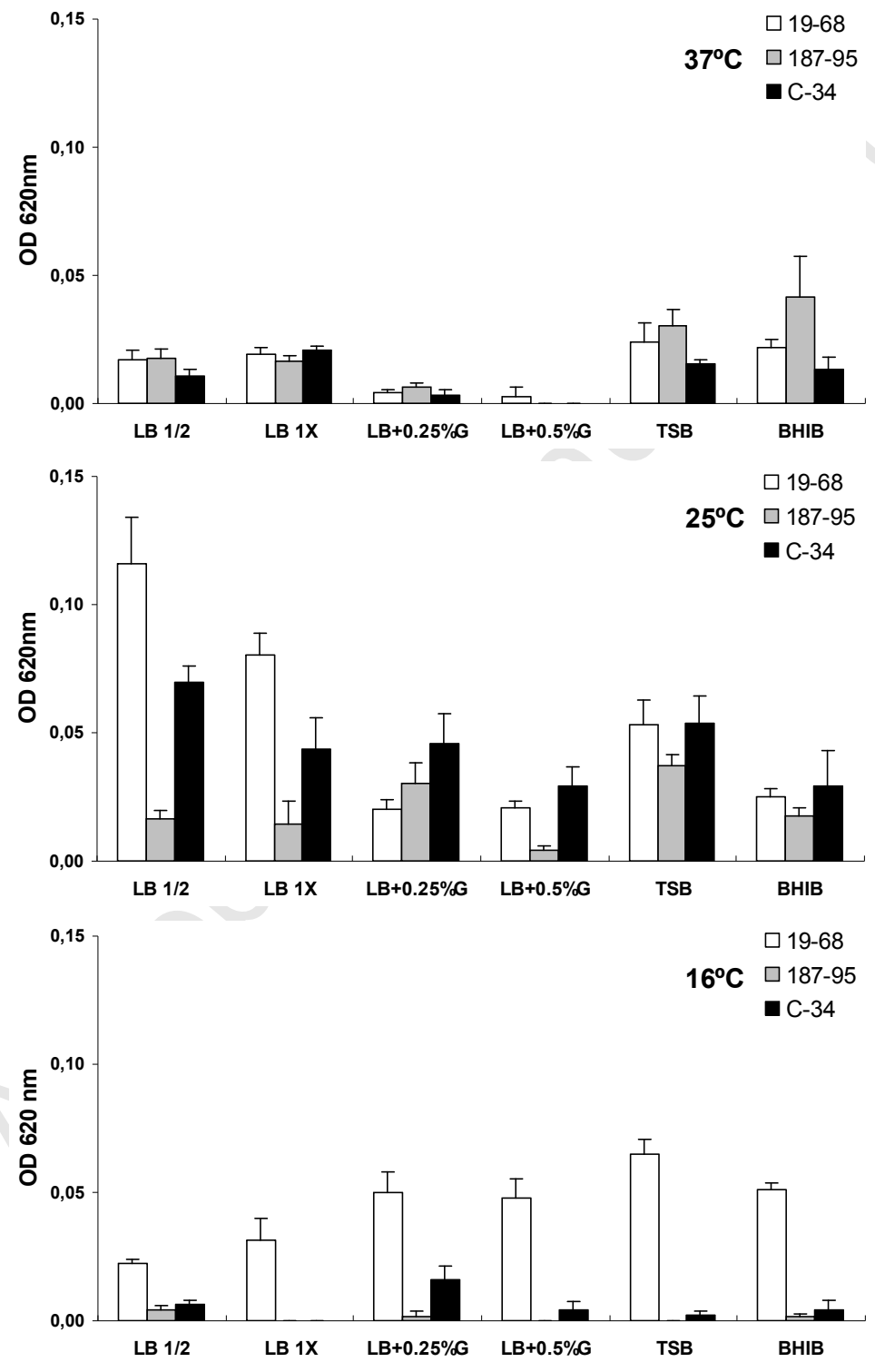


Fig. 2

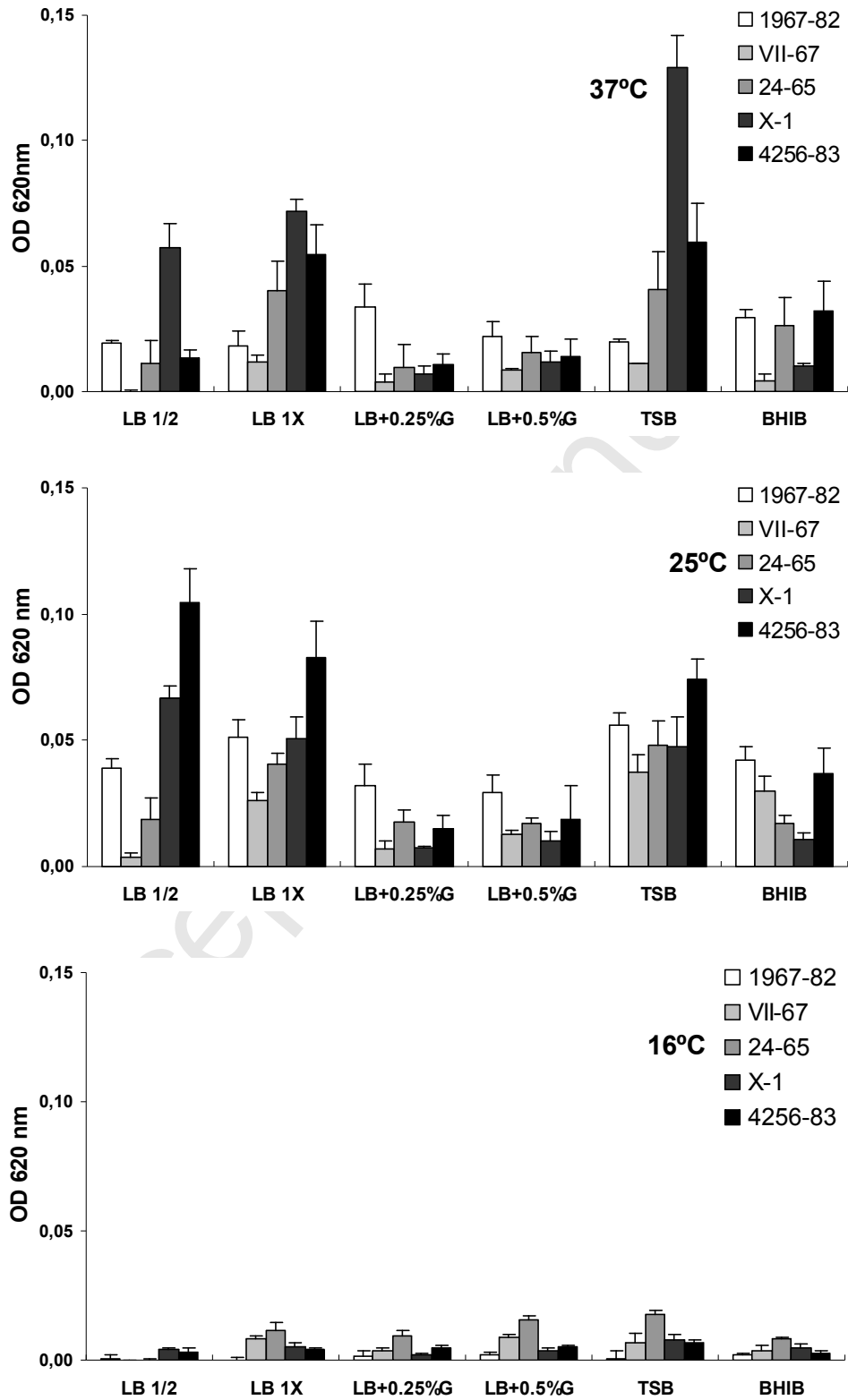


Fig. 3.

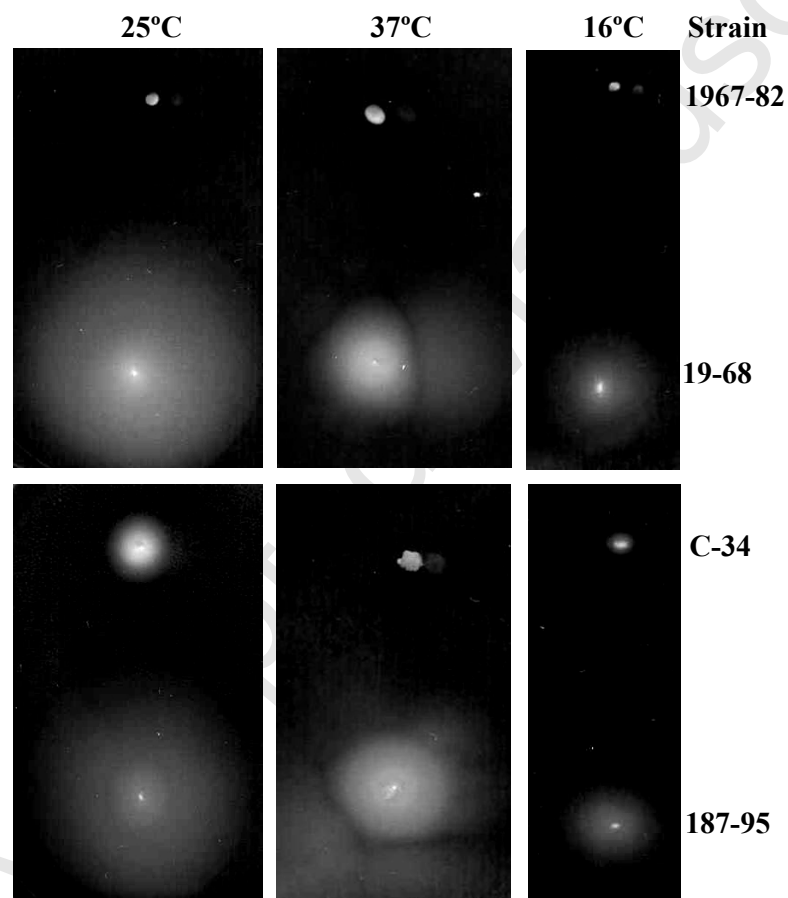
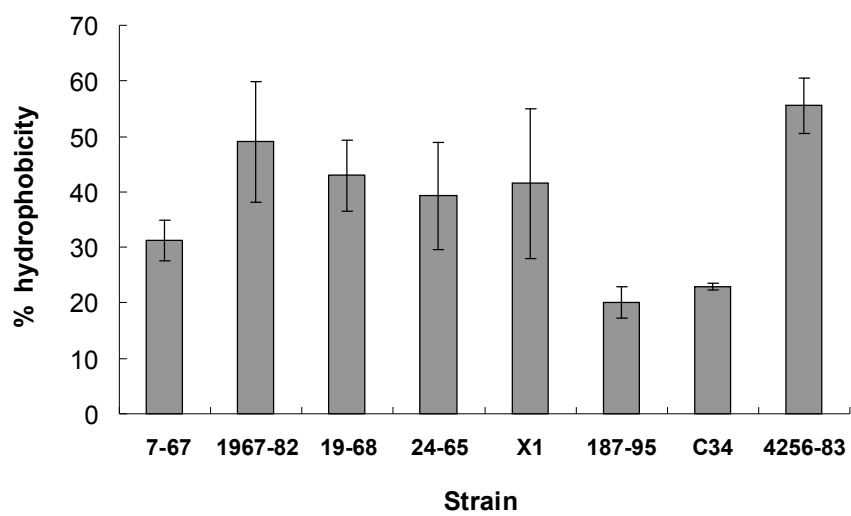


Fig. 4.



Accepted M