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1 **Phenotypic heterogeneity in attachment of marine bacteria**

2 **towards antifouling copolymers unraveled by AFM**

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25 **Keywords:** Marine bacteria, adhesion, heterogeneity, AFM, copolymers surfaces

26 **Running title:** Phenotypic heterogeneity in bacterial adhesion

27

28 **Summary**

29 Up to recent years, bacterial adhesion have mostly been evaluated at the population level. Single
30 cell level have improved in the past few years allowing a better comprehension of the
31 implication of individual behaviors as compared to the one of a whole community. A new
32 approach using atomic force microscopy to measure adhesion forces between a live bacterium
33 attached via a silica microbead to the AFM tipless cantilever and the surface have been recently
34 developed. The objectives of this study is to examine the bacterial adhesion to a surface
35 dedicated to ship hulls at the population and the cellular level to understand to what extent these
36 2 levels could be correlated. Adhesion of marine bacteria on inert surfaces are poorly studied
37 in particular when substrata are dedicated to ship hulls. Studying these interactions in this
38 context are worthwhile as they may involve different adhesion behaviors, taking place in salty
39 conditions, using different surfaces than the ones usually utilized in the literacy. FRC (fouling
40 release coatings)–SPC (self-polishing coatings) hybrids antifouling coatings have been used as
41 substrata and are of particular interest for designing environmentally-friendly surfaces,
42 combining progressive surface erosion and low adhesion properties. In this study, a hybrid
43 coating has been synthetized and used to study the adhesion of three marine bacteria, displaying
44 different surface characteristics, using microplate assays associated with confocal scanning
45 laser microscopy and, AFM. This study shows that the bacterial strain that appeared to have the
46 weakest adhesion and biofilm formation abilities when evaluated at the population level using
47 microplates assays and CSLM, displayed stronger adhesion forces on the same surfaces at the
48 single cell level using AFM. In addition, one of the strains tested which presented a strong
49 ability to adhere and to form biofilm at the population level, displayed a heterogeneous
50 phenotypic behaviour at the single cell level. Therefore, these results suggest that the evaluation
51 of adhesion at the population level cannot always be correlated with adhesion forces measured

52 individually by AFM and that some bacteria are prone to phenotypic heterogeneity among their
53 population.
54
55 (339 words)

56 **Introduction**

57

58 Little is known on adhesion of marine bacteria on surfaces in particular when they are dedicated
59 to ship hulls. The comprehension of bacterial adhesion on these surfaces should help finding
60 potential environmentally less toxic anti-adhesion or anti-fouling strategies. The specific
61 intrinsic nature of marine bacteria, that are poorly studied and characterized, may modify the
62 type of interactions that can be observed between a cell and its surface, particularly when the
63 interactions take place in marine conditions, making them worthwhile studying. Overall,
64 molecular or cellular mechanisms of bacterial adhesion have been in fact mostly evaluated at
65 the population level but very rarely at the single cell level. Indeed, very few information are
66 factually available on individual behaviors of bacteria regarding adhesion. Due to the
67 development of new single cell level approaches, individual cells can be studied with the
68 purpose of understanding how a single cell behaves as compared to its population of origin and
69 if bacterial cells behave all similarly within a supposedly clonal population, after
70 synchronization in growth culture, or if important behavioral differences exist between them.
71 Recently, the idea that bacterial population could be composed of heterogeneous individuals
72 has emerged, even when coming from a single cell or a group of clonal or genetically identical
73 individuals (Grote et al., 2015; Martins and Locke, 2015). Differential gene expression could
74 explain these phenotypic fluctuations. Some bacterial strains are also more prone to allelic
75 variations than others (Davis and Isberg, 2016). In adhesion studies, AFM approaches have
76 been used to study adhesion forces at the single cell level between a cell and a surface. They
77 have been improved during the past few years, making it possible to study these interactions
78 with alive bacteria (Kang and Elimelech, 2009; Loskill et al., 2012; Beaussart et al., 2013;
79 Beaussart et al., 2014; El-Kirat-Chatel et al., 2014b). Indeed, a new approach using a silica
80 microbead fixed on the tipless cantilever allow the attachment of a single cell that can stay alive

81 during the time of the measurement. These approaches have been proven very useful to decipher
82 adhesion of bacteria such as *Staphylococcus aureus*, *Escherichia coli* toward glass and
83 functionalized surfaces.

84
85 In the marine context, all artificial surfaces immersed in seawater are subjected to the
86 accumulation of marine organisms such as microorganisms and macrofoulers, known as marine
87 biofouling. Current antifouling strategies rely on the widely use of self-polishing coatings
88 (SPC), which releases toxic biocides with a constant rate controlled by the coating erosion
89 process (Yebra et al., 2004). The erosion of the coating is achieved through the hydrolysis of
90 the polymeric binder in seawater making the polymer water-soluble. Fouling Release Coatings
91 (FRC) represent a second type of antifouling coatings, which are able to release organisms
92 settled on the surface while boats are navigating (Lejars et al., 2012). Their efficacy relies on
93 hydrophobicity, low surface energy and low elastic modulus of its poly(dimethylsiloxane)
94 (PDMS) cross-linked matrix, which decreases the adhesion strength of marine organisms and
95 enhance their removal. Despite, the clear environmentally friendly advantage of this antifouling
96 solution, FRCs are inefficient when vessels are docked. During navigation, the coating is able
97 to release the macrofouling but retains a microfouling film (composed mainly of bacteria and
98 diatoms), which is still responsible for 10% of drag resistance (Schultz, 2007). An attractive
99 option in developing such coatings is the synthesis of new polymers which are both
100 hydrolyzable and hydrophobic/low-surface energy materials. Poly(dimethylsiloxane) blocks
101 could be inserted in silylated-based polymers to provide access to a wide variety of materials
102 with tunable hydrophobicity, water resistance and mechanical properties. Bressy and coworkers
103 have synthesized tri-alkylsilylester-based statistical copolymers by conventional radical
104 polymerization and several diblock copolymers using the reversible addition-fragmentation
105 chain transfer (RAFT) polymerization for developing erodible binders for marine antifouling

106 coatings (Bressy and Margailan, 2009; Bressy et al., 2010; Bressy et al., 2014; Lejars et al.,
107 2014). Hybrid copolymers with PDMS blocks or side-chains and silylated side groups have
108 been reported to exhibit surface erosion and hydrophobic surfaces depending on the relative
109 content of the two components (Lejars et al., 2013). These hybrid surfaces displaying SPC and
110 FRC properties have been characterized including for their antifouling efficacy (Duong et al.,
111 2014; Duong et al., 2015).

112

113 In this study, three strains isolated from the Mediterranean sea, presenting different
114 phenotypical traits, have been used to evaluate their ability to adhere on a new antifouling
115 coating dedicated to ship hulls at the population and the cellular level (Brian-Jaisson et al.,
116 2014). TC5 belonging to the *Polaribacter* genus, a non-motile marine bacteria, is the most
117 hydrophobic of the 3 strains according to Microbial Adhesion to Solvents (MATS) assays and
118 has a poor ability to form biofilm on polystyrene when studied in microplates (Brian-Jaisson et
119 al., 2014). TC10 and TC11 are 2 different strains of *Shewanella*, which are overall more
120 hydrophilic and are motile. TC11 is able of a stronger adhesion and a faster capacity to form a
121 biofilm on polystyrene while for TC10, it takes more time to form its biofilm. In this context,
122 adhesion have been tested on an hybrid block copolymer SPC-FRC coating called MC3MB6
123 (PDMS-*b*-p(SiMA-*stat*-BMA)). The results have been compared to its SPC block alone called
124 MB6 (SiMA-*stat*-BMA). In contrast with conventional SPC, MB6 has no biocide but retaining
125 the ability of self-hydrolysis. Both surfaces have been synthesized and their properties
126 characterized similarly as previously done (Duong et al., 2014; Duong et al., 2015). The
127 adhesion of these 3 marine strains on the hybrid coatings have been performed through a
128 microplate assay associated with CLSM and AFM, in order to verify if the adhesion forces
129 measured at the single cell level could be correlated with the evaluation of the population
130 adhesion. Bacterial adhesion has been very rarely evaluated at the same time, at the population

131 and the cellular level to understand to what extent these 2 levels could be correlated for each of
132 the strain.

133

134 **Material and Methods**

135 Substrates

136 Two copolymers based on *Tert*-butyldimethylsilyl methacrylate (SiMA) were synthesized as
137 previously reported (Duong et al., 2014) . Butyl methacrylate was used as co-monomer of SiMA
138 to prepare films without cracking (Table 1). Each copolymer was dissolved in toluene, at a 40–
139 50 wt% solid content, and applied on abraded poly(vinyl chloride) (PVC) substrates with a bar-
140 coater resulting in about 100 μm dried thickness coatings. The surfaces of the samples for the
141 contact angle measurement and for AFM measurements were 25mm x 45 mm and 10 mm x 10
142 mm, respectively. The coated plates were left to dry in the open air for 15 days.

143

144 Characterization methods

145 The number-average molar mass (M_n) and dispersity (\mathcal{D}) of polymers were determined by triple
146 detection size exclusion chromatography (TD-SEC). Analyses were performed on a Viscotek
147 apparatus, composed of a GPC Max (comprising a degasser, a pump and an autosampler) with
148 a TDA-302 (RI refractive index detector, right and low angle light scattering detector at 670
149 nm and viscometer) and an UV detector ($\lambda = 298$ nm). The following columns were used: a
150 Viscotek HHR-H precolumn and two Viscotek ViscoGel GMHHR-H columns. THF was used
151 as the eluent with a flow rate of 1.0 mL min⁻¹ at 30 °C. For each precipitated polymer, the
152 refractive index increment (dn/dc) was determined using the OmniSec software, from a solution
153 of known concentration (ca. 10 mg mL⁻¹) filtered through a 0.2 mm PTFE filter.

154 Differential scanning calorimetric (DSC) measurements were performed on a DSC Q10
155 apparatus from TA Instruments calibrated with indium. Polymer samples weighing 15-20 mg
156 were run at equal heating and cooling rates, $10\text{ }^{\circ}\text{C min}^{-1}$, under a constant stream of nitrogen.
157 The MC3MB6 sample was first scanned from room temperature to $100\text{ }^{\circ}\text{C}$ (PDMS-*block*-
158 P(SiMA-*stat*-BMA)). The sample was then cooled to $-165\text{ }^{\circ}\text{C}$. This temperature was held for 5
159 min to allow the system to attain thermal equilibrium before the second heating scan. The first
160 heating ramp of each sample was discarded for this work. The glass transition temperature (T_g)
161 values were determined as the midpoint between the onset and the end of a step transition using
162 the TA Instruments Universal Analysis 2000 software.

163 Static contact angle measurements were carried out at room temperature using a sessile drop
164 method with a DIGIDROP contact angle meter from GBX Instruments. Two test liquids:
165 deionized water and diiodomethane (Sigma-Aldrich) were used. The liquid drop volume was 1
166 μL and $0.5\text{ }\mu\text{L}$ for water and diiodomethane, respectively. A picture of the liquid drop on the
167 surface was taken 4s after its formation for contact angle measurement. The reported contact
168 angles were an average of five individual measurements in different regions of the same coating
169 ($\pm\sigma$). Surface free energies of the coatings (γ_s) and their dispersive (γ_s^D) and polar components
170 (γ_s^P) were calculated using the Owens-Wendt method. Dynamic contact angle measurements
171 were carried out under ambient conditions by using the dynamic sessile drop technique. A water
172 drop with a volume of around $1\text{ }\mu\text{L}$ is growing on a syringe tip and picked up by the surface.
173 The syringe tip never leaves the liquid drop. The water was inflated and sucked up from the
174 surface and the advancing and receding angles were obtained.

175

176 AFM characterization of the surface

177 AFM measurements were performed on a Nanoscope V controller equipped with a Multimode
178 V Atomic Force Microscope, with a 8610 JVLR type scanner. Tapping mode cantilever probes

179 (RTESP model from BRUKER) were used to show the topography of the supported polymer
180 films and to evaluate their Young modulus values. The system sensitivity and cantilever spring
181 constant k_c are successively determined from force measurements on a rigid sample and from
182 the thermal tune method (Butt et al., 2005), implemented in Bruker Nanoscope (V7.3) software.
183 The topography was initially scanned in tapping mode with a cantilever spring constant around
184 48 N/m and a resonance frequency of ~ 380 kHz. AFM force curves were performed with
185 maximum forces lower than $1.5 \mu\text{N}$ (The slope of the force–displacement approach curve in the
186 linear elastic range gives an apparent stiffness k_{eff} which is directly linked to the sample
187 stiffness k_s knowing the cantilever stiffness (Butt et al., 2005). In the case of a perfectly elastic
188 tip with a spherical end and a homogeneous sample, with no adhesive effects, the Hertz model
189 can give an estimation of Young's modulus from k_s measurement (Butt et al., 2005; Belec et
190 al., 2015). The slope was calculated on the approach curves (between 400 and 450 nm of
191 deflection). The standard deviation is calculated on 7 measurements.

192

193 Microorganisms and growth conditions.

194 Bacterial strains used in this study (TC for Toulon Collection) are listed in Table 4. They were
195 isolated from biofilms formed on inert surfaces immersed in the Mediterranean Sea (bay of
196 Toulon, France, $43^{\circ}06'23''\text{N}$ - $5^{\circ}57'17''\text{E}$) (Camps et al., 2011; Briand et al., 2012). TC strains
197 were grown in Vaatanen nine salt solution (VNSS) at 20°C in a rotary shaker (120rpm)
198 (Mardén, 1985) up to post-exponential phase prior to analysis.

199

200 Adhesion assay on polystyrene

201 Post exponential phase grown cells were centrifuged and resuspended in artificial sea water
202 (ASW). Then $200 \mu\text{L}$ of cells were inoculated at $\text{OD}_{600\text{nm}} 0.3$ in triplicate in black microplates
203 (sterile black PS; Nunc, Fisher Scientific, Illkirch, france). After 24h of incubation at 20°C , the

204 non-adhered bacteria were eliminated by three successive washes (36g.L⁻¹ sterile NaCl
205 solution). The adhered bacteria were stained by both Syto 61 Red and Syto 9 Green fluorescent
206 markers targeting bacterial DNA (Life technology). After 10 min, the excess stain was
207 eliminated by one wash. Fluorescence intensity (FI) was measured using an Infinit 200
208 microplate fluorescence reader (Tecan, Lyon, France). A fluorescent intensity was calculated
209 per well: Fluorescent intensity (FI) = FI average assay / FI average negative control. Three
210 independent assays were done for each strain tested. Same results were found with both stains
211 (data not shown).

212

213 Adhesion assay on copolymers

214 Adhesion assay on copolymers was performed as described for adhesion assay on polystyrene
215 excepted for the following points. PVC coverslips of 13 mm of diameter were coated with the
216 MB6 and MC3MB6 polymers. Each copolymer previously dissolved in toluene, at a 40–50
217 wt% solid content, were applied on PVC coverslips with a bar-coater resulting in about 100 µm
218 dried thickness coatings. Coverslips were inserted in 24 well microplates (sterile transparent
219 PS; VWR) and sterilized 15 minutes with UV. Post-exponential bacterial strains were
220 resuspended in ASW and inoculated at OD_{600nm} 1 in the microplates. After 24h of incubation
221 at 20°C strains were labelled with 5µM of Syto 9 Green fluorescent nucleic acid stain (Life
222 technology). After 10 min, the excess stain was eliminated by 3 washes. Fluorescence intensity
223 (FI) was measured using an Infinit 200 microplate fluorescence reader (Tecan, Lyon, France).
224 A fluorescent intensity was calculated per well: Fluorescent intensity (FI) = FI average assay /
225 FI average negative control. Three independent assays were done for each strain tested.

226

227 CSLM observation

228 The same coverlips were used for the observation of the bacteria on the surfaces using CSLM
229 Zeiss LSM 510. Briefly, the coated coverlips were glued onto a glass slide and covered with
230 prolong antifade (Life technology) and a new glass coverslips. After 48 h drying, the samples
231 were stored at 4°C until use for CSLM observation.

232

233 Atomic force microscopy imaging

234 AFM contact mode images were obtained in air, at room temperature, using a Nanoscope VIII
235 Multimode AFM (Nano Surfaces Business, Bruker Corporation, Santa Barbara, CA), MSCT
236 cantilevers with a nominal spring constant of ~0.01 N/m (calculated with the thermal noise
237 method), and a scanning rate of 2 Hz. One hundred μ l of cell suspension from post-exponential
238 growth phase was put in contact with freshly cleaved mica supports mounted on steel pucks.
239 The samples were incubated for 2 h at 30 °C, gently rinsed in three successive baths of ultrapure
240 water (Elga, purelab), and allowed to dry at 30°C overnight.

241

242 Cell probes

243 For single-bacterial cell force spectroscopy, cell probes were prepared using a recently
244 developed protocol that combines colloidal probe cantilevers and bioinspired polydopamine
245 wet adhesives (Beaussart et al., 2013). Briefly, silica microspheres (6.1 μ m diameter, bangs
246 laboratories) were attached on triangular shaped tipless cantilevers (NP-O10, Microlevers,
247 Bruker Corporation) using UV-curable glue (NOA 63, Norland Edmund Optics). The
248 cantilevers were then immersed for 1 h in a 10 mM Tris Buffer solution (pH 8.5) containing 4
249 mg ml⁻¹ dopamine hydrochloride (99%, Sigma), and dried with N₂ flow. Single bacteria were
250 then attached onto polydopamine-coated colloidal probes using a Bioscope Catalyst AFM

251 (Bruker corporation, Santa Barbara, CA). To this end, 2 μ l of a cell suspension were added to
252 4 ml of ASW solution (pH 8, Sea salts, Sigma) in a glass Petri dish containing MB6 and
253 MC3MB6 substrates. A single probe was brought into contact with an isolated cell for 3 min,
254 and the obtained cell probe was then transferred over a solid substrate for further force
255 measurements. Viability of the attached bacteria was checked using a Live-dead *BacLight*
256 viability kit (Invitrogen, kit L7012) following the manufacturer instructions.

257

258 Single-cell force spectroscopy measurements

259 SCFS measurements were performed at room temperature (20 °C) in in ASW solution pH 8
260 and using a Bioscope Catalyst AFM (Bruker AXS Corporation, Santa Barbara, CA). The
261 nominal spring constant of the colloidal probe cantilever was $\sim 0.06 \text{ N m}^{-1}$, as determined by
262 the thermal noise method. Multiple force-distance curves were recorded on various spots of
263 MB6 and MC3MB6 substrates using a maximum applied force of 250 pN, a contact time of 100
264 ms or 1s, and constant approach and retraction speeds of 1000 nm s^{-1} . For each condition, the
265 interaction forces of 3 bacterial cells from independent cultures were measured and $n > 400$
266 force curves were recorded for each bacteria.

267

268 **Results and discussion**

269

270 **Polymers synthesis and characterization of copolymer surfaces**

271 Well-defined diblock copolymers combining a *tert*-butyldimethylsilyl methacrylate (SiMA)-
272 based block, as hydrolyzable “SPC-type” monomer, with a poly(dimethylsiloxane) (PDMS)
273 block, as hydrophobic, “FRC-type” monomer have been investigated. The synthesis of the
274 PDMS-*b*-p(SiMA-*stat*-BMA) block copolymer called MC3MB6 was achieved from
275 copolymerizations of *tert*-butyldimethylsilyl methacrylate (SiMA) and butyl methacrylate on

276 PDMS macro-RAFT agents. The methodology relies on the synthesis of PDMS monofunctional
277 chain transfer agents easily available in one synthetic step from commercially available
278 hydroxylated PDMSs (Duong et al., 2014). A statistical copolymer P(SiMA-*stat*-BMA) called
279 MB6, with a composition similar to the second block of MC3MB6, has also been prepared
280 (Table 1). As these copolymers might be used in marine environment as coatings, their ability
281 to form films without cracking is required. Good film properties have been displayed for MB6
282 and MC3MB6 due to their low glass transition temperature of 45-46°C corresponding to
283 P(SiMA-*stat*-BMA) block. In the case of MC3MB6, the T_g of the PDMS block (from -127°C
284 to -124°C) was not visible because of a low amount of DMS monomer units within the
285 copolymer (Duong et al., 2014). Surface properties including wetting properties and
286 smoothness have been investigated. Table 2 shows that the water contact angle values increased
287 and the polar component of the surface free energy (γ_s^P) decreased when the PDMS block was
288 added within the copolymer. Taken together these results show that MC3MB6 is more
289 hydrophobic than its MB6 counterpart which could suggest according to the literature a close
290 packing of the pendant methyl groups of the flexible siloxane chain at the film/air interface
291 (Lejars et al., 2012). Tapping-mode AFM analysis shows the topography of the PDMS-based
292 films to be smoother than the MB6 PDMS-free coating (Figure 1). In addition, the flexibility
293 of the PDMS block coming from its low T_g value and the flexibility of the methacrylic block
294 coming from the presence of BMA monomer units provided soft samples. A lower Young's
295 modulus value and a higher indentation were found for the PDMS-based sample (Table 3).
296 Taken together these results show that MC3MB6 surface is softer than MB6 one.
297 When immersing these silylester-based polymers in artificial seawater, the hydrophilic
298 character of the two coating surfaces increased with time as the water contact angle θ_{H_2O}
299 decreases with immersion time (Figure 2). This result is in agreement with the well-known
300 hydrolysis reaction of the hydrophobic silyl ester groups of SiMA units into hydrophilic sodium

301 carboxylate groups in artificial seawater (Bressy and Margailan, 2009). Nevertheless, the
302 surface of the PDMS-based coating MC3MB6 remains more hydrophobic than the MB6 one.

303

304 **Adhesion tests of marine bacteria on polystyrene**

305 Biofilm formation has been previously evaluated in different rich marine media for a number
306 of marine bacteria isolated from the Mediterranean sea (Camps et al., 2011; Brian-Jaisson et
307 al., 2014). Five of these marine bacterial strains (Table S1), which were all isolated from
308 biofilms formed on immersed supports in the bay of Toulon (France), were analyzed for their
309 adhesion ability in artificial sea water (ASW) on polystyrene (Figure 3). Three of them (TC9-
310 TC10 and TC11) belong to *Shewanella* genus. TC5 and TC8 belong respectively to
311 *Polaribacter* and *Pseudoalteromonas* genus (Table S1). All strains except TC5, were able to
312 form a biofilm in laboratory conditions (Brian-Jaisson et al., 2014). In this study, strains
313 exhibited different adhesion patterns on polystyrene after 24h (Figure 3) in a poor medium,
314 artificial sea water (ASW). Bacterial adhesion of TC11 was the strongest. TC8 adhered to
315 polystyrene but fluorescent intensity was 1.6 times less than TC11. Adhesion on polystyrene
316 was weak for TC5 and very weak for the TC10 and TC9 strains. Three profiles based on
317 adhesion on polystyrene and biofilm formation pattern can be identified : i) a weak adhesion
318 profile in ASW with an incapacity to form biofilm in rich media (Brian-Jaisson et al., 2014) for
319 TC5; ii) a strong adhesion on polystyrene with a strong ability to form biofilm in rich media for
320 TC8 and TC11; iii) a weaker ability to adhere on polystyrene in ASW and a slower capacity to
321 form a biofilm in rich media for TC9 and TC10, corresponding thus to an intermediary
322 phenotypic between the 2 first groups. For the following approaches, we therefore chose to
323 work with one strain of each group. TC5 belonging to the *Polaribacter* genus, a non-motile
324 marine bacteria, is the most hydrophobic of the 3 strains according to Microbial Adhesion to
325 Solvents (MATS) assays, has a weak adhesion profile and has a poor ability to form biofilm

326 (Brian-Jaisson et al., 2014). TC10 and TC11 are 2 different strains of *Shewanella*, which are
327 overall more hydrophilic and are motile. TC11 is able of a stronger adhesion and a faster
328 capacity to form a biofilm on polystyrene while for TC10, it takes more time to form its biofilm
329 (Brian-Jaisson et al., 2014).

330

331 **AFM imaging to unravel morphological features of bacteria species.**

332 We used AFM contact mode imaging in air to visualize the general cell topography of TC5,
333 TC10 and TC11. For all strains, bacteria were small rod-shaped, which agree well with
334 observations performed previously (Brian-Jaisson et al., 2014). TC5 and TC10 were about 3.4
335 micrometer long. TC11 seemed to be smaller and was about 2.6 micrometers long (Figure 4).
336 Pili and flagella are major contributors to mobility, adhesion and biofilm formation (Mattick,
337 2002; Telford et al., 2006; Pelicic, 2008; Belas, 2014; Laverty et al., 2014). As suspected
338 flagella were clearly present on the surface of TC10 and TC11, while they were not seen for
339 TC5, previously described as non-motile (Figure 4) (Brian-Jaisson et al., 2014). Furthermore,
340 in few images of TC10, we observed a smaller and thinner structure, which could be pili, on
341 the surface of this strain (Figure 4D, white thin arrow). Overall, pili were more difficult to
342 observe than flagella. It is possible that pili were broken during the preparation of the cells for
343 the AFM observation. Despite the presence of small residues particles most likely coming from
344 the culture medium, spherical particles, which surround TC5 strain seem to be of different
345 nature (Figure 4B, black triangle). We hypothesized that this strain produces outer membrane
346 vesicles (OMV). A wide variety of Gram negative bacteria secrete OMV including marine
347 bacteria such as *Prochlorococcus* or *Shewanella vesiculosa* (Beveridge, 1999; Perez-Cruz et
348 al., 2013; Biller et al., 2014). OMV are implicated in many functions such as bacterial survival,
349 pathogenicity, enzyme delivery and biofilm formation (Beveridge, 1999; Schooling and

350 Beveridge, 2006; Lee et al., 2008; Yonezawa et al., 2009; Baumgarten et al., 2012; van Hoek,
351 2013; Altindis et al., 2014; Murphy et al., 2014; Orench-Rivera and Kuehn, 2016). Taken
352 together, these observations show that the *Polaribacter* TC5 strain presents different features
353 from the 2 *Shewanella* strains as it has no flagella and seems to present OMV at its surface.

354

355 **Evaluation of adhesion on copolymers at the population level**

356 In order to evaluate bacterial adhesion on the hybrid MC3MB6 and its control MB6, these
357 polymers were coated onto round PVC coverslips (as they did not stick well on glass) and
358 inserted in 24 well plates. Glass coverslips, widely used in fluorescence or CLSM microscopy
359 experiments, served as a reference. Bacteria were then left to seed onto the surface for 24 h,
360 washed off to remove non adherent bacteria and then stained using the fluorescent marker
361 Syto9. Because some polymers can present an autofluorescence, a direct observation of the
362 same samples was performed using CLSM. This double approach is rarely undergone when
363 such coatings are used. Figure 5A and B show that for each coating tested, TC11 was the strain
364 displaying stronger adhesive properties, in particular with glass alone. TC11 adhered 2 and 1.5
365 times more than TC5 or TC10 on MB6 or MC3MB6 respectively (Figure 5B). All 3 strains
366 adhered better on glass coverslips than on the polymers (Figure 5A and C), with no significant
367 difference in adhesion between MB6 and MC3MB6 (Figure 5A). These results have been
368 corroborated with the CSLM observation as very few bacteria can be seen on either MC3MB6
369 or MB6 surfaces in sharp contrast with the glass surface (Figure 5C). Taken together, these
370 results suggest that copolymers MB6 and MC3MB6 prevent bacterial adhesion. While the
371 adhesion was overall very weak, TC11 appears to adhere slightly better on these 2 surfaces.
372 MB6 alone, composed of SiMA-*stat*-BMA with self-hydrolysis properties (with no biocide) is
373 self-sufficient for the inhibition of bacterial adhesion in these conditions. The hydrophobic
374 PDMS blocks, do not add in efficacy whichever bacteria studied despite their different

375 hydrophilic surface properties (Brian-Jaisson et al., 2014). As previously described,
376 hydrophobic/hydrophilic interactions can be easily overcome by the presence of extracellular
377 appendages and covalent bindings, in particular in the stage of the “irreversible adhesion”
378 (Garrett et al., 2008). Bacteria can adapt to their environment i.e. the presence of a surface, very
379 rapidly by, temporarily and in coordinated manner, specifically expressing numbers of proteins
380 anchored in the membrane or being part of the extracellular appendages that can modify and
381 overcome such interactions.

382

383 **Single-cell adhesion force analysis.**

384 The results of the previous experiments reflect the behavior of the bacteria at the population
385 level. In order to understand how each bacterium behaves on these surfaces at the single cell
386 level, AFM was used in single-cell force spectroscopy (SCFS) mode (Helenius et al., 2008;
387 Muller et al., 2009) to quantify the adhesive properties of individual TC5, TC10 and TC11
388 bacterial cells towards surfaces MB6 and MC3MB6 (Beaussart et al., 2013). Briefly, a colloidal
389 probe cantilever coated with polydopamine bioadhesive was used to pick up single cells without
390 altering their viability (assessed using the Live-dead *BacLight* viability kit) and to measure
391 force-distance curves between the bacterium and the surfaces MB6 and MC3MB6 (Figure 6).
392 The 3 bacteria tested stayed alive during the course of the experiment.

393 We first used SCFS to investigate the adhesion force of single cells towards MB6 surfaces and
394 the effect of contact time between cells and substrates. Figure 7 shows the adhesion force and
395 rupture length histograms, together with representative force curves, obtained between TC5
396 (Figure 7A and B), TC10 (Figure 7C and D) and TC11 (Figure 7E and F) cells and MB6
397 surfaces at short (Figure 7A, C, and E) and prolonged (Figure 7B, D, and F) contact times.
398 Consecutive force curves were recorded on different spots of the substrate and no changes were
399 observed regarding the general features of the curves, indicating the cells were not damaged

400 and cell surface properties were not altered by force measurements. Cell from independent
401 cultures were analysed and generally yielded similar behaviour although sometimes one cell
402 showed differences (Figure 7C, D, and F) that we attribute to heterogeneity of the bacterial
403 population. This phenotypic heterogeneity was less pronounced for TC5, whereas it was more
404 obvious for TC11 whether at a short or long contact time. At short contact time, the adhesion
405 frequency of TC5 cells on MB6 surfaces was ~30-35 % with adhesive force curves presenting
406 force of 50-400 pN and rupture distances of 100-900 nm (Figure 7A). Prolonged contact time
407 (1 s) led to increased adhesion frequencies (75-90 %), increased adhesion forces (from 300 to
408 2400 pN) and rupture lengths in the same range as at short contact time, yet with higher
409 frequencies of short rupture distances (Figure 7B). At short contact time, most force distance
410 curves presented multiple well-defined individual peaks of 50-100 pN (Figure 7A, right
411 histogram inset). According to previous observations, we attribute those multiple peaks
412 signatures with flat regions preceding each peak to type IV pili interaction with MB6 surfaces
413 (Touhami et al., 2006; Biais et al., 2010). The absence of such structures on bacterial images
414 (Figure 4A and B) suggest that TC5 pili are fragile, short or could be retracted during sample
415 drying. On 1 s contact time force curves, similar peaks were sometimes observed but the short
416 distances interaction at higher forces (>300 pN) seemed to govern the adhesion of cell on MB6
417 surfaces. Such first large force and short distance adhesive events phenomenon could be
418 attributed to the outer membrane surface property itself that needs longer contact time for
419 interaction rather than appendages or adhesives molecules that would lead to longer rupture
420 lengths. Analysis of TC10 cells led to similar results, still with few differences. Increasing the
421 contact time did not significantly increases the adhesion frequency or the range of forces. As
422 for TC5, force curves signatures obtained for TC10 suggested type IV pili interaction and this
423 conclusion was confirmed by AFM images (Figure 4D). For TC11 cells, the adhesion frequency
424 did not increase with contact time. However, adhesion forces of some cells significantly

425 increased up to 10 nN (Figure 7F). These high forces corresponded to short rupture distances
426 peak suggesting strong hydrophobic interaction between the cell surface and the MB6 substrate.
427 At short contact time, force curves frequently showed a first adhesive event with sometimes a
428 sawtooth pattern (Figure 7E, inset in right histogram, upper curve). This first peak may
429 correspond to cell surface proteins interacting with MB6 surface and strengthened in force and
430 number under prolonged contact time. On short contact time curves, although no pili were
431 detected on bacterial images (Figure 4E and F), peaks following the initial adhesive event
432 presented signatures that could be attributed to pili and as for TC5, we hypothesize that those
433 pili were fragile, short or could be retracted on image samples. Force curves obtained after
434 prolonged contact time revealed sawtooth pattern with regular peaks and long rupture distances.
435 Based on previous observations, these signatures could correspond to proteins interacting with
436 the surface and containing multiple repeats that are unfolded upon bacterial pulling from the
437 substrate (Alsteens et al., 2009; Beaussart et al., 2014; El-Kirat-Chatel et al., 2014a).

438

439 The MC3MB6 surface was used similarly to evaluate the impact of the surface chemistry
440 change on bacterial adhesion of TC5, TC10 and TC11 cells. Figure 8 shows the adhesion force
441 and rupture length histograms, together with representative force curves, obtained between TC5
442 (Figure 8A and B), TC10 (Figure 8C and D) and TC11 (Figure 8 E and F) cells and surfaces
443 MC3MB6 at short (Figure 8A, C, and E) and prolonged (Figure 8B, D, and F) contact times.
444 TC5 cells presented high frequency adhesion towards surface MC3MB6 (more than 70 % at
445 short contact times and about 100 % at prolonged contact times). Increasing contact time
446 resulted in higher adhesion force (from 300-2000 pN to 1200-4800 pN). Force curves recorded
447 for TC5 on surface MC3MB6 presented large initial force peaks followed by smaller forces that
448 may correspond to stretching of cell surface molecules. Compared to results obtained on
449 surfaces MB6, force curves recorded on surface MC3MB6 rarely presented pili signature,

450 suggesting that TC5 pili are mostly involved in interaction with surface MB6 and that the
451 interaction with surface MC3MB6 is governed by the cell wall itself together with surface
452 adhesive molecules. TC10 cells presented slightly similar adhesive profile on surface MC3MB6
453 and surface MB6. Its adhesion to both surfaces is lower in term of frequency and force than the
454 adhesion of TC5. Based on force curves shape, this adhesion seems to be mainly controlled by
455 pili at short contact time (small peaks at long distance and visualisation of pili on image Figure
456 4) and at longer contact time, the cell adhesion through pili seems to be reinforced by cell wall
457 and surface molecule (large initial peak and sawtooth pattern of molecules unfolding). TC11
458 cells were slightly more adhesive to surface MC3MB6 than what was observed for surfaces
459 MB6. Still, the interaction looks similar with almost no pili signature but rather protein
460 unfolding and large initial peaks suggesting that TC11 adhere mainly through cell wall
461 hydrophobicity and adhesive macromolecules containing repeated domains unfolded upon
462 pulling. Phenotypic heterogeneity in adhesion was also more obvious on MC3MB6 for TC11
463 than for the 2 other strains, whether on short or long contact time.

464

465 To validate the specificity of the measured adhesion forces and rule out the possibility of
466 artefact associated with the cell probe preparation, a control experiment was performed using a
467 1s contact time (Figure S2). Use of polydopamine-coated probes instead of bacterial probes led
468 to a major reduction of adhesion frequency and no signatures similar to what we observed for
469 cells were present. This control indicates that the adhesive events measured above reflect the
470 interactions between bacteria and coatings.

471

472 Taken together, these results show first that TC5 was the most adhesive of the 3 strains on both
473 surfaces in terms of frequency and presented large adhesion forces, in particular on the
474 hydrophobic surface, MC3MB6, while TC10 showed a weaker adhesion on both surfaces with

475 adhesion forces averaging 1000 or 1200 pN. The influence of surface chemistry is mostly
476 observed for TC5. This comes in contrast with the results found at the population level, as TC11
477 was the bacteria that adhered the most efficiently on polystyrene, glass as well as on MB6 and
478 MC3MB6, even though adhesion on the antifouling surfaces was overall very low (Figure 4).
479 Second, different extracellular components seem to be involved in the 3 strains adhesion on the
480 surfaces. Short distance interactions at higher forces govern adhesion of TC5 and TC10 on the
481 surfaces (Dufrene, 2015). Adhesion seems to be controlled by pili, cell wall on MB6 (and
482 MC3MB6 for TC10) and by cell wall and stretching of surface molecules on MC3MB6. TC11
483 adheres mainly through cell wall hydrophobicity and adhesive macromolecules containing
484 repeated domains unfolded upon pulling. Third, some TC11 cells were slightly more adhesive
485 to both surfaces (with long rupture distances) than others. While TC5 and in a lesser extent
486 TC10 showed a more homogenous response towards the surfaces, TC11 presented
487 heterogeneous adhesion profiles toward both surfaces, with some bacterial cells presenting
488 weak adhesion forces and some of them presenting very strong ones. Phenotypic heterogeneity
489 within a population, which corresponds to the expression of substantial phenotypical
490 differences by individuals when they are in a similar context, is thought to allow better chance
491 of survival for the population as a whole entity. A subpopulation may be then better equipped
492 to face stressful situations and settle in new environmental niches. This heterogeneity can come
493 from variations of gene expression at the single cell level but also from allelic variations (Davis
494 and Isberg, 2016). Some bacteria are more susceptible to genetic variations than others (for
495 instance the ones undergoing phase variation). These phenomena have been described for
496 instance in biofilm with the apparition of persisters as well as in relation with QS dependent
497 mechanisms due to highly heterogeneous gene expression at a single cell level (Grote et al.,
498 2015). This is most likely a widespread phenomenon, which just started to be highlighted in the
499 literature with the development of single cell approaches, even though this variability may

500 differs from a bacterium to another. This emerging evidence of phenotypical variability need to
501 be studied more precisely at the molecular and cellular level in order to understand how these
502 variations can make a subpopulation adapt and survive in an environmental niche. It is possible
503 that TC11 fits to this description and can present at the population level a better fitness when
504 facing a new environment than TC5 or TC10. Phenotypic heterogeneity, and differential
505 bacterial-bacterial collaborative interactions involved in biofilm formation could explain the
506 reason why, despite a weaker adhesion at the single cell level, TC11 is able to form more biofilm
507 than TC5 including in ASW (data not shown). Further studies would be required using, for
508 instance, other single cell techniques, such as the newly developed single cell RNA-seq,
509 allowing a broader vision of the variability to confirm these results (Davis and Isberg, 2016).

510

511

512 **Supporting Information**

513 Additional supporting information may be found in the online version of this article at the
514 publisher's web-site:

515 Detailed experimental procedures

516 Table S1

517 Figure S1

518 Figure S2

519

520 **Conflict of interests**

521 The authors declare that they have no conflict of interests.

522

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651

652 **Illustrations and figures**

653

654 **Tables and figures legends**

655 Table 1. Characteristics of diblock and statistical copolymers prepared by RAFT
 656 polymerization of SiMA and BMA from PDMS-macro RAFT agent at 70 °C in toluene.

Polymer	% mol	% mass	%	M_n ($g \cdot mol^{-1}$)*	D^*
	(DMS/SiMA/BMA)	(DMS/SiMA/BMA)	vol_{PDMS}		
MC3MB6	31/10/59	18/16/66	19	59,700	1.1
MB6	0/14/86	0/18/82	0	49,500	1.1

657 * assessed by triple detection size exclusion chromatography (TD-SEC)

658

659 Table 2. Wetting properties of the coating surfaces.

Polymer	Contact angle (°)				Surface energies ($mJ \cdot m^{-2}$)*		
	θ_{H_2O}	σ	$\theta_{CH_2I_2}$	σ	γ_s	γ_s^D	γ_s^P
MC3MB6	101.9	1	71.5	0.6	22	20.9	1.1
MB6	91.3	0.5	64.3	4.6	26.7	23.3	3.3

660 *using Owens-Wendt's method (Owen and Wendt, 1969)

661

662 Table 3. Young's modulus at the coating surfaces measured by AFM.

Polymer	E (MPa)	Indentation (nm)
MC3MB6	73 ± 4	207 ± 4
MB6	88 ± 7	145 ± 5

663

664

665 **Figure 1. Height AFM images of A) MB6 and B)MC3MB6**

666 Rms is 2.3 ± 0.10 and 5.4 ± 1 for MC3MB6 and MB6 respectively with Ra of 8.6
667 ± 0.6 and 14 ± 7 respectively.

668

669 **Figure 2. Evolution of the static water contact angle of MC3MB6 and MB6 with ASW**
670 **immersion time.**

671

672 **Figure 3. Evaluation of the adhesion of five marine bacterial strains on polystyrene**

673 After 24h of incubation at 20°C , bacteria were stained by Syto 9 Green and fluorescent intensity
674 was measured as a representation of bacterial adhesion. Bars represent the standard deviation
675 obtained from three independent measures.

676

677 **Figure 4. Imaging bacterial surface in air.** AFM deflection and height (insets) images
678 (contact mode) of post-exponentially growing cells that were directly deposited on mica and
679 dried prior analysis. Vertical cross sections taken in the height images (dashed lines) are also
680 shown to emphasize sizes of cellular structures.

681

682 **Figure 5. Adhesion of TC5, TC10 and TC11 on MB6 and MC3MB6 surfaces.** Adhesion
683 was first measured at the population level in 24 well plates. A and B are different statistical
684 analysis displays of the same measurement. After 24h of incubation at 20°C , bacteria were
685 stained by Syto 9 green fluorescent nucleic and fluorescent intensity was measured using a
686 TECAN microplate reader as a representation of bacterial adhesion. Bars represent the standard

687 deviation obtained from three independent measures. Statistical significance was accepted at
688 $p < 0.05$. * for $p < 0.05$, **for $p < 0.01$ and *** for $p < 0.001$. C show the CSLM visualization of
689 adhered bacteria on the surfaces. The same coverslips were used in the microplate assay and in
690 CSLM. Glass coverslips were used as a control.

691

692 **Figure 6. The use of a microbead for single-cell force spectroscopy analysis.** A. Principle
693 of single cell force spectroscopy with tip less cantilevers modified with colloidal beads and
694 coated with polydopamine to attach a single bacteria (green) and probe it towards surfaces. B.
695 Optical microscope image of a single bacterium attached to the colloidal cantilever probes
696 documenting that the cell is properly located and alive (green fluorescence).

697

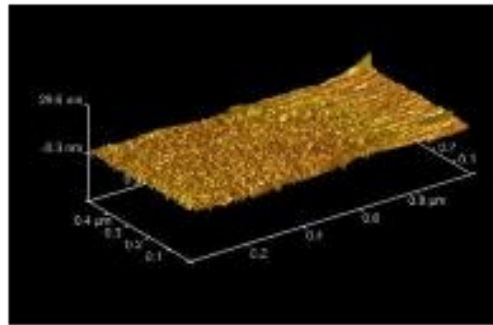
698 **Figure 7. Single-cell force spectroscopy analysis on surface MB6.** Adhesion force (left) and
699 rupture length histograms with representative retraction force profiles (right) obtained by
700 recording multiple force–distance curves between single TC5 (A and B), TC10 (C and D) or
701 TC11 (E and F) bacteria and surface MB6 at short (100 ms, A, C and E) or prolonged (1s, B,
702 D, F) contact times. Black, red, and blue colors represent results from three cells from
703 independent cultures ($n > 400$ force–distance curves for each cell).

704

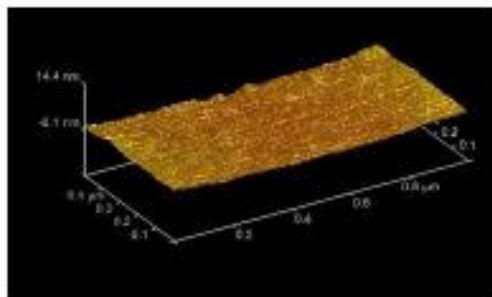
705 **Figure 8. Single-cell force spectroscopy analysis on surface MC3MB6.** Adhesion force (left)
706 and rupture length histograms with representative retraction force profiles (right) obtained by
707 recording multiple force–distance curves between single TC5 (A and B), TC10 (C and D) or
708 TC11 (E and F) bacteria and surface MC3MB6 at short (100 ms, A, C and E) or prolonged (1s,
709 B, D, F) contact times. Black, red, and blue colors represent results from three cells from
710 independent cultures ($n > 400$ force–distance curves for each cell).

711

A.



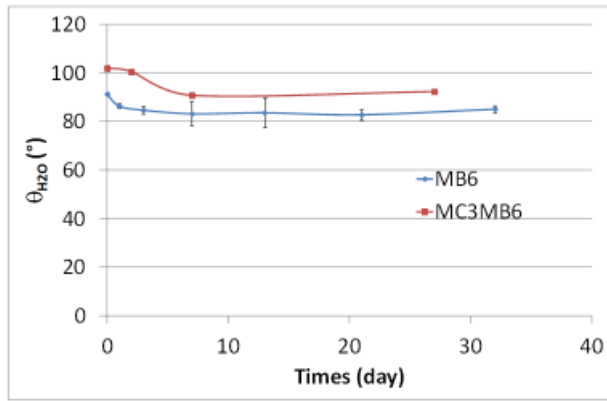
B.

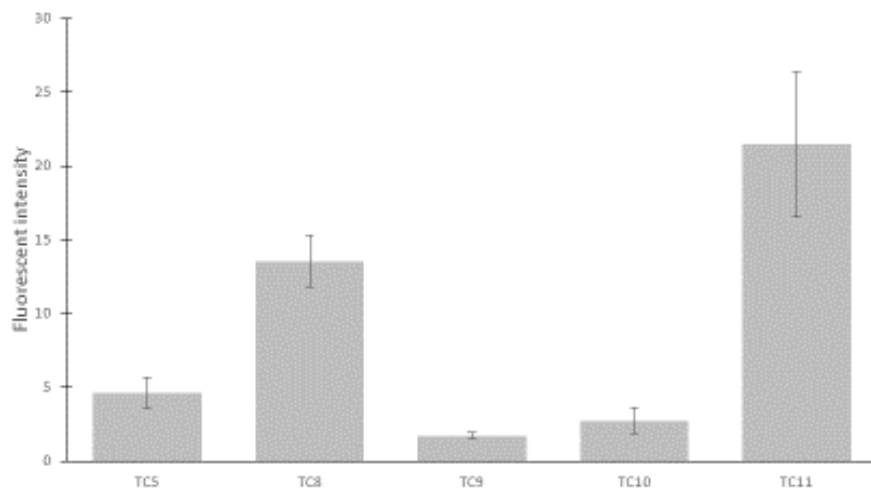


712

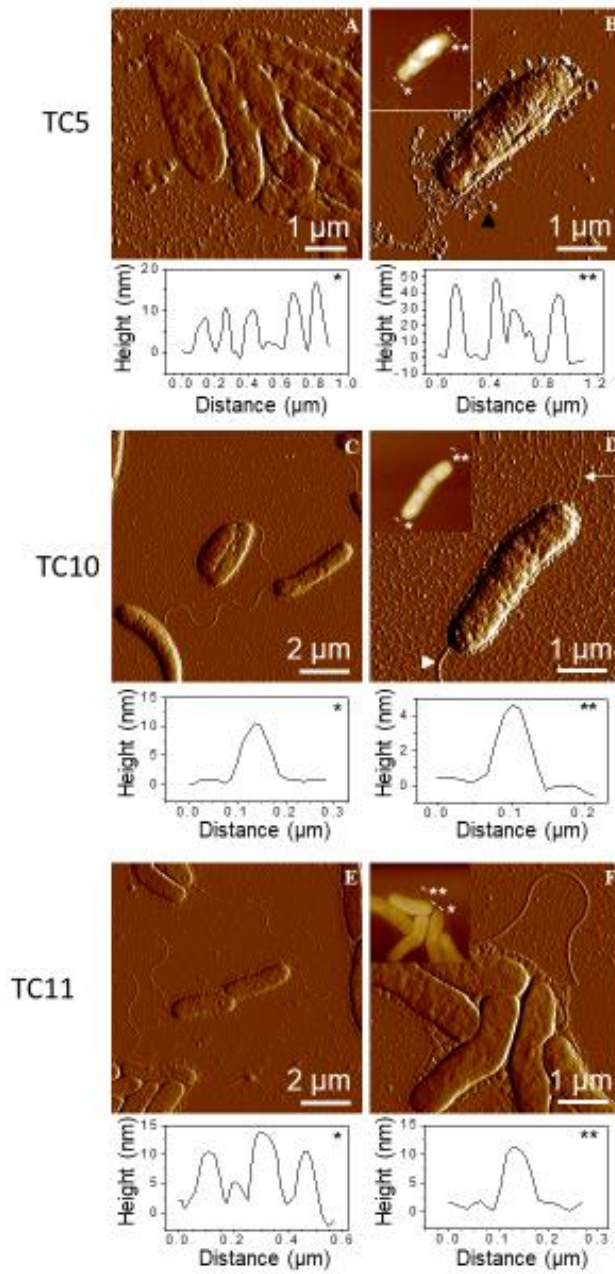
713

El-Kirat-Chatel et al. 2017 Figure 1.

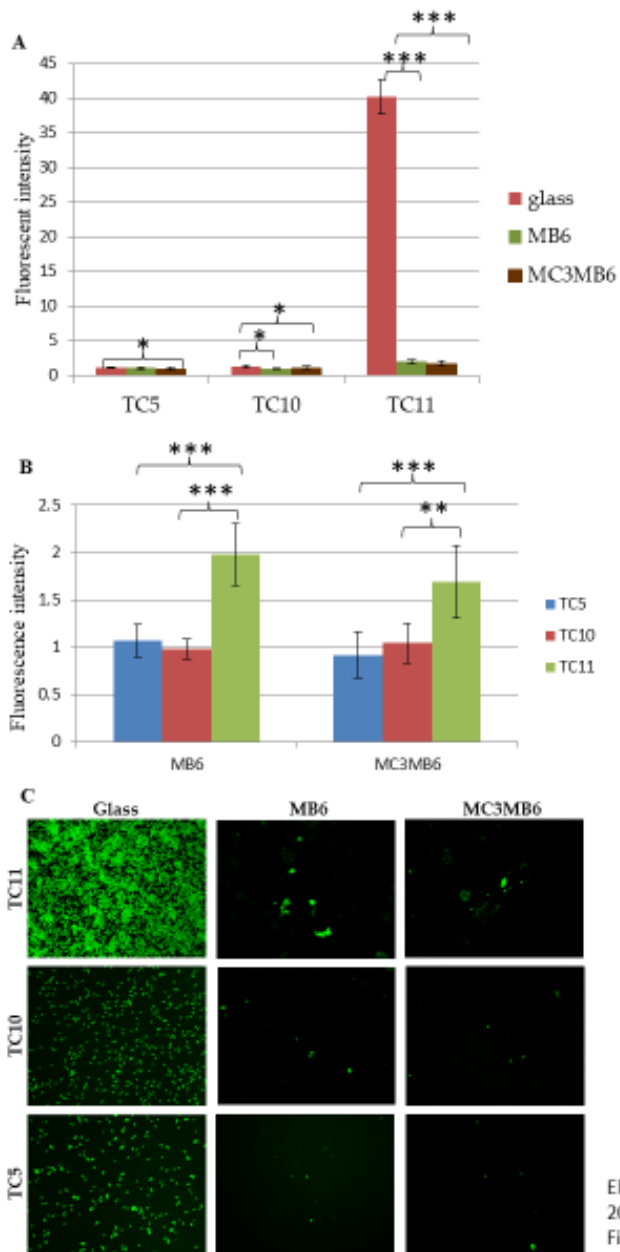


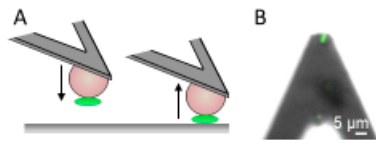


El-Kirat-Chatelet *et al.* 2017 Figure 3.

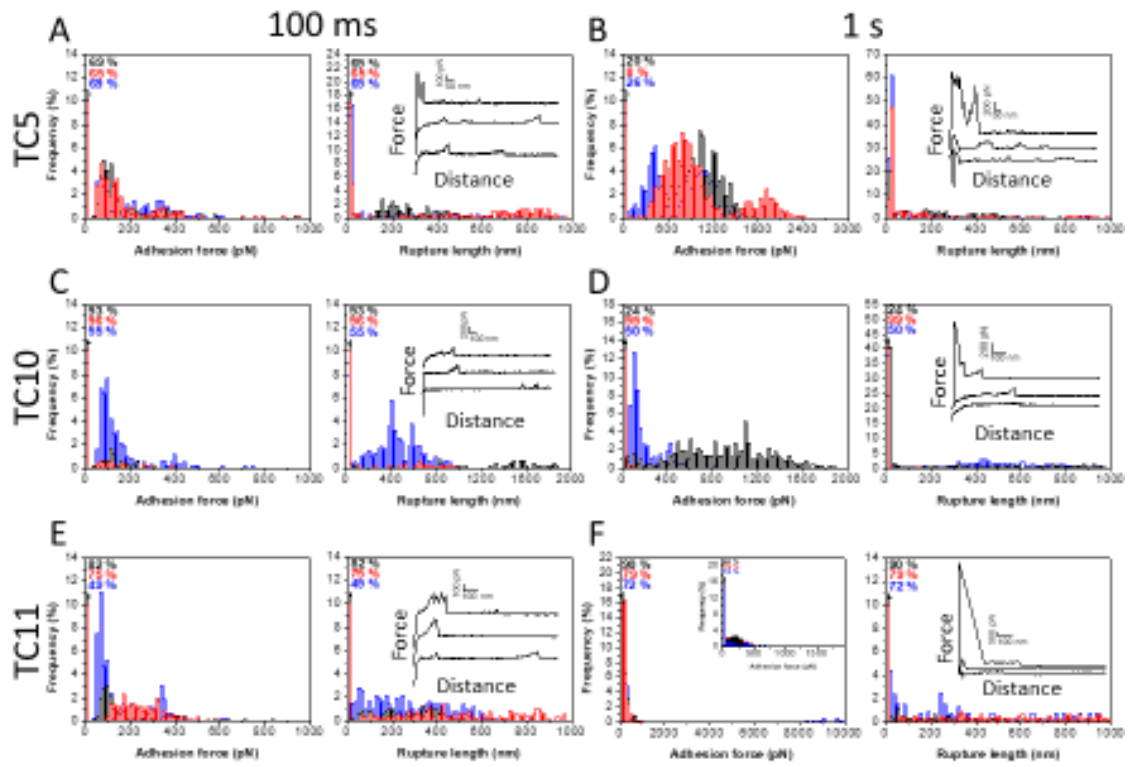


El-Kirat-Chatel *et al.*
2017 Figure 4.



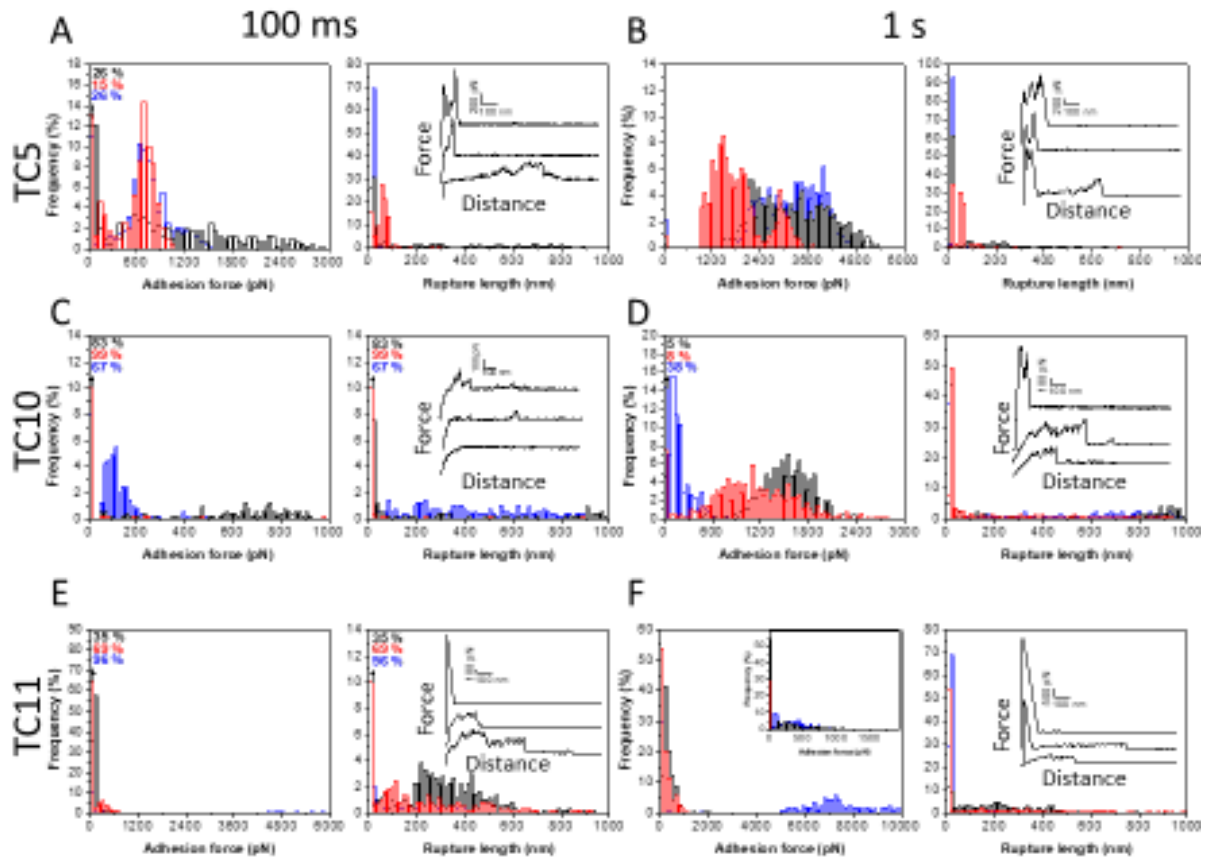


Surface MB6 (p(MASI-Co-BMA))

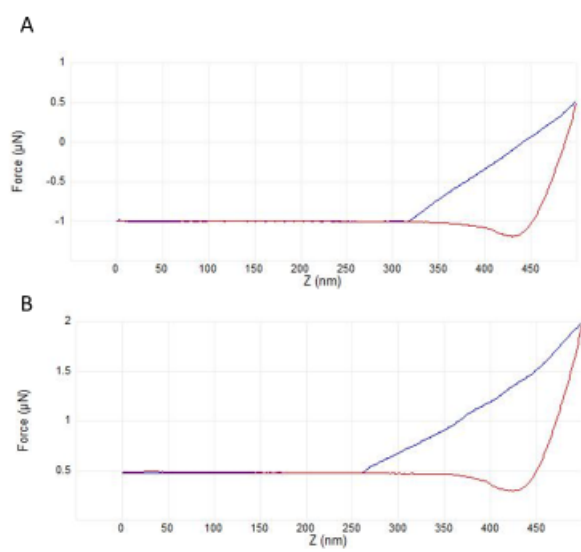


El-Kirat-Chatel *et al.* 2017 Figure 7.

Surface MC3MB6 (PDMS-b-P(MASI-Co-BMA))

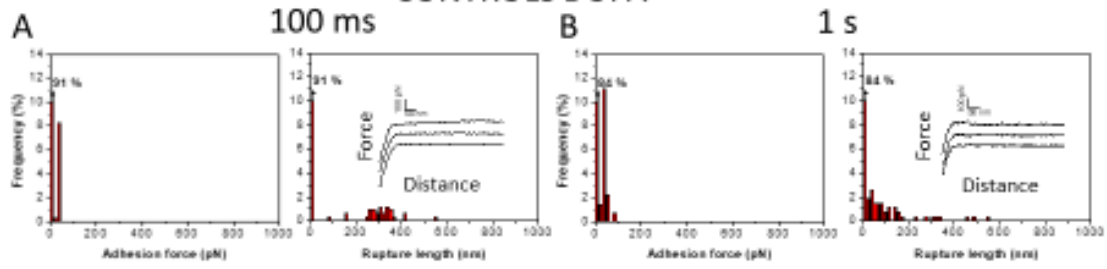


El-Kirat-Chatel *et al.* 2017 Figure 8.



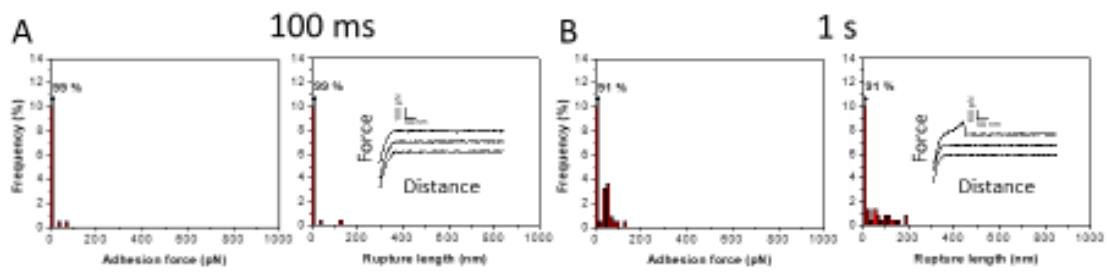
MB6 surface (P(SIMA-*stat*-BMA))

CONTROLS DOPA



MC3MB6 surface (PDMS-*b*-p(SIMA-*stat*-BMA))

CONTROLS DOPA



El-Kirat-Chatel *et al.* 2017 Figure S2.