



Influence of environmental conditions on biofilm formation by strains

J. Ramos-Vivas, D. Padilla, F. Real, J. Bravo, V. Grasso, F. Acosta

► To cite this version:

J. Ramos-Vivas, D. Padilla, F. Real, J. Bravo, V. Grasso, et al.. Influence of environmental conditions on biofilm formation by strains. *Veterinary Microbiology*, 2008, 129 (1-2), pp.150. 10.1016/j.vetmic.2007.11.007 . hal-00532358

HAL Id: hal-00532358

<https://hal.science/hal-00532358>

Submitted on 4 Nov 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

Title: Influence of environmental conditions on biofilm formation by *Hafnia alvei* strains

Authors: J. Ramos-Vivas, D. Padilla, F. Real, J. Bravo, V. Grasso, F. Acosta



PII: S0378-1135(07)00547-0
DOI: doi:10.1016/j.vetmic.2007.11.007
Reference: VETMIC 3883

To appear in: *VETMIC*

Received date: 18-6-2007
Revised date: 6-11-2007
Accepted date: 8-11-2007

Please cite this article as: Ramos-Vivas, J., Padilla, D., Real, F., Bravo, J., Grasso, V., Acosta, F., Influence of environmental conditions on biofilm formation by *Hafnia alvei* strains, *Veterinary Microbiology* (2007), doi:10.1016/j.vetmic.2007.11.007

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

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

**Ramos-Vivas J^{1,2}, Padilla D¹, Real F¹, Bravo, J¹,
Grasso, V¹, Acosta F^{1*}.**

¹ Institute of Animal Health, IUSA, University of Las Palmas de Gran Canaria, 35416
Arucas, Las Palmas, Spain.

² Present Address: Department of Microbial Biotechnology, National Center of
Biotechnology CNB-CSIC, 28049 Madrid, Spain.

*** Correspondence:** Felix Acosta Ph.D., University Institute of Animal Health, IUSA,
University of Las Palmas de Gran Canaria, 35416 Arucas, Las Palmas, Spain
Tel./fax: +34 928 451093; +34 928 451142. e-mail: facosta@dpac.ulpgc.es

Abstract

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

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

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

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

2. Materials and Methods

2.1. Bacterial strains

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

2.2 Culture conditions and biofilm formation

Tryptic soy broth (TSB), Luria-Bertani (LB), LB diluted ½ (LB ½), LB supplemented with 0.25% and 0.5% of glucose, and brain heart infusion broth (BHIB) were employed 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

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

2.4 Test for surface hydrophobicity

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

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

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

230

231

232

233 **References**

234 Absolom, D.R., 1988. The role of bacterial hydrophobicity in infection: bacterial
235 adhesion and phagocytic ingestion. *Can. J. Microbiol.* 34, 287-298.

236

237 Brenner, D.J. 1981. Introduction to the family *Enterobacteriaceae*. In: Starr, M.P.,
238 Stolp, H., Trüper, H.G., Balows, A., Schlegel, H.G. (Eds.), *The prokaryotes*. Berlin,
239 Springer-Verlag, pp. 1105-1127.

240

241 Casanova-Román, M., Sanchez-Porto, A., Casanova-Bellido, M. 2004. Late-onset
242 neonatal sepsis due to *Hafnia alvei*. *Scand. J. Infect. Dis.* 36, 70-72.

243

244 Crandall, C., Abbott, S.L., Zhao, Y.Q., Probert, W., Janda, J.M. 2006. Isolation of
245 toxigenic *Hafnia alvei* from a probable case of hemolytic uremic syndrome. *Infection*.
246 34, 227-229.

247

248 Dewanti, R., Wong, A.C.L. 1995. Influence of culture conditions on biofilm formation
249 by *Escherichia coli* O157:H7. *Int. J. Food. Microbiol.* 26, 147-164.

250

- 251 Domka, J., Lee, J., Wood, T.K. 2006. YliH (BssR) and YceP (BssS) regulate
252 *Escherichia coli* K-12 biofilm formation by influencing cell signalling. Appl. Environ.
253 Microbiol. 72, 2449-2459.
- 254 Gamage, S.D., Luchansky, J.B., Ingham, S.C. 1998. Pulsed-field gel electrophoresis
255 typing of *Hafnia alvei* isolated from chub-packed and retail ground beef. Lett. Appl.
256 Microbiol. 26, 105-109.
- 257
- 258 García-Fontán, M.C., Lorenzo, J.M., Parada, A., Franco, I., Carballo, J. 2007.
259 Microbiological characteristics of "androlla", a spanish traditional pork sausage. Food
260 Microbiol. 24, 52-58.
- 261
- 262 Guinée, P.A.M., Valkenburg, J.J. 1968. Diagnostic value of a *Hafnia*-specific
263 bacteriophage. J. Bacteriol. 96, 564.
- 264
- 265 Gelev, I., Gelev, E., Steigerwalt, A.G., Carter, G.P., Brenner, D.J. 1990. Identification
266 of the bacterium associated with haemorrhagic septicaemia in rainbow trout as *Hafnia*
267 *alvei*. Res. Microbiol. 141, 573-576.
- 268
- 269 González-Rodríguez, M.N., Sanz, J.J., Santos, J.A., Otero, A., García-López, M.L.
270 2001. Bacteriological quality of aquacultured freshwater fish portions in prepackaged
271 trays stored at 3 degrees C. J. Food Prot. 64, 1399-1404.
- 272
- 273 Hall-Stoodley, L., Costerton, J.W., Stoodley, P. 2004. Bacterial biofilms: from the
274 natural environment to infectious diseases. Nat. Rev. Microbiol. 2:95-108.
- 275

- 276 Janda, J.M., Abbott, S.L., Khashe, S., Probert, W. 2002. Phenotypic and genotypic
277 properties of the genus *Hafnia*. J. Med. Microbiol. 51, 575-580.
278
- 279 Janda, J.M., Abbott, S.L. 2006. The Genus *Hafnia*: from soup to nuts. Clin. Microbiol
280 Rev. 19, 12-28.
281
- 282 Jakowski, S., Rowinski, S., Cisowska, A., Gamian, A. 1996. The sensitivity of *Hafnia*
283 *alvei* strains to the bactericidal effect of serum. FEMS Immunol. Med. Microbiol. 13,
284 59-64.
285
- 286 Kelly, W.R. 1993. Patterns of hepatic necrosis. In: Jubb, K.V.F., Kennedy, P.C.,
287 Palmer, N. (Eds.), Pathology of domestic animals, vol. II, 4th ed. Academic Press, San
288 Diego, CA, pp. 337-346.
289
- 290 Kirov, S.M. 2003. Bacteria that express lateral flagella enable dissection of the
291 multifunctional roles of flagella in pathogenesis. FEMS Microbiol. Lett. 224, 151-159.
292
- 293 Klapholz, A., Lessnau, K.D., Huang, B., Talavera, W., Boyle, J.F. 1994. *Hafnia alvei*.
294 Respiratory tract isolates in a community hospital over a three-year period and a
295 literature review. Chest. 105, 1098-1100.
296
- 297 Morales, P., Feliu, I., Fernandez-García, E., Nuñez, M. 2004. Volatile compounds
298 produced in cheese by *Enterobacteriaceae* strains of dairy origin. J. Food Prot. 67, 567-
299 573.
300

- 301 O'Toole, G.A., Kolter, R. 1998. Initiation of biofilm formation in *Pseudomonas*
 302 *fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic
 303 analysis. *Mol. Microbiol.* 28, 449-461.
- 304 Padilla, D., Real, F., Gómez, V., Sierra, E., Acosta, B., Déniz, S., Acosta, F. 2005.
 305 Virulence factors and pathogenicity of *Hafnia alvei* for gilthead seabream, *Sparus*
 306 *aurata* L. *J. Fish. Dis.* 28, 411-417.
- 307
- 308 Pillai, S.K., Sakoulas, G., Eliopoulos, G.M., Moellering, R.C., Murria, B.E., Intuye,
 309 R.T. 2004. Effects of glucosa on *fsd*-mediated biofilm formation in *Enterococcus*
 310 *faecalis*. *J. Infect. Dis.* 190, 967-970.
- 311
- 312 Podschun, R., Fischer, A., Ullmann, U. 2001. Characterisation of *Hafnia alvei* isolates
 313 from human clinical extra-intestinal specimens: haemagglutinins, serum resistance and
 314 siderophore synthesis. *J. Med. Microbiol.* 50, 208-214.
- 315
- 316 Proietti, C.P. Passamonti, F., Pia-Franciosini, M., Asdrubali, G. 2004. *Hafnia alvei*
 317 infection in pullets in Italy. *Avian Pathol.* 33, 200-204.
- 318
- 319 Ramos, A., Damaso, D. 2000. Extraintestinal infection due to *Hafnia alvei*. *Eur. J. Clin.*
 320 *Microbiol. Infect Dis.* 19, 708-710.
- 321
- 322 Real, F., Fernández, A., Acosta, F., Acosta, B., Castro, P., Déniz, S., Orós, J. 1997.
 323 Septicemia associated with *Hafnia alvei* in laying hens. *Avian Diseases* 41, 741-747.
- 324

- 325 Reisner, A, Krogfelt, K.A., Klein, B.M., Zechner, E.L., Molin, S. 2006. *In vitro* biofilm
326 formation of commensal and pathogenic *Escherichia coli* strains: impact of
327 environmental and genetic factors. J Bacteriol. 188, 3572-3581.
- 328
- 329 Ridell, C. 1987. Bacterial hepatitis. In: American Association of Avian Pathologists
330 (Eds.), Avian histopathology, 1st ed., Allen Press, Lawrence, KS, p. 61.
- 331
- 332 Rodriguez LA, Vivas J, Gallardo CS, Acosta F, Barbeyto L, Real F. 1999. Identification
333 of *Hafnia alvei* with the MicroScan WalkAway system. J. Clin. Microbiol. 37, 4186-
334 4188.
- 335
- 336 Rodríguez, L.A., Gallardo, C.S., Acosta, F., Nieto, T.P., Acosta, B., Real, F. 1998.
337 *Hafnia alvei* is an opportunistic pathogen causing mortality in brown trout, *Salmo trutta*
338 L. J. Fish Dis. 21, 365-370.
- 339
- 340
- 341 Rosenberg, M., Gutnick, D., Rosenberg, E. 1980. Adherence of bacteria to
342 hydrocarbons: a simple method for measuring cell-surface hydrophobicity. FEMS
343 Microbiol Lett 9, 29-33.
- 344
- 345 Sharma, R.K., Boro, B.R., Borah, P. 1991. Incidence of caprine pneumonia and
346 associated bacterial species. Indian J. Anim. Sci. 61, 54-55.
- 347
- 348 Smibert, R.M., Krieg, N.R. 1981. Systematics: general characterization. In: Gerhardt,
349 P., Murray, R.G.E., Costilow, R.N., Nester, E.W., Wood, N.A., Krieg, N.R., Phillips,

350 G.B. (Eds.), Manual of methods for general bacteriology American Society for
351 Microbiology, Washington, D.C., pp. 409-443.

352

353 Stanley, N.R., Lazazzera, B.A. 2004. Environmental signals and regulatory pathways
354 that influence biofilm formation. Mol. Microbiol. 52, 917-924.

355

356 Tornadijo, E., Fresno, J.M., Carballo, J., Martin-Sarmiento, R. 1993. Study of
357 *Enterobacteriaceae* throughout the manufacturing and ripening of hard goats' cheese. J.
358 Appl. Bacteriol. 75, 240-246.

359

360 Van Loosdrecht, M.C.M., Lyklema, J., Norde, W., Schraa, G., Zehnder, A.J.B. 1987.
361 The role of bacterial cell wall hydrophobicity in adhesion. Appl. Environ. Microbiol.
362 53, 1893-1897.

363

364 Wang, T.K., Yam, W.C., Yuen, K.Y., Wong, S.S. 2006. Misidentification of a mucoid
365 strain of *Salmonella enterica* serotype *choleraesuis* as *Hafnia alvei* by the Vitek GNI+
366 card system. J. Clin. Microbiol. 44, 4605-4608.

367

368

369

370

371

372

373

374

375

376

377

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.

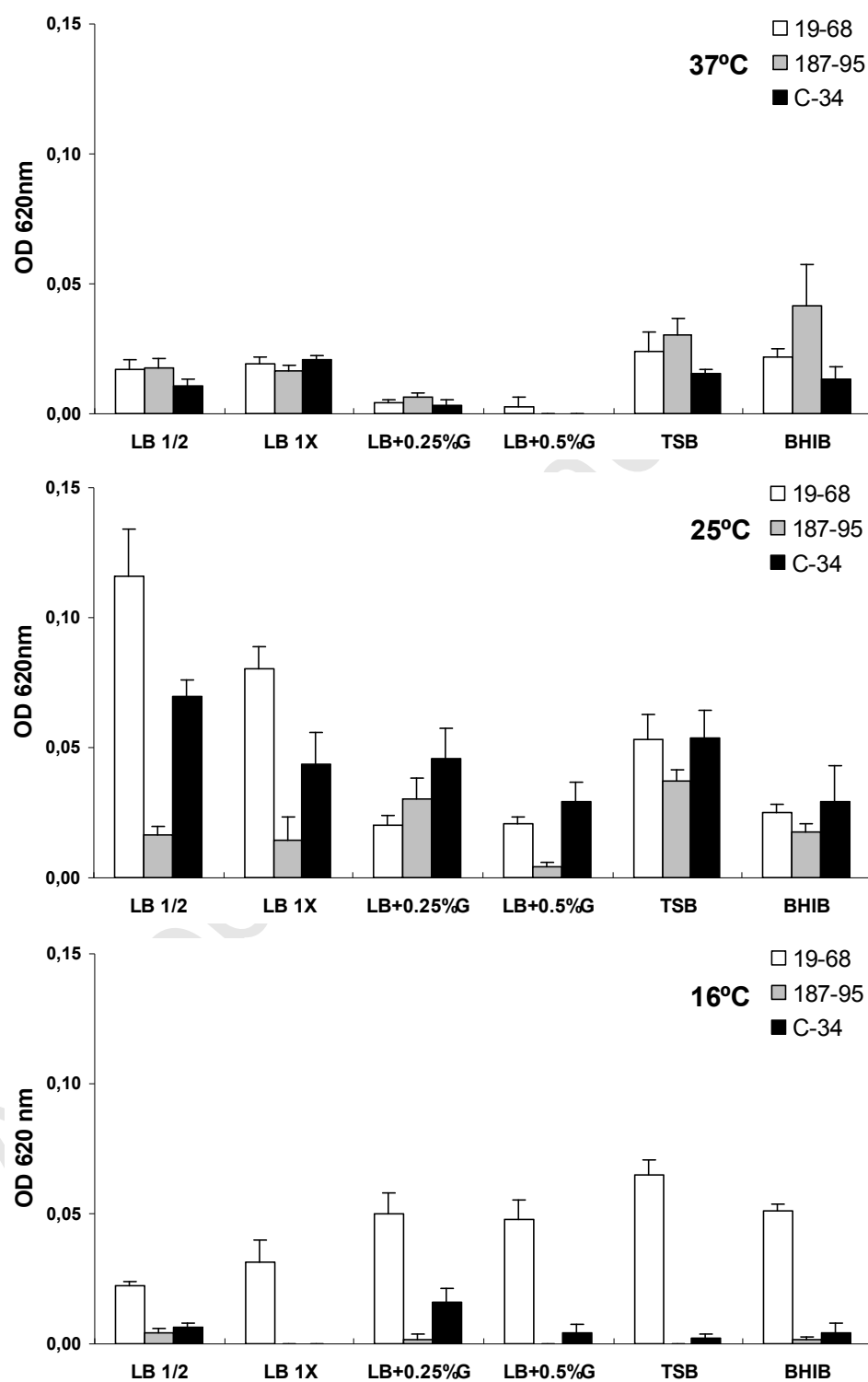


Fig. 2

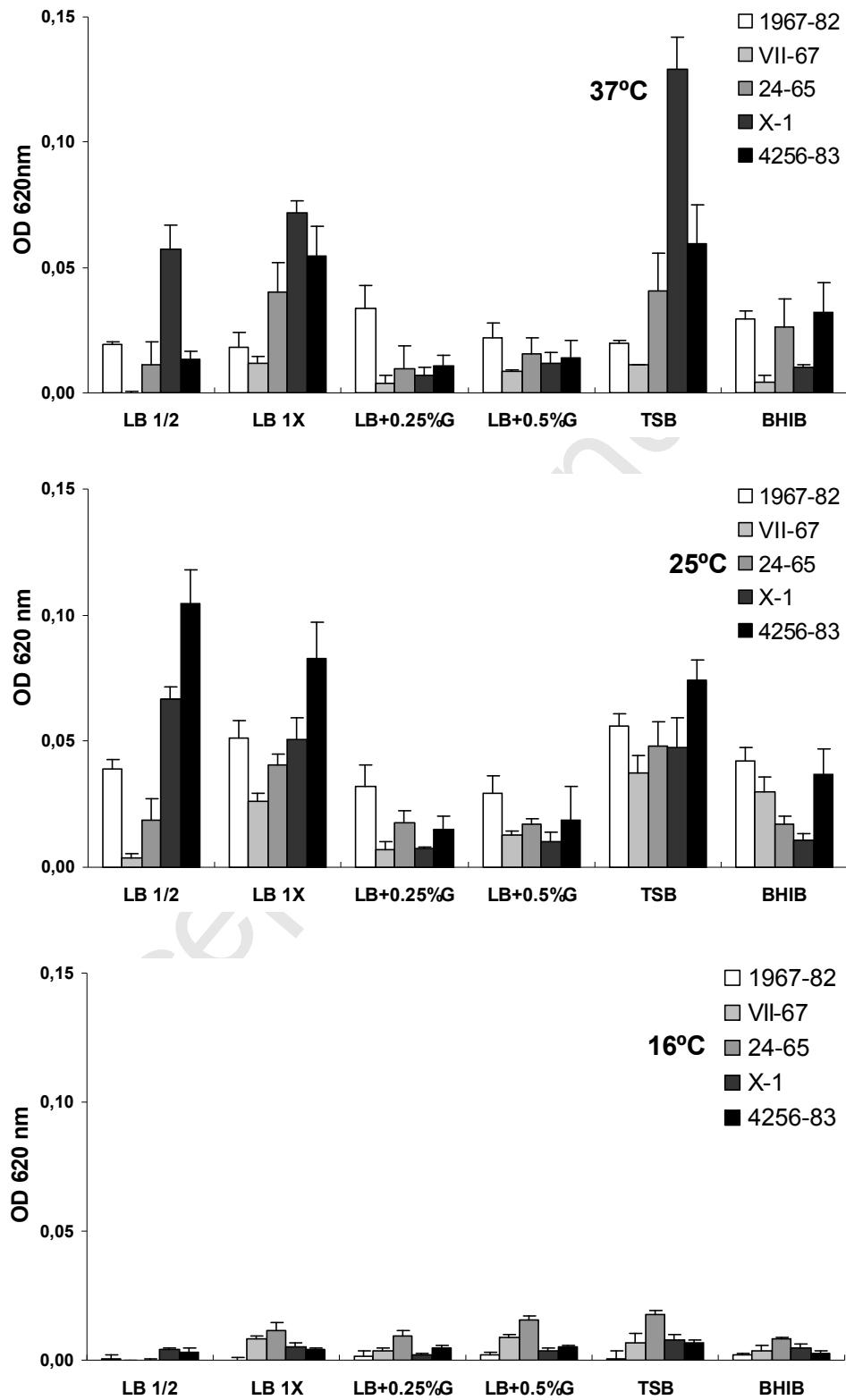


Fig. 3.

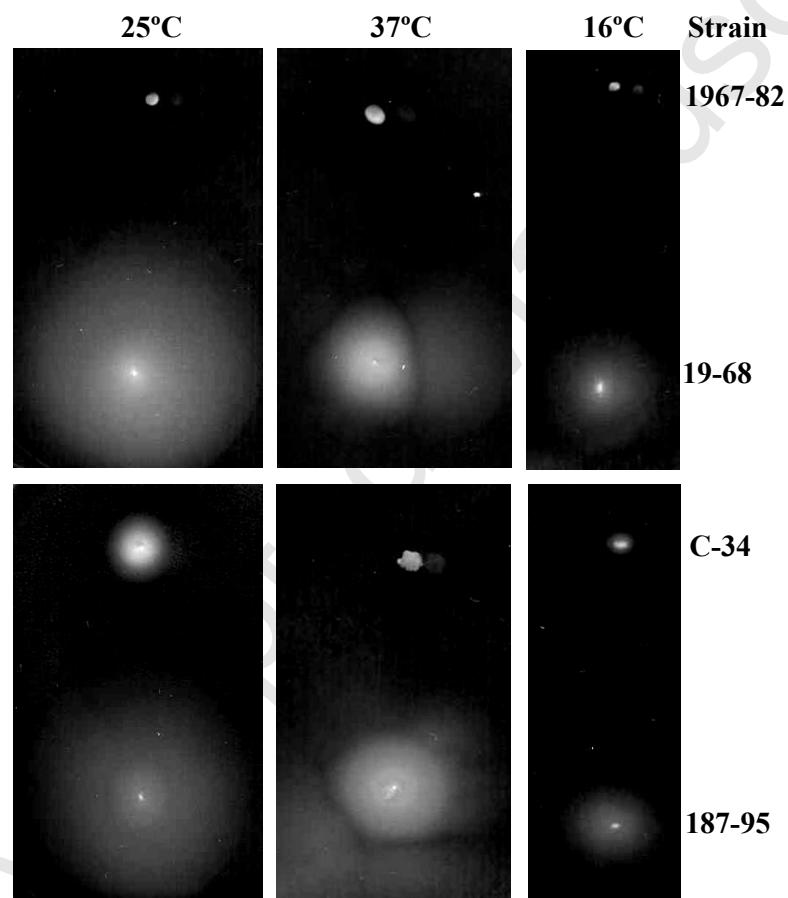


Fig. 4.

