

1 **Title:**

2 **Type IV pilus shapes a ‘bubble-jet’ pattern opposing spatial**
3 **intermixing of two interacting bacterial populations**

4 **Running title:**

5 **Type IV pilus opposes spatial intermixing**

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21

Abstract

22 Microbes are social organisms that commonly live in sessile biofilms. Spatial patterns
23 of populations within biofilms can be an important determinant of community-level
24 properties. The best-studied characteristics of spatial patterns is spatial intermixing of
25 different populations. The specific levels of spatial intermixing critically contribute to
26 how the dynamics and functioning of such communities are governed. However, the
27 precise factors that determine spatial patterns and intermixing remain unclear. Here,
28 we investigated the spatial patterning and intermixing of an engineered synthetic
29 consortium composed of two *Pseudomonas stutzeri* strains that degrade salicylate via
30 metabolic cross-feeding. We found that the consortium self-organizes across space to
31 form a previously unreported spatial pattern (referred to here as a ‘bubble-jet’ pattern)
32 that exhibits a low level of intermixing. Interestingly, when the genes encoding for
33 type IV pili were deleted from both strains, a highly intermixed spatial pattern
34 developed and increased the productivity of the entire community. The intermixed
35 pattern was maintained in a robust manner across a wide range of initial ratios
36 between the two strains. Our findings show that the type IV pilus plays a role in
37 mitigating spatial intermixing of different populations in surface-attached microbial
38 communities, with consequences for governing community-level properties. These
39 insights provide tangible clues for the engineering of synthetic microbial systems that
40 perform highly in spatially structured environments.

41

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Importance

43 When growing on surfaces, multi-species microbial communities form biofilms that
44 exhibit intriguing spatial patterns. These patterns can significantly affect the overall
45 properties of the community, such as enabling otherwise impermissible metabolic
46 functions to occur, as well as driving the evolutionary and ecological processes acting
47 on communities. The development of these patterns is affected by several drivers,
48 including cell-cell interactions, nutrient levels, density of founding cells and surface
49 properties. The type IV pilus is commonly found to mediate surface-associated
50 behaviors of microorganism, but its role on pattern formation within microbial
51 communities is unclear. Here we report that in a cross-feeding consortium, the type
52 IV pilus affects the spatial intermixing of interacting populations involved in pattern
53 formation, and ultimately influences overall community productivity and robustness.
54 This novel insight assists our understanding of the ecological processes of
55 surface-attached microbial communities and suggests a potential strategy to engineer
56 high-performance synthetic microbial communities.

57

58 In addition to the planktonic lifestyle, microorganisms also form intricate multispecies
59 communities on surfaces (1, 2). These surface-attached communities play important
60 roles in ecosystem processes (3), pollutant removal (4) and human health (5).
61 Biofilms are spatially well-organized, with different populations interacting with each
62 other, arranging themselves non-randomly across space, and ultimately developing
63 well-organized spatial patterns (referred to as ‘spatial self-organization’ hereafter (6,
64 7)). Spatial patterns of a community reflect the distribution of different populations
65 across the habitat, and this distribution profoundly influences the interactions that
66 occur among these populations. For example, spatial mixing of different interacting
67 cells (referred to as a ‘mixed pattern’ thereafter) benefits their metabolic exchanges (8)
68 and alleviates antibiotic stress (9). On the other hand, spatial demixing (referred to as
69 ‘segregated pattern’ thereafter) stabilizes intransitive interactions among
70 antibiotic-producing, -sensitive and -resistant species (10), and also protects cells
71 from contact-dependent killing by its competitors (11). These outcomes can
72 eventually be magnified to determine community-level properties, such as overall
73 community productivity (8), resistance to invaders (12, 13), and robustness to initial
74 conditions (7, 14) .

75 Recently, several abiotic and biotic factors have been reported to influence the
76 intermixing level of spatial patterns. For instance, genetic surfing during range
77 expansion generally demixes populations during growth, resulting in a segregated
78 pattern (15). However, the degrees of intermixing can be increased by increasing the
79 nutrient levels (16), increasing the density of founding cells (17), the presence of

80 physical objects (18) and promoting metabolic interactions (19, 20). As a result, the
81 community will self-organize into a more intermixed pattern. In addition, several
82 studies reported cell appendages, such as Type IV pilus, play an essential role in
83 microbial colonization on surface (21). On the one hand, Type IV pilus mediates
84 twitching motility of bacterial cells (21). On the other hand, it also contributes to
85 stabilizing interactions between cells and the abiotic surface (22), as well as
86 facilitating cell-to-cell adhesion (23, 24), which is required for aggregate formation.
87 Nevertheless, whether this functional cell appendage impacts the intermixing level of
88 the formed spatial patterns still remains to be elucidated.

89 Here, we explored a selection of factors potentially affecting spatial self-organization
90 of a well-defined two-strain consortium using the strains *Pseudomonas stutzeri*
91 AN0010 and *P. stutzeri* AN0001. While strain AN0010 degrades salicylate into the
92 intermediate catechol, strain AN0001 further degrades catechol to pyruvate and
93 acetyl-CoA (Figure 1A). We previously showed that when paired, these two strains
94 engage in a cross-feeding interaction in the presence of salicylate (25).

95 To investigate the spatial self-organization of this community, we cultured the
96 community on an agarose surface using salicylate as the sole carbon source. Based on
97 findings from previous studies, we expected our cross-feeding community to
98 self-organize into a highly mixed pattern (14, 19, 20, 26). Surprisingly, we observed
99 instead a segregated pattern, where the cells of strain AN0010 formed bubble-like
100 structures inside the colony, with cells of strain AN0001 subsequently surrounding
101 these bubbles (Figure 1B; Figure S1A). During range expansion, cells of strain

102 AN0010 expanded from these ‘bubble’ structures, similar to the cells being ‘jetted’
103 outside of the bubbles to form the expanding sectors. We therefore refer to this
104 previously undescribed spatial pattern as the ‘bubble-jet’ pattern. Our analysis using
105 three-dimensional confocal microscopy showed that cells of strain AN0010 assembled
106 in the ‘bubble’ structure, exhibiting a ‘bowl’-like geometrical morphology (Figure
107 S1B). Further analysis of the fluorescence intensities of the two genotypes showed
108 that cells of strain AN0001 were mostly distributed around the bubble formed by
109 strain AN0010 (Figure S1C), suggesting that the metabolic interaction between the
110 two populations still necessitated that the two populations were located in close
111 spatial proximity.

112 To quantitatively test whether the levels of mixing between the two populations
113 within the ‘bubble-jet’ pattern are lower than the previously reported patterns
114 developed by other cross-feeding consortia, we calculated the intermixing indexes of
115 all these patterns (26) (see Supplementary Information S1.5 for details). We found
116 that the intermixing levels of the ‘bubble-jet’ pattern are significantly lower than those
117 generated by cross-feeding consortia performing toluene degradation (14)
118 (Mann–Whitney test, $p = 1.07e-23$) and denitrification (26) (Mann–Whitney test, $p =$
119 $2.64e-28$; Figure 1C). In addition, previous studies reported that spatial intermixing in
120 those cross-feeding communities was reduced when the strains become metabolically
121 independent (14, 19, 20, 26). We thus set out to test whether eliminating
122 metabolic cross-feeding in our synthetic community also reduce the intermixing levels
123 of the ‘bubble-jet’ pattern. We cultured our synthetic community on an agar surface

124 using pyruvate as the sole carbon source. Pyruvate constitutes one of the final
125 products of the salicylate degradation pathway, and is directly utilized by both strains
126 for growth (Figure 1A). When the two strains directly competed for this limited
127 resource, a clear segregated pattern formed (Figure 1B). However, our analysis of this
128 pattern suggested that the ‘bubble-jet’ pattern formed in the ‘cross-feeding’ scenario is
129 even less mixed than the pattern formed in the ‘competition’ scenario (Figure 1C;
130 Mann–Whitney test, $p = 4.61e-9$), in disagreement with previous observations (14, 26)
131 (Figure 1B-C). Moreover, we also tested whether the ‘bubble-jet’ pattern is observed
132 regardless of the magnitude of known key drivers, for example, nutrient levels (16)
133 and density of founder cells (17). These investigations indicated that although these
134 factors quantitatively affected the detailed morphology of the pattern, alteration of
135 these factors failed to qualitatively affect the development of the ‘bubble-jet’ pattern
136 (Figure S2-S3).

137 It has previously been reported that cell appendages, such as type IV pilus and
138 flagellum, are critically involved in the formation of *Pseudomonas* biofilms (22, 27).
139 To investigate whether these cell appendages are also involved in the formation of our
140 ‘bubble-jet’ pattern, we introduced loss-of-function deletions in genes encoding key
141 proteins involved in pilus and flagellum assembly of both strains (Figure S4).
142 Interestingly, while the deactivation of flagellar genes did not change the development
143 of the ‘bubble-jet pattern’. In comparison, deactivation of the type IV genes encoding
144 pilus caused the ‘bubble-jet’ pattern to disappear and significantly increased the
145 spatial intermixing of the two interacting populations in the developed pattern (Figure

146 2A-B; Mann–Whitney test, $p = 3.96e-27$). The mixed pattern that formed also better
147 resembled the ones that developed in previous similar studies ((14, 26); Figure 2 A-B;
148 Mann–Whitney test of the intermixing indexes: $p = 0.026$ compared with the pattern
149 formed by the toluene-degrading community, and $p = 0.017$ compared with that of the
150 denitrification community), and showed higher intermixing compared to the pattern
151 formed by the same community in the ‘competition’ scenario (Mann–Whitney test, p
152 = $6.06e-17$). To test whether the disappearance of the ‘bubble-jet’ pattern only
153 requires the pili mutation in a single strain, we examined the patterns formed by
154 mixing the Δ pili mutant of one strain to the wild-type of the other strain. We found
155 that the ‘bubble-jet’ pattern completely disappeared once the pili of strain AN0010
156 were knocked out (Figure S5A). Moreover, the size of ‘bubbles’ significantly reduced
157 when the pili mutant of AN0001 was mixed with the strain AN0010 (Figure S5D-E).
158 Together, these results strongly suggest that the presence of type IV pilus is a
159 determining factor in the formation of the to ‘bubble-jet’ pattern and controls the
160 intermixing level of the spatial pattern.

161 To investigate whether increased intermixing following removal of type IV pili
162 influences community-level properties, we compared the biomass of the colonies
163 developed by the communities composed of wild type strains and the Δ pili mutant
164 strains. Intriguingly, although the two communities grew similarly in liquid culture
165 (25), colonies developed by the Δ pili mutants produced more biomass than the wild
166 type strains (Figure 2C, unpaired two-tailed Student's t-test, $p = 0.011$; Figure S5C).
167 This result suggested that Δ pili mutants of the two strains interact better with each

168 other than the wild type strains, possibly due to the spatial proximity to each other in
169 the more mixed pattern, leading to increased productivity at the community level. We
170 next tested the robustness of community structure and productivity to the initial strain
171 ratio, which was previously reported to constitute a key feature of the cross-feeding
172 community (14, 19). Despite the fact that the two communities exhibited similar
173 robustness to the initial strain ratio in liquid cultivation (Figure 2D; see Table S1 for
174 variation comparisons), we found that on agarose surface, the community composed
175 of pili mutants was more robust to the initial conditions than the community that
176 formed the ‘bubble-jet pattern’ (Figure 2E; Table S1). Together, these results implied
177 that removing the pili increased the overall productivity, and renders the community
178 for robust against varied initial ratios between the two strains.

179 Several studies based on microscopic investigation directly observed that
180 *Pseudomonas* cells harboring Type IV pilus could migrate along the surface and
181 gather together by recruiting adjacent and homologous cells, resulting in the
182 formation of multicellular aggregates (22, 28). Therefore, we hypothesize that the
183 observed ‘bubble’-like structures in our study may be derived from pilus-mediated
184 formation of cell aggregates. However, this hypothesis requires further testing on the
185 single-cell level.

186 Here, we found that a cross-feeding community self-organized into a previously
187 unknown ‘bubble-jet’ pattern in the presence of pili structures, which opposes spatial
188 mixing of the different populations involved. Our findings also demonstrated that the
189 reduced spatial mixing is associated with a decrease in community productivity and

190 robustness. In conclusion, our findings add critical insights to our current
191 understanding of the ecology of surface-attached microbial communities. In addition,
192 our results strongly suggest a potential strategy to engineer artificial communities
193 with optimized spatial patterns – engineer the pili of the interacting strains to
194 modulate interspecific distances in a surface-attached community, and thus we can
195 promote its performance.

196

197

198 **Competing Interests**

199 The authors declare that they have no conflict of interest.

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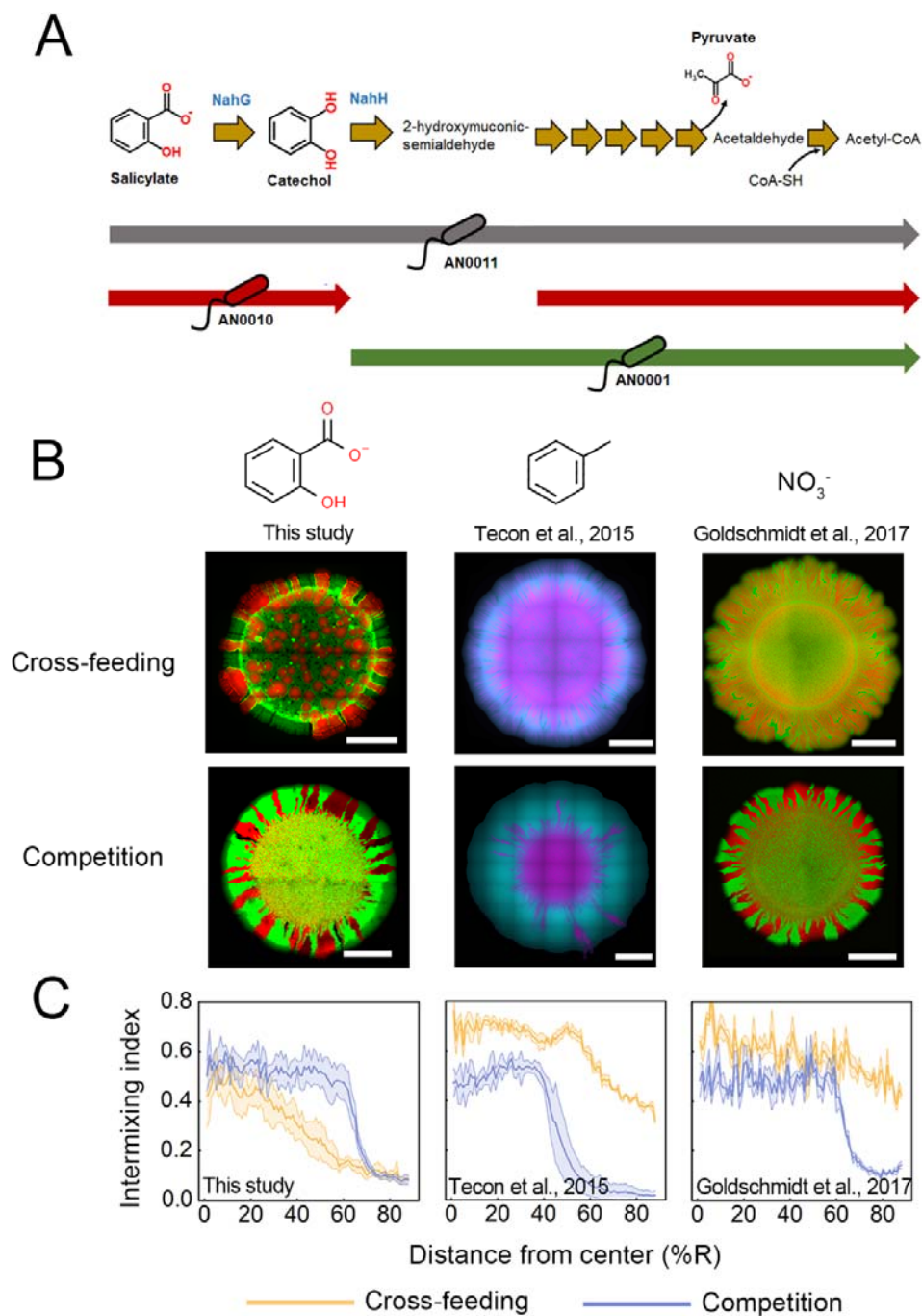
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215 **Figures**



216

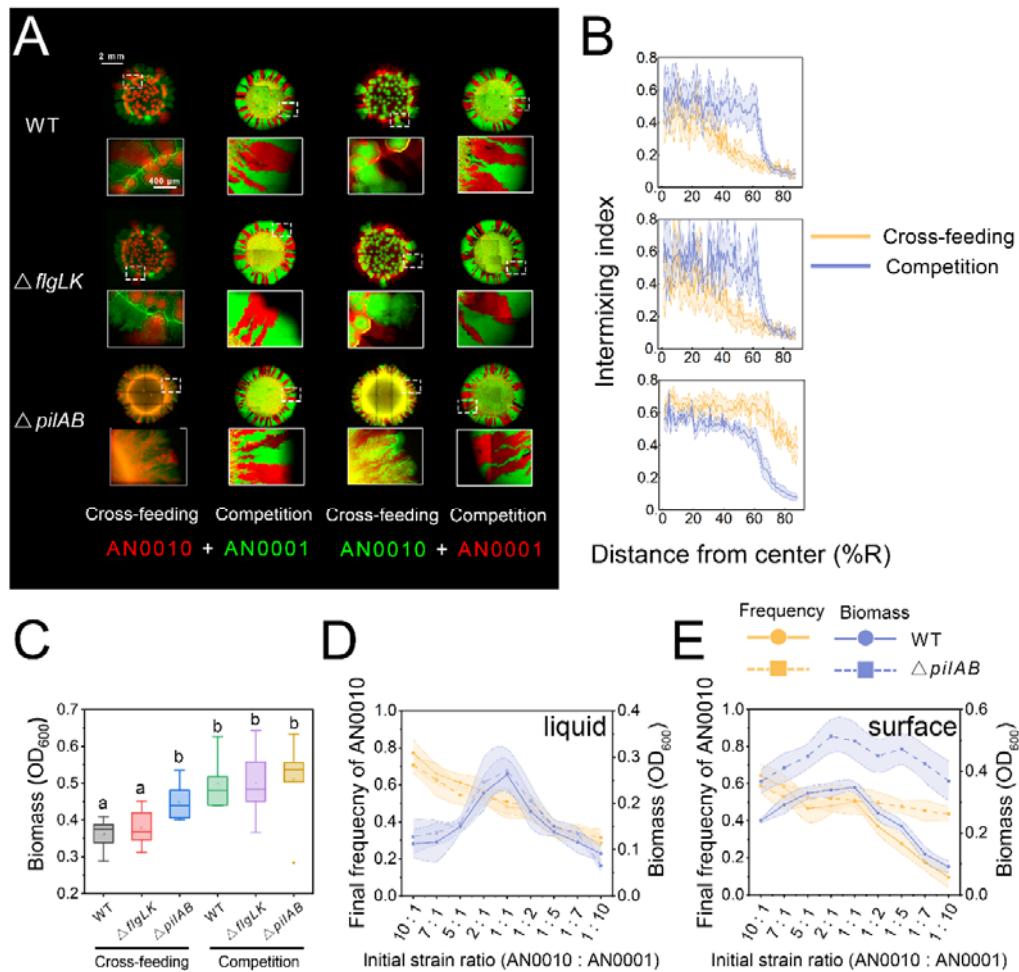
217 **Figure 1** The salicylate-degrading community self-organized into a ‘bubble’ jet

218 pattern characterized by lower intermixing level. (A) Pathway of salicylate

219 degradation in strain *P. stutzeri* AN0011, as well as partial pathways carried out by

220 strain AN0010 and strain AN0001. (B) Representative colony patterns developed by
221 our salicylate-degrading community, as well as patterns developed by the similar
222 cross-feeding communities previously constructed. Overlay fluorescence images of
223 these patterns are shown. For the patterns formed by our salicylate-degrading
224 community (left), strain AN0010 was tagged with mCherry (shown as pseudo-color
225 Red), while strain AN0001 were labeled with eGFP (shown as pseudo-color Green).
226 Shown in the middle panels are the patterns developed by a toluene-degrading
227 cross-feeding community built by Tecon (14) *et al.* (2015), in which strain *P. putida*
228 PpF4 (tagged with eGFP and shown as cyan) degrades toluene to 3-methylcatechol,
229 before it being transformed to acetate and pyruvate by strain *P. putida* PpF107 (tagged
230 with mCherry and shown in magenta). Shown in the right panels are the patterns
231 developed by a denitrification cross-feeding community built by Goldschmidt (26) *et*
232 *al.* (2017), in which strain *P. stutzeri* A1603 (tagged with eCFP and shown as green)
233 converts NO_3^- to NO_2^- , and strain *P. stutzeri* A1602 (tagged with mCherry and shown
234 as red) further reduces NO_2^- to N_2 . For all three synthetic communities, pattern
235 formation assays in ‘Cross-feeding’ scenario (supplying salicylate, toluene or NO_3^- in
236 the media, respectively) and ‘Competition’ scenario (supplying pyruvate, benzoate as
237 the sole carbon source, or grow two complete NO_3^- degraders together on LB agar
238 surface). The scale bar in each image corresponds to 1 mm. (C) Analysis of
239 intermixing indexes of these patterns. Higher values indicate higher levels of local
240 spatial intermixing of the two strains. These values were assessed through image
241 analysis following the protocol modified from a previous study (26) (see

242 Supplementary Information 1.5 for details). To obtain the patterns formed by our
243 salicylate-degrading community, six experimental replicates were performed. Images
244 of the patterns formed by the toluene-degrading community were published (14),
245 courtesy of Dr. Robin Tecon. In addition, the microscopic images of the patterns
246 formed by denitrification community were obtained by performing three replicated
247 pattern formation assays following a protocol previously reported (26).
248



249

250 **Figure 2** Type IV pili are required for formation of the ‘bubble’ structures, while
251 flagella are dispensable. (A) Images showing that the colony patterns formed by
252 co-culturing the wild type strain of AN0010 and AN0001 (top), their flagellum-mutant
253 strains (middle), as well as their pili-mutant strains (bottom) on agarose surface.
254 Patterns obtained in both ‘Cross-feeding’ scenario (supplying salicylate as the sole
255 carbon source) and ‘Competition’ scenario (supplying pyruvate as the sole carbon
256 source) were shown. Alternative fluorescence labelling was used to eliminate
257 potential effects caused by expression of different fluorescent proteins. Typical
258 morphology of colony edges (five-time zoom). Images were obtained after 120-h

259 incubation. (B) Analyses of intermixing index of these patterns. (C) Biomass analysis
260 of colonies. Lower-case letters indicate significant differences between these
261 conditions at 0.05 level (unpaired, two-tailed, Student's t-test). (D-E) The alterations
262 of the salicylate-degrading community composed of the wild type strains (solid lines),
263 as well as the community composed of the pili-mutant (dashed lines), against the
264 initial strain ratios between the two populations. We cultured these communities
265 initiated with nine different strain ratios. After incubation (144-h for liquid cultivation
266 and 120-h for cultivation on agarose surface), final community structures (yellow line)
267 and biomass (blue line) were analyzed. In liquid cultivation, both communities
268 exhibited similar alterations in community structures and the biomass against different
269 strain ratios (D). However, when the communities grew in agarose surface, for the
270 community composed of pili-mutant strains, the final ratio of the two strains
271 converged to similar values ($\approx 1:1$) regardless of initial strain ratio (E). In comparison,
272 the final strain ratio for the community composed of wild type strains exhibited larger
273 variations. Furthermore, the initial strain ratio also showed smaller effects on the final
274 productivity (biomass) of the pili-mutant community than that of the wild-type
275 community. These results strongly suggested that the community formed by pili
276 mutants is more robust against any fluctuations in the initial conditions. For each
277 initial strain ratio, six replicated experiments were performed. See Table S1 for
278 quantitative comparison of variations in different conditions.
279

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