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Author(s)	Okubo, Torahiko; Matsushita, Mizue; Nakamura, Shinji; Matsuo, Junji; Nagai, Hiroki; Yamaguchi, Hiroyuki
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1 ***Acanthamoeba* S13WT relies on its bacterial endosymbiont to backpack human**
2 **pathogenic bacteria and resist *Legionella* infection on solid media**

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5 Torahiko Okubo¹, Mizue Matsushita¹, Shinji Nakamura², Junji Matsuo¹, Hiroki Nagai³,
6 and Hiroyuki Yamaguchi^{1*}

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8
9 ¹Department of Medical Laboratory Science, Faculty of Health Sciences, Hokkaido
10 University, North-12, West-5, Kita-ku, Sapporo 060-0812, Japan

11 ²Division of Biomedical Imaging Research, Juntendo University Graduate School of
12 Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan

13 ³Department of Microbiology, Gifu University School of Medicine, Yanagido 1-1, Gifu,
14 Gifu 501-1194, Japan

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17 Running title: Backpacking of bacteria by amoebae

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20 *Corresponding author:

21 Hiroyuki Yamaguchi

22 Department of Medical Laboratory Science, Faculty of Health Sciences, Hokkaido
23 University, Nishi-5 Kita-12 Jo, Kita-ku, Sapporo, Hokkaido 060-0812, Japan

24 Tel: +81-11-706-3326

25 Fax: +81-11-706-3326

26 E-mail: hiroyuki@med.hokudai.ac.jp

27

28

29 E-mail addresses

30 Torahiko Okubo: t.okubo@hs.hokudai.ac.jp

31 Mizue Matsushita: all-around@eis.hokudai.ac.jp

32 Shinji Nakamura: shinji-n@juntendo.ac.jp

33 Junji Matsuo: matsuo@hs.hokudai.ac.jp

34 Hiroki Nagai: hnagai@gifu-u.ac.jp

35 Hiroyuki Yamaguchi: hiroyuki@med.hokudai.ac.jp

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38 **Summary**

39 Soil-borne amoeba *Acanthamoeba* S13WT has an endosymbiotic relationship with an
40 environmental *Neochlamydia* bacterial strain. However, regardless of extensive
41 experiments in liquid media, the biological advantage of the symbiosis remained elusive.
42 We therefore explored the role of the endosymbiont in predator-prey interactions on
43 solid media. A mixed culture of the symbiotic or aposymbiotic amoebae and
44 GFP-expressing *Escherichia coli* or *Salmonella* Enteritidis was spotted onto the center
45 of a LB or B-CYE agar plate pre-inoculated with a ring of mCherry-expressing
46 *Legionella pneumophila* (*Legionella* “wall”). The spread of the amoebae on the plate
47 was assessed using a fluorescence imaging system or scanning electron microscopy. As
48 a result, in contrast to the aposymbiotic amoebae, the symbiotic amoebae backpacked
49 these GFP-expressing bacteria and formed flower-like fluorescence patterns in an
50 anticlockwise direction. Other bacteria (*Pseudomonas aeruginosa* and
51 *Stenotrophomonas maltophilia*), but not *Staphylococcus aureus*, were also backpacked
52 by the symbiotic amoebae on LB agar, although lacked the movement to anticlockwise
53 direction. Furthermore, in contrast to the aposymbiotic amoebae, the symbiotic amoebae
54 backpacking the *E. coli* broke through the *Legionella* “wall” on B-CYE agar plates.
55 Thus, we concluded that *Acanthamoeba* S13WT required the *Neochlamydia*
56 endosymbiont to backpack human pathogenic bacteria and resist *Legionella* infection on
57 solid agar.

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60 **Keywords:** *Acanthamoeba*, bacterial endosymbiont, *Neochlamydia*, backpack,

61 *Legionella*, solid media

62

63 **Introduction**

64 Free-living *Acanthamoeba* species cause keratitis or granulomatous amoebic
65 encephalitis in humans (De Jonckheere, 1991; Marciano-Cabral and Cabral, 2003;
66 Schuster and Visvesvara, 2004; Khan, 2006; Visvesvara *et al.*, 2007), and are readily
67 isolated from a wide range of natural and built environments (soil, river water, domestic
68 tap water, seawater, pond water, and dust) where they are an important predator of
69 bacteria (Rivera *et al.*, 1987; Sawyer, 1989; Ettinger *et al.*, 2003; Kilic *et al.*, 2004;
70 Górnik and Kuzna-Grygiel, 2004; Tsvetkova *et al.*, 2004; Lorenzo-Morales *et al.*,
71 2005a). Like many other amoebae, *Acanthamoeba* often harbor endosymbiotic bacteria.
72 Generally, approximately 20–25% of environmental amoeba isolates contain
73 endosymbionts, indicating that endosymbiosis occurs ubiquitously among free-living
74 amoebal species (Fritsche *et al.*, 1993; Horn and Wagner, 2004; Thomas *et al.*, 2006;
75 Heinz *et al.*, 2007; Schmitz-Esser *et al.*, 2008). It is therefore relevant to investigate the
76 stable interaction between endosymbionts and their host amoebae to better understand
77 the evolution of symbiosis and the survival mechanisms of endosymbionts.

78 With this in mind, we previously isolated several environmental *Acanthamoeba*
79 strains infected with endosymbiotic bacteria from natural and built environments,
80 including soil, hot spring water, and hospital floors. These strains have proven useful as
81 models for understanding host-parasite relationships (Matsuo *et al.*, 2010; Nakamura *et*
82 *al.*, 2010; Sampo *et al.*, 2014; Fukumoto *et al.*, 2016). Among them, soil-borne
83 *Acanthamoeba* strain S13WT, harboring environmental non-pathogenic chlamydial
84 strain *Neochlamydia* S13, was of particular interest for several reasons (Matsuo *et al.*,
85 2010). First, although the endosymbiont does not cause amoebal lysis and lacks

86 transferability to other amoebae, it has an amoebal infection rate of 100% (Matsuo *et al.*,
87 2010; Nakamura *et al.*, 2010). Second, while the aposymbiotic amoebae grow well, the
88 symbiotic amoebae show growth restriction (Okude *et al.*, 2012). Third, amoebae
89 harboring the *Neochlamydia* endosymbiont can resist *Legionella*, a natural pathogen of
90 amoebae (Ishida *et al.*, 2014). Thus, the presence of the bacterial endosymbiont
91 obviously hinders in the growth of the host amoebae, and despite extensive experiments
92 in liquid media (Matsuo *et al.*, 2010; Nakamura *et al.*, 2010; Sampo *et al.*, 2014;
93 Fukumoto *et al.*, 2016; Okude *et al.*, 2012; Ishida *et al.*, 2014), the biological advantage
94 of maintaining the endosymbiont remains to be fully understood.

95 Therefore, in the present study, we explored the role of the endosymbiont in
96 predator-prey interactions using a simple experiment on agar plates more closely mimic
97 natural surface conditions rather than in liquid media. For the first time, we were able to
98 show that the symbiotic amoebae could backpack human pathogenic bacteria
99 (*Escherichia coli*, *Salmonella* Enteritidis, *Pseudomonas aeruginosa* and
100 *Stenotrophomonas maltophilia*) on solid media, and break through a “wall” of
101 *Legionella*. This activity was dependent on the presence of the *Neochlamydia*
102 endosymbiont, and confirmed that the endosymbiont was advantageous for
103 predator-prey interactions.

104

105

106 **Results and discussion**

107 *Symbiotic amoebae spotted onto agar plates with green fluorescent protein*
108 *(GFP)-expressing E. coli and Salmonella Enteritidis formed flower-like fluorescence*

109 ***patterns consisting of each of the colonies***

110 GFP-expressing bait bacteria (*E. coli* DH5 α) were used to track the movements of the
111 *Nechlamydia* S13 symbiotic amoebae (S13WT), the aposymbiotic amoebae (S13RFP),
112 reconstructed symbiotic amoebae containing *Protochlamydia* HS-T3 (S13HS-T3), and
113 C3 reference strain amoebae on Luria-Bertani (LB) agar plates. Surprisingly, while no
114 movement was noted for the combinations of S13RFP or C3 amoebae (*Acanthamoeba*
115 *castellanii*) plus GFP-expressing *E. coli*, in the presence of the symbiotic amoebae, the
116 *E. coli* spread out towards the edges of the agar plates (Fig. 1A). Furthermore, with
117 increasing incubation time, the symbiotic amoebae also formed flower-like fluorescence
118 patterns consisting of the GFP-expressing *E. coli* in an anticlockwise direction (Fig. 1A,
119 Also, see enlarged upper image). Interestingly, the reconstructed symbiotic amoebae
120 containing the *Protochlamydia* HS-T3 endosymbiont showed partial construction of the
121 flower-like formations (Fig. 1A, Also, see enlarged upper image). To objectively
122 measure these traces, the fluorescence in the areas containing the bacterial colonies was
123 measured using Image J software. As a result, a significant increase in fluorescence was
124 recorded in areas containing the S13WT amoebae in the presence of *E. coli*, supporting
125 the results obtained from observation of the agar plates (Fig. 1B). This ability of the
126 symbiotic amoebae to move the fluorescent bacteria was confirmed using a second
127 human pathogenic bacterium, GFP-expressing *Salmonella enterica* serovar Enteritidis
128 YH0815. Again, in the presence of the S13WT symbiotic amoebae, fluorescent bacterial
129 colonies could be observed spreading towards the edges of the agar plates with the
130 movement of anticlockwise (Fig. 2AB). Furthermore, it was also confirmed that other
131 bacteria (*P. aeruginosa* ATCC 27853 and *S. maltophilia* DA185), but not

132 *Staphylococcus aureus* ATCC 29213 (Fig. S1), were backpacked by the symbiotic
133 amoebae on LB agar, although lacked the movement to anticlockwise direction (Fig. 3).
134 Thus, the results showed that the S13WT amoebae could backpack human pathogenic
135 bacteria, moving faster with a flower-like pattern on the surface of the agar (in the cases
136 of the *E. coli* and *Salmonella*), which appeared to depend on the presence of the
137 endosymbiont.

138 At present, the exact reason for the faster movement with flower-like tracks in an
139 anticlockwise direction by the symbiotic amoebae, which depended on the
140 endosymbiotic bacteria, remains unknown. Meanwhile, actin filament bundling is one
141 of the key regulators contributing to actin-dependent motility in amoebae (Knecht *et al.*,
142 2010; Westendorf *et al.*, 2013). The direction of polarized actin protrusion can alter the
143 walking speed or crawling direction of amoebae (Westendorf *et al.*, 2013). Furthermore,
144 directional amoebal locomotion against resistive forces requires a turgid
145 forward-pointing actin filament, most likely sustained by cortical actomyosin II (Van
146 Haastert, 2000). In this regard, it is well known that chlamydiae can subvert host
147 cytoskeletal and membrane trafficking pathways via secretion machinery (Scidmore,
148 2011). Thus, the structural change of actin on the amoebal surface might be responsible
149 for the faster walking of the symbiotic amoebae in a specific direction, resulting in
150 anticlockwise movement, presumably regulated with the effector proteins secreted by
151 the bacterial endosymbiont genetically possessing type III secretion machinery (Ishida
152 *et al.*, 2014; Yamaguchi *et al.*, 2015). In addition, infection of the aposymbiotic
153 amoebae with endosymbiont *Protochlamydia* HS-T3, generating a reconstructed
154 symbiotic amoebal strain, partially reinstated the ability to produce the flower-like

155 locomotion with the backpacking of *E. coli*, but not *Salmonella*, on the agar plates.
156 Since *Salmonella* strains possess virulence islands that aid in the manipulation of the
157 host's systems (Fàbrega, 2013; Tezcan-Merdol *et al.*, 2004; Bleasdale *et al.*, 2009),
158 backpacking of this pathogen would likely be fatal with apoptosis-like cell death (Feng
159 *et al.*, 2009), possibly explaining why the flower-like locomotion was not observed for
160 the reconstructed strain in the presence of *Salmonella*.

161

162 ***Backpacking of human pathogenic bacteria (E. coli) by the symbiotic amoebae were***
163 ***dependent of the presence of the Neochlamydia endosymbiont***

164 To confirm that the symbiotic amoebae could backpack human pathogenic bacteria, we
165 observed the tracks of amoebae on the agar plates for a longer period of time. As
166 expected, in contrast to the aposymbiotic S13RFP amoebae, the tracks of the symbiotic
167 S13WT amoebae showed that a large number of the amoebae had crawled away from
168 the central spot by 12 h post-inoculation (Fig. 4A). Interestingly, the reconstructed
169 symbiotic amoebae (S13HS-T3) showed tracks similar to the S13WT amoebae (Fig.
170 4A). Thus, the symbiotic amoebae crawled faster than the aposymbiotic amoebae,
171 indicating that the phenomenon depended on the presence of the endosymbionts. Next,
172 to confirm the ability of the amoebae to backpack the human pathogenic bacteria, we
173 used GFP signals to search for *E. coli* micro-colonies along the amoebal tracks. Assays
174 revealed micro-colonies irregularly spaced along the tracks (Fig. 4B), while scanning
175 electron microscopy (SEM) observation revealed that the symbiotic S13WT amoebae
176 appeared to smear the surface of the agar plates with *E. coli* clusters (Fig. 5). No
177 micro-colonies were observed along the tracks of the aposymbiotic amoebae. Taken

178 together, these results indicated that the endosymbiotic bacteria allowed the S13WT
179 amoebae to backpack the GFP-expressing *E. coli* around the agar plates.

180 SEM observation of the aposymbiotic amoebae revealed that the backpacked
181 bacteria did not appear healthy, displaying disrupted or coccid forms, presumably
182 indicating a decrease in the number of viable bacteria on the surface of the amoebae.
183 This finding suggests that effective backpacking also depends on the presence of the
184 endosymbionts. Although studies on the interaction between amoebae and
185 surface-carried bacteria are very limited, a previous study interestingly showed that
186 motility is a pre-requisite for backpack formation by *Listeria monocytogenes* and other
187 motile bacteria on the surface of *Acanthamoeba* trophozoites (Doyscher et al., 2013). In
188 this regard, our findings supportively revealed that in contrast to *S. aureus* lacking
189 motility, the other motile bacteria (*E. coli*, *Salmonella*, *P. aeruginosa* and *S. maltophilia*)
190 were backpacked by the symbiotic amoebae (See Fig. 1-3, Fig. S1). Thus, the
191 backpacking that we found might be required for pre-interaction of the symbiotic
192 amoebae with motile bacteria in the mixed culture spotted on agar plate.

193

194 ***The Neochlamydia-carrying symbiotic amoebae could break through a “wall” of***
195 ***amoebal pathogenic bacterium L. pneumophila***

196 To explore the biological significance of the relationship between *Neochlamydia* and the
197 host amoebae, we investigated the interaction between the different amoebal strains and
198 *L. pneumophila*, an important pathogen of amoebae. Specifically, symbiotic or
199 aposymbiotic amoebae and GFP-expressing *E. coli* were spotted into the center of a ring
200 of mCherry-expressing *L. pneumophila* on a buffered charcoal yeast extract (B-CYE)

201 agar plate. The effects of the wall on the spread of the amoebae were then monitored by
202 visualizing the amoebal tracks using the GFP-expressing *E. coli* as a tracer. As was
203 observed on LB agar plates, the S13WT amoebae spread towards the edges of the
204 B-CYE agar plate (Fig. 6), despite the fact that the *Legionella* wall was completely
205 established (Fig. 6). Between 144 and 196 h post-inoculation, a wave of green
206 fluorescence, corresponding to the symbiotic S13WT amoebae backpacking the
207 GFP-expressing *E. coli*, could be seen mixing with the red fluorescence of the
208 *Legionella* wall, changing the color from green to yellow (Fig. 6). Interestingly, the
209 yellow color could be observed moving through the *Legionella* wall between 288 and
210 ~336 h post-inoculation (Fig. 6), before the signal began to return to green at ~312–336
211 h post-inoculation, indicating that the amoebae had successfully broken through the
212 *Legionella* wall (Fig. 6). Meanwhile, while the aposymbiotic amoebae (S13RFP) also
213 moved towards the *Legionella* wall, their movement was delayed compared with the
214 symbiotic amoebae (Fig. S2-S3). A color change to yellow was also observed near the
215 wall between 144 and 196 h post-inoculation, showing the amoebae mixing with *L.*
216 *pneumophila* (Fig. S2); however, few yellow signals were observed within the
217 *Legionella* wall, indicating that the aposymbiotic amoebae could not break through the
218 wall. Although a small number of green signals were detected outside the wall, we
219 suspect that a small number of aposymbiotic amoebae were able to pass through the
220 wall by chance (Fig. S2). Thus, the results indicated that the *Neochlamydia*
221 endosymbiont helped the symbiotic amoebae to successfully break through the wall of *L.*
222 *pneumophila*.

223 *Legionella* is a well-documented pathogen of amoebae, with bacterial growth

224 resulting in disruption of the host amoebae (Greub and Raoult, 2004). However, we
225 have previously shown that the *Neochlamydia* symbiont helps protect the amoebal host
226 from *Legionella* in liquid media (Ishida et al., 2014). Assays showed that the symbiotic
227 amoebae successfully broke through a wall of *L. pneumophila*, and that this activity
228 depended on the presence of the endosymbiotic *Neochlamydia*. Interestingly, we
229 observed that the symbiotic amoebae backpacking the GFP-expressing *E. coli* formed
230 channel-like structures through the walls of mCherry-expressing *Legionella*. As
231 mentioned above, it is possible that the presence of the GFP-expressing bacteria on the
232 surface of the amoebae limits the number of sites through which *L. pneumophila* can
233 enter the amoebal cell. Interestingly, a small number of aposymbiotic amoebae were
234 noted beyond the *Legionella* wall. However, very few yellow color signals (indicating
235 the presence of the amoebae backpacking the GFP-expressing bacteria) were seen
236 within the mCherry-expressing *Legionella*, indicating that the aposymbiotic amoebae a
237 failed to break through the wall. We therefore concluded that the external aposymbiotic
238 amoebae accidentally climbed over the wall, rather than passing through the *L.*
239 *pneumophila* barrier. At present, the exact role of the symbiotic bacteria in breaking
240 through the *Legionella* “wall” in a natural ecological niche remains unknown. However,
241 in their natural environment, *Legionella* are known to form biofilms when they
242 encounter amoebae, which provide a rich environment for the replication of *Legionella*
243 (Lau and Ashbolt, 2009). It is therefore likely that maintaining the endosymbiont is
244 advantageous to the amoebae as it allows the backpacking of feed and protects the host
245 against *Legionella*, allowing them to pass through the “wall”.

246 Taken together, the results of the current study suggested that the symbiotic

247 amoebae could backpack human pathogenic bacteria on solid agar plates, breaking
248 through a “wall” of *Legionella*, and that the activity depended on the presence of the
249 amoebal endosymbiont *Neochlamydia*. This confirmed a role for the endosymbiont in
250 the predator-prey interactions of the host. Finally, we strongly propose observing
251 predator-prey interactions of such free-living amoebae on solid surfaces as they better
252 mimic the natural environments of the amoebae.

253

254

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398 **Legends to figures**

399

400 **Fig. 1.** Movement of amoebae carrying GFP-expressing *E. coli* DH5 α on agar plates. **A.**

401 Changes in fluorescence patterns up to 14 day post-inoculation. S13WT; symbiotic

402 amoeba strain S13WT harboring *Neochlamydia* . S13RFP; aposymbiotic amoebae

403 established from strain S13WT by treatment with rifampicin. S13HS-T3; S13RFP

404 amoebae infected with *Protochlamydia* HS-T3, resulting in a reconstructed symbiotic

405 stain. C3; reference strain *Acanthamoeba castellanii* C3 (ATCC 50739). Enlarged

406 images of S13WT (dashed square with red color) and S13RFP (dashed square with blue

407 color) amoebal tracks. Symbiotic *Acanthamoeba* strain S13WT, harboring

408 *Neochlamydia* strain S13 and originally isolated from Sapporo City, Japan, was used in

409 this study (Matsuo *et al.*, 2010). An aposymbiotic strain was established from S13WT

410 by treatment with rifampicin (RFP; 64 μ g/mL), and designated S13RFP (Okude *et al.*,

411 2012). *Acanthamoeba* strain HS-T3, persistently infected with endosymbiont

412 *Protochlamydia* HS-T3, was originally isolated from a hot spring located in Kanagawa

413 Prefecture, Japan (Sampo *et al.*, 2014). We generated a reconstructed S13RFP symbiont

414 strain containing *Protochlamydia* HS-T3, designated S13HS-T3, for use in this study. In

415 addition, *A. castellanii* C3 (ATCC 50739) (C3 amoebae), purchased from the American

416 Type Culture Collection, was used as a reference strain. All amoebae were maintained

417 in peptone-yeast extract-glucose (PYG) medium at 18°C as previously described

418 (Matsuo *et al.*, 2010). *E. coli* DH5 α (our own laboratory collection) was also used in

419 this study. The bacterial strain was genetically modified to carry a GFP-expressing

420 plasmid (pBBR122 encoding *gfp*) for ease of visualization. *E. coli* was cultured on LB

421 agar for 1 day, at 37°C. Agar were supplemented with chloramphenicol (10 µg/mL) to
422 maintain the plasmid. Amoebae (1×10^5 cells, counted using a cell counting chamber)
423 were mixed with GFP-expressing *E. coli* (approximately 1×10^7 cells, adjusted to a
424 McFarland turbidity of 0.5, followed by 1/10 dilution) in 1 mL of Page's amoeba saline
425 containing chloramphenicol (10 µg/mL). A 20-µL aliquot of the mixture was then spotted
426 onto the center of LB (diameter, 6 cm) agar plates. The plates were incubated at 30°C in a
427 moist environment (about 100% humidity) for up to 14 days, and amoebal walking was
428 then visualized using the fluorescent bacterial signals as a tracer under a transilluminator.

429 **B.** Changes in the fluorescence of areas corresponding to GFP-expressing *E. coli*. These
430 areas were determined using Image J software. Briefly, colors (RGB channel type) were
431 split into red, green, and blue, and the blue and red colors were omitted. The remaining
432 color was adjusted against the background of the dish edge using the threshold adjuster.
433 Finally, the total fluorescent area was recorded as a pixel value. Data were expressed as a
434 ratio of each value versus the control after 24 h of incubation. Data are expressed as
435 average pixel values \pm SD. Comparisons among groups were performed using a multiple
436 comparison test for parametric analysis using the Bonferroni/Dunn method. Asterisks
437 indicate a *p*-value < 0.05 (considered significant) vs. other values at the same time
438 point.

439

440 **Fig. 2.** Movement of amoebae carrying GFP-expressing *Salmonella* Enteritidis YH0815
441 on agar plates. **A.** Changes in fluorescence patterns up to 14 day post-inoculation.
442 S13WT; symbiotic amoeba strain S13WT harboring *Neochlamydia* . S13RFP;
443 aposymbiotic amoebae established from strain S13WT by treatment with rifampicin.

444 S13HS-T3; S13RFP amoebae infected with *Protochlamydia* HS-T3, resulting in a
445 reconstructed symbiotic stain. C3; reference strain *A. castellanii* C3 (ATCC 50739).
446 Enlarged images of S13WT (dashed square with red color) and S13RFP (dashed square
447 with blue color) amoebal tracks. See the legend for Fig. 1. **B.** Changes in the
448 fluorescence of areas corresponding to GFP-expressing *Salmonella* Enteritidis. The
449 bacterial strain was genetically modified to carry a GFP-expressing plasmid (pBBR122
450 encoding *gfp*) for ease of visualization. See the legend for Fig. 1. Data are expressed as
451 average pixel values \pm SD. Comparisons among groups were performed using a multiple
452 comparison test for parametric analysis using the Bonferroni/Dunn method. Asterisks
453 indicate a *p*-value < 0.05 (considered significant) vs. other values at the same time
454 point.

455

456 **Fig. 3.** Movement of amoebae carrying other bacteria (**A.** *P. aeruginosa* ATCC 27853; **B.**
457 *S. maltophilia* DA185) on LB agar plates. Changes in fluorescence patterns up to 8 day
458 post-inoculation. S13WT; symbiotic amoeba strain S13WT harboring *Neochlamydia* .
459 S13RFP; aposymbiotic amoebae established from strain S13WT by treatment with
460 rifampicin. S13HS-T3; S13RFP amoebae infected with *Protochlamydia* HS-T3,
461 resulting in a reconstructed symbiotic stain. C3; reference strain *A. castellanii* C3
462 (ATCC 50739). See the legend for Fig. 1.

463

464 **Fig. 4.** Amoebal tracks on LB agar plates in the first 48 h post-simultaneous inoculation
465 with GFP-expressing *E. coli*. **A.** Microscopic observation of the amoebal tracks at 12 h
466 post-inoculation. S13WT; symbiotic amoeba strain S13WT harboring *Neochlamydia*.

467 S13RFP; aposymbiotic amoeba strain established from S13WT. S13HS-T3; S13RFP
468 amoebae infected with *Protochlamydia* HS-T3. For more detail see the legend of Fig. 1.
469 Right-hand panels are enlargements of the dashed red squares shown in the left-hand
470 panels. See the legend for Fig. 1. Magnification, $\times 100$. **B.** Micro-colonies formed by *E.*
471 *coli* along the tracks of S13WT amoebae at 48 h post-inoculation. Arrowheads show
472 identical localization of micro-colonies with GFP signals. Magnification, $\times 100$.

473

474 **Fig. 5.** Representative SEM images showing the ultrastructure of amoeba strains
475 S13WT and S13RFP during spreading. Asterisks indicate amoebae. The dashed red
476 square shown in the left-hand panel is enlarged in the right-hand panel. The dashed blue
477 square in enlarged in the lower panel. **A.** S13WT; symbiotic amoeba strain S13WT
478 harboring *Neochlamydia*. **B.** S13RFP; aposymbiotic amoebae established from S13WT
479 amoebae. SEM analysis was performed according to a previous method (Okude *et al.*,
480 2012). In brief, amoebae were fixed with 2.5% (v/v) glutaraldehyde in
481 phosphate-buffered saline (pH 7.4) for 2 h at room temperature, and subsequently
482 soaked in 2% (w/v) osmium tetroxide for 1 h at 4°C. The samples were then dehydrated
483 in ethanol, freeze-dried, and coated with osmium using a plasma osmium coater.
484 Ultimately, treated samples were analyzed using a Hitachi S-4800 scanning electron
485 microscope.

486

487 **Fig. 6.** Time course showing the movement of S13WT amoebae on the surface of
488 B-CYE agar plates. Amoebae were spotted with GFP-expressing *E. coli* into the center
489 of a ring of mCherry-expressing *L. pneumophila* Lp01. *L. pneumophila* Lp01

490 (T4ASS⁻/T4BSS⁺/Tra⁺) (Rao *et al.*, 2013) strain was genetically modified to carry a
491 mCherry-expressing plasmid (pAM239 encoding mCherry *gene*) for ease of
492 visualization (Coers *et al.*, 2000). Dashed white squares in the upper panels are enlarged
493 in the lower panel. Green color corresponds to GFP-expressing *E. coli*. Red color
494 corresponds to the *L. pneumophila*. Yellow color indicates a mixture of the *E. coli* and *L.*
495 *pneumophila* bacteria. Amoebae (1×10^5 cells, counted using a cell counting chamber)
496 were mixed with GFP-expressing *E. coli* DH5 α (approximately 1×10^7 cells, adjusted
497 to a McFarland turbidity of 0.5, followed by 1/10 dilution) in 1 mL of Page's amoeba
498 saline containing chloramphenicol (10 μ g/mL). A 20- μ L aliquot of the mixture was then
499 spotted onto the center of B-CYE (diameter, 10 cm) agar plates. mCherry-expressing *L.*
500 *pneumophila* Lp01 was simultaneously stamped onto the agar plate, surrounding the
501 central spot (diameter, 6.6 cm). The plates were incubated at 30°C in a moist environment
502 (about 100% humidity) for up to 14 days (336 h), and amoebal walking was then
503 visualized using the fluorescent bacterial signals as a tracer under a transilluminator.

504

505

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507

508 **Supporting information**

509

510 **Supplementary Fig. 1 (Fig. S1)** Movement of amoebae inoculated with *S. aureus*
511 ATCC 29213 onto LB agar plates. Changes in colony patterns were monitored for up to
512 8 day. S13WT; symbiotic amoeba strain S13WT harboring *Neochlamydia*. S13RFP;
513 aposymbiotic amoebae established from strain S13WT. S13HS-T3; S13RFP amoebae
514 infected with *Protochlamydia* HS-T3. See the legend of Fig. 1.

515

516 **Supplementary Fig. 2 (Fig. S2)** Time course assay showing changes in the tracks
517 produced by C3 amoebae and *E. coli* DH5 α (A) and S13RFP amoebae (B) on B-CYE
518 agar plates. The dashed white square in the lower left-hand panel is enlarged in the
519 lower right-hand panels. Green color corresponds to the GFP-expressing *E. coli*. Red
520 color corresponds to mCherry-expressing *L. pneumophila* Lp01. Yellow color
521 corresponds to areas containing a mixture of the *E. coli* and *L. pneumophila* bacteria.
522 Dashed white square is enlarged in the right panel. The plates were incubated at 30°C in
523 a moist environment (about 100% humidity) for up to 14 days (in the case of S13RFP
524 amoebae), and amoebal walking was then visualized using the fluorescent bacterial
525 signals as a tracer under a transilluminator. See the legend for Fig. 6.

526

527 **Supplementary Fig. 3 (Fig. S3)** Time course showing the movement of amoebae on the
528 surface of B-CYE agar plates for a prolonged period. Amoebae were spotted with
529 GFP-expressing *E. coli* into the center of a ring of mCherry-expressing *L. pneumophila*
530 Lp01. S13WT; symbiotic amoeba strain S13WT harboring *Neochlamydia*. S13RFP;

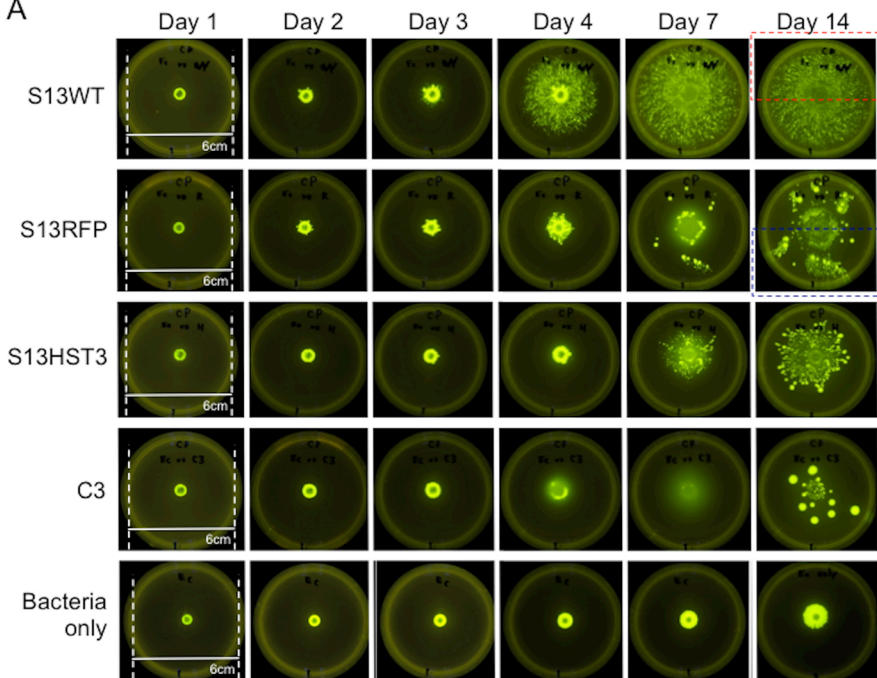
531 aposymbiotic amoebae established from strain S13WT. Green color corresponds to
532 GFP-expressing *E. coli*. Red color corresponds to the *L. pneumophila*. Yellow color
533 indicates a mixture of the *E. coli* and *L. pneumophila* bacteria. The plates were
534 incubated at 30°C in a moist environment (about 100% humidity) for up to 20 days, and
535 amoebal walking was then visualized using the fluorescent bacterial signals as a tracer
536 under a transilluminator. See the legend for Fig. 6.

537

538

Fig. 1

A



B

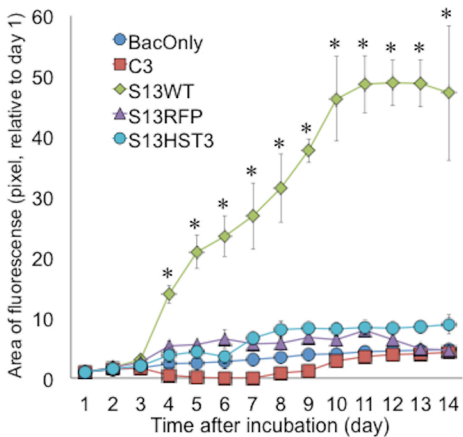
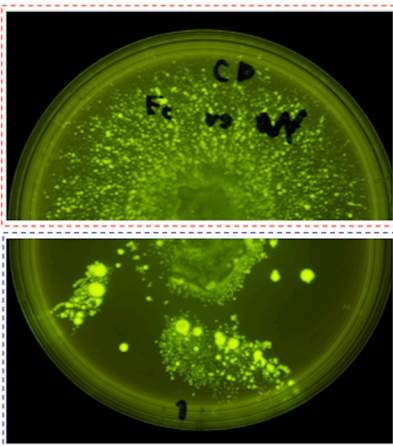


Fig. 2

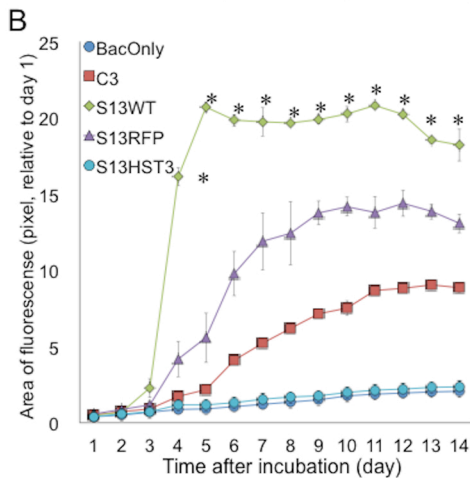
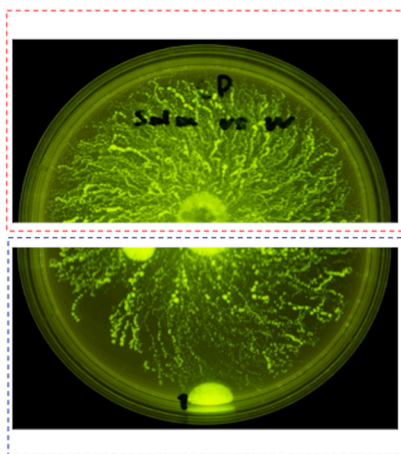
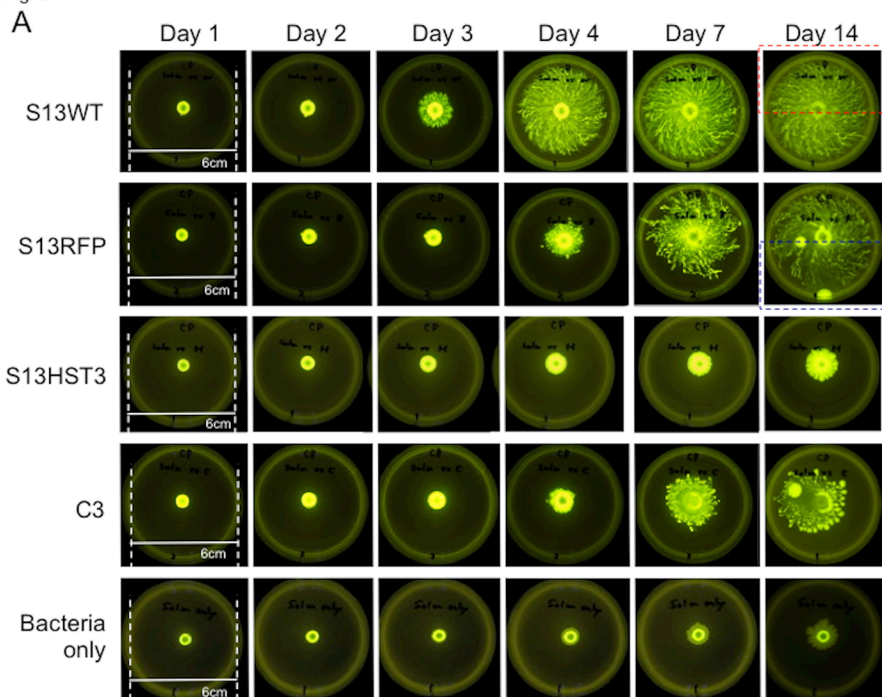
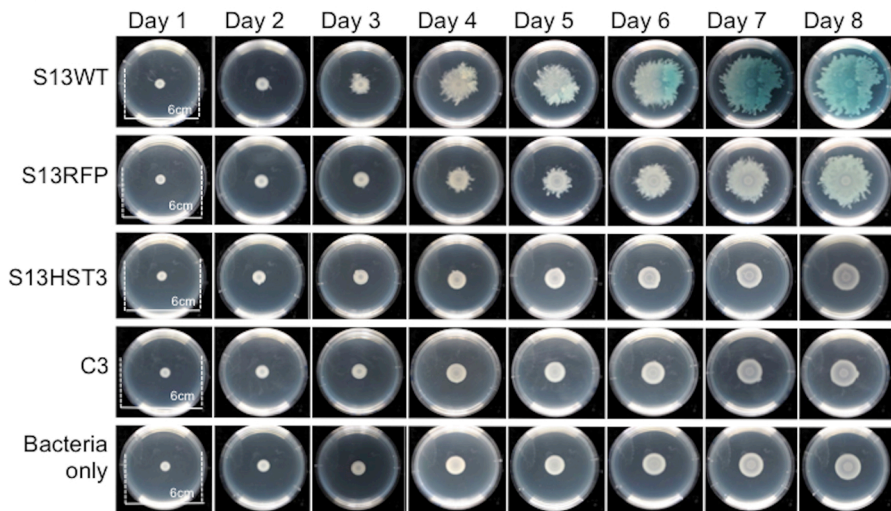


Fig. 3

A



B

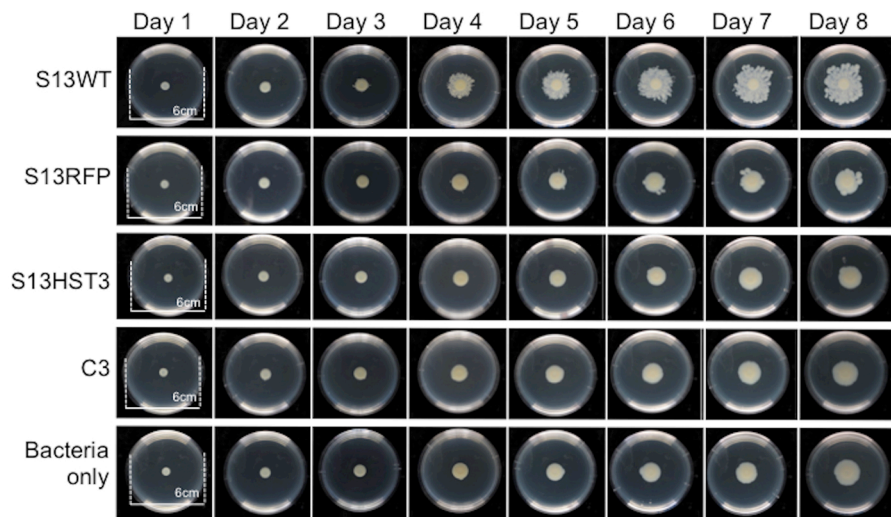
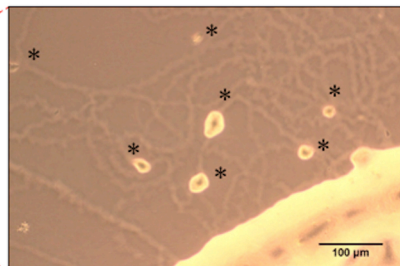
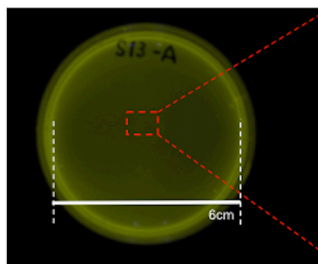


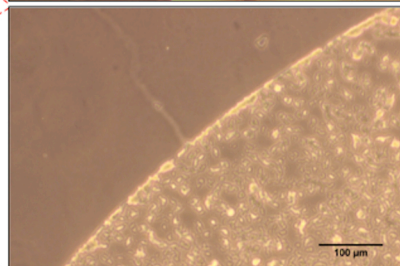
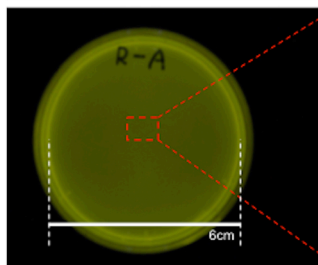
Fig. 4

A

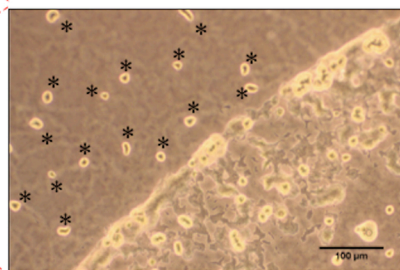
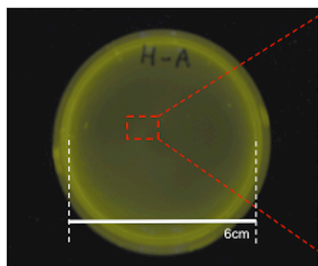
S13WT
(12h)



S13RFP
(12h)



S13HS-T3
(12h)



B

S13WT
(48h)

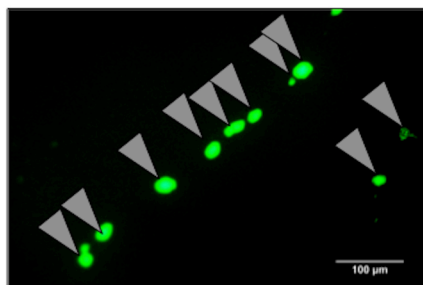
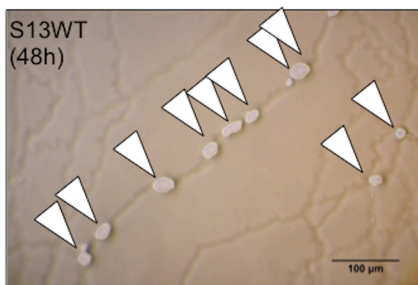
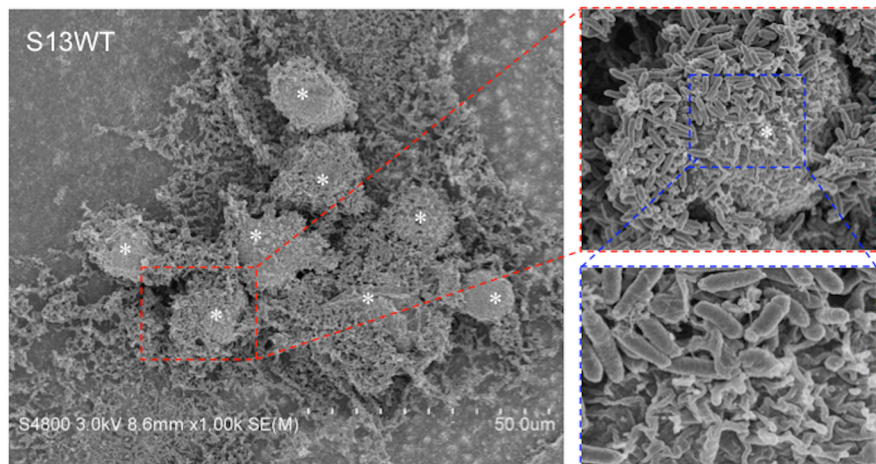


Fig. 5

A



B

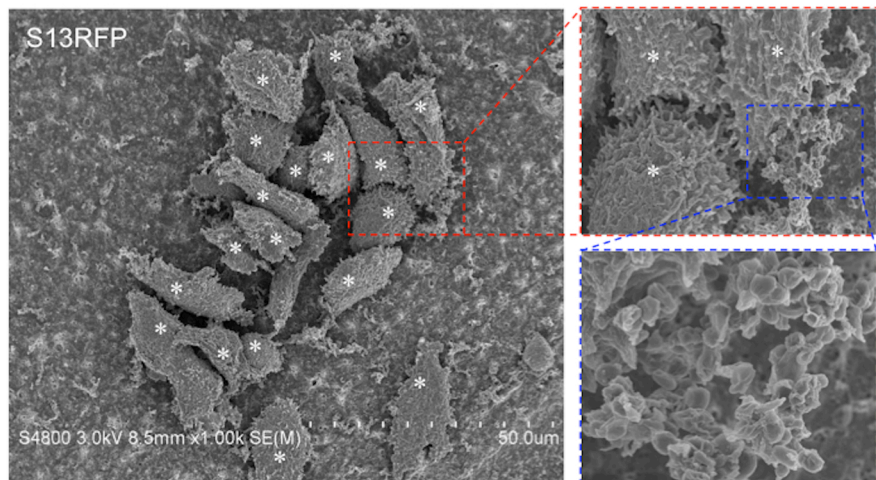


Fig. 6

