Acanthamoeba S13WT relies on its bacterial endosymbiont to backpack human pathogenic bacteria and resist *Legionella* infection on solid media

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Running title: Backpacking of bacteria by amoebae

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Summary

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

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

61 *Legionella*, solid media

62

Introduction

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

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

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

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

Results and discussion

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

patterns consisting of each of the colonies

GFP-expressing bait bacteria (*E. coli* DH5α) were used to track the movements of the *Nechlamydia* S13 symbiotic amoebae (S13WT), the aposymbiotic amoebae (S13RFP), reconstructed symbiotic amoebae containing *Protochlamydia* HS-T3 (S13HS-T3), and C3 reference strain amoebae on Luria-Bertani (LB) agar plates. Surprisingly, while no movement was noted for the combinations of S13RFP or C3 amoebae (*Acanthamoeba castellanii*) plus GFP-expressing *E. coli*, in the presence of the symbiotic amoebae, the *E. coli* spread out towards the edges of the agar plates (Fig. 1A). Furthermore, with increasing incubation time, the symbiotic amoebae also formed flower-like fluorescence patterns consisting of the GFP-expressing *E. coli* in an anticlockwise direction (Fig. 1A, Also, see enlarged upper image). Interestingly, the reconstructed symbiotic amoebae containing the *Protochlamydia* HS-T3 endosymbiont showed partial construction of the flower-like formations (Fig. 1A, Also, see enlarged upper image). To objectively measure these traces, the fluorescence in the areas containing the bacterial colonies was measured using Image J software. As a result, a significant increase in fluorescence was recorded in areas containing the S13WT amoebae in the presence of *E. coli*, supporting the results obtained from observation of the agar plates (Fig. 1B). This ability of the symbiotic amoebae to move the fluorescent bacteria was confirmed using a second human pathogenic bacterium, GFP-expressing *Salmonella enterica* serovar Enteritidis YH0815. Again, in the presence of the S13WT symbiotic amoebae, fluorescent bacterial colonies could be observed spreading towards the edges of the agar plates with the movement of anticlockwise (Fig. 2AB). Furthermore, it was also confirmed that other bacteria (*P. aeruginosa* ATCC 27853 and *S. maltophilia* DA185), but not

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

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

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

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

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

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

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

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

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396
397

Legends to figures

Fig. 1. Movement of amoebae carrying GFP-expressing *E. coli* DH5α on agar plates. **A.** Changes in fluorescence patterns up to 14 day post-inoculation. S13WT; symbiotic amoeba strain S13WT harboring *Neochlamydia* . S13RFP; aposymbiotic amoebae established from strain S13WT by treatment with rifampicin. S13HS-T3; S13RFP amoebae infected with *Protochlamydia* HS-T3, resulting in a reconstructed symbiotic stain. C3; reference strain *Acanthamoeba castellanii* C3 (ATCC 50739). Enlarged images of S13WT (dashed square with red color) and S13RFP (dashed square with blue color) amoebal tracks. Symbiotic *Acanthamoeba* strain S13WT, harboring *Neochlamydia* strain S13 and originally isolated from Sapporo City, Japan, was used in this study (Matsuo *et al*., 2010). An aposymbiotic strain was established from S13WT by treatment with rifampicin (RFP; 64 μg/mL), and designated S13RFP (Okude *et al*., 2012). *Acanthamoeba* strain HS-T3, persistently infected with endosymbiont *Protochlamydia* HS-T3, was originally isolated from a hot spring located in Kanagawa Prefecture, Japan (Sampo *et al*., 2014). We generated a reconstructed S13RFP symbiont strain containing *Protochlamydia* HS-T3, designated S13HS-T3, for use in this study. In addition, *A. castellanii* C3 (ATCC 50739) (C3 amoebae), purchased from the American Type Culture Collection, was used as a reference strain. All amoebae were maintained in peptone-yeast extract-glucose (PYG) medium at 18°C as previously described (Matsuo *et al*., 2010). *E. coli* DH5α (our own laboratory collection) was also used in this study. The bacterial strain was genetically modified to carry a GFP-expressing 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 ± 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 ± 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*.

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

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

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

(T4ASS$^-$/T4BSS$^+$/Tra$^+$) (Rao *et al*., 2013) strain was genetically modified to carry a mCherry-expressing plasmid (pAM239 encoding mCherry *gene*) for ease of visualization (Coers *et al*., 2000). Dashed white squares in the upper panels are enlarged in the lower panel. Green color corresponds to GFP-expressing *E. coli*. Red color corresponds to the *L. pneumophila*. Yellow color indicates a mixture of the *E. coli* and *L. pneumophila* bacteria. Amoebae (1×10^5 cells, counted using a cell counting chamber) were mixed with GFP-expressing *E. coli* DH5α (approximately 1×10^7 cells, adjusted to a McFarland turbidity of 0.5, followed by 1/10 dilution) in 1 mL of Page's amoeba saline containing chloramphenicol (10 μg/mL). A 20-μL aliquot of the mixture was then spotted onto the center of B-CYE (diameter, 10 cm) agar plates. mCherry-expressing *L. pneumophila* Lp01 was simultaneously stamped onto the agar plate, surrounding the central spot (diameter, 6.6 cm). The plates were incubated at 30°C in a moist environment (about 100% humidity) for up to 14 days (336 h), and amoebal walking was then visualized using the fluorescent bacterial signals as a tracer under a transilluminator.

Supporting information

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

Supplementary Fig. 2 (Fig. S2) Time course assay showing changes in the tracks produced by C3 amoebae and *E. coli* DH5α (**A**) and S13RFP amoebae (**B**) on B-CYE agar plates. The dashed white square in the lower left-hand panel is enlarged in the lower right-hand panels. Green color corresponds to the GFP-expressing *E. coli*. Red color corresponds to mCherry-expressing *L. pneumophila* Lp01. Yellow color corresponds to areas containing a mixture of the *E. coli* and *L. pneumophila* bacteria. Dashed white square is enlarged in the right panel. The plates were incubated at 30°C in a moist environment (about 100% humidity) for up to 14 days (in the case of S13RFP amoebae), and amoebal walking was then visualized using the fluorescent bacterial signals as a tracer under a transilluminator. See the legend for Fig. 6.

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

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

Fig. 1

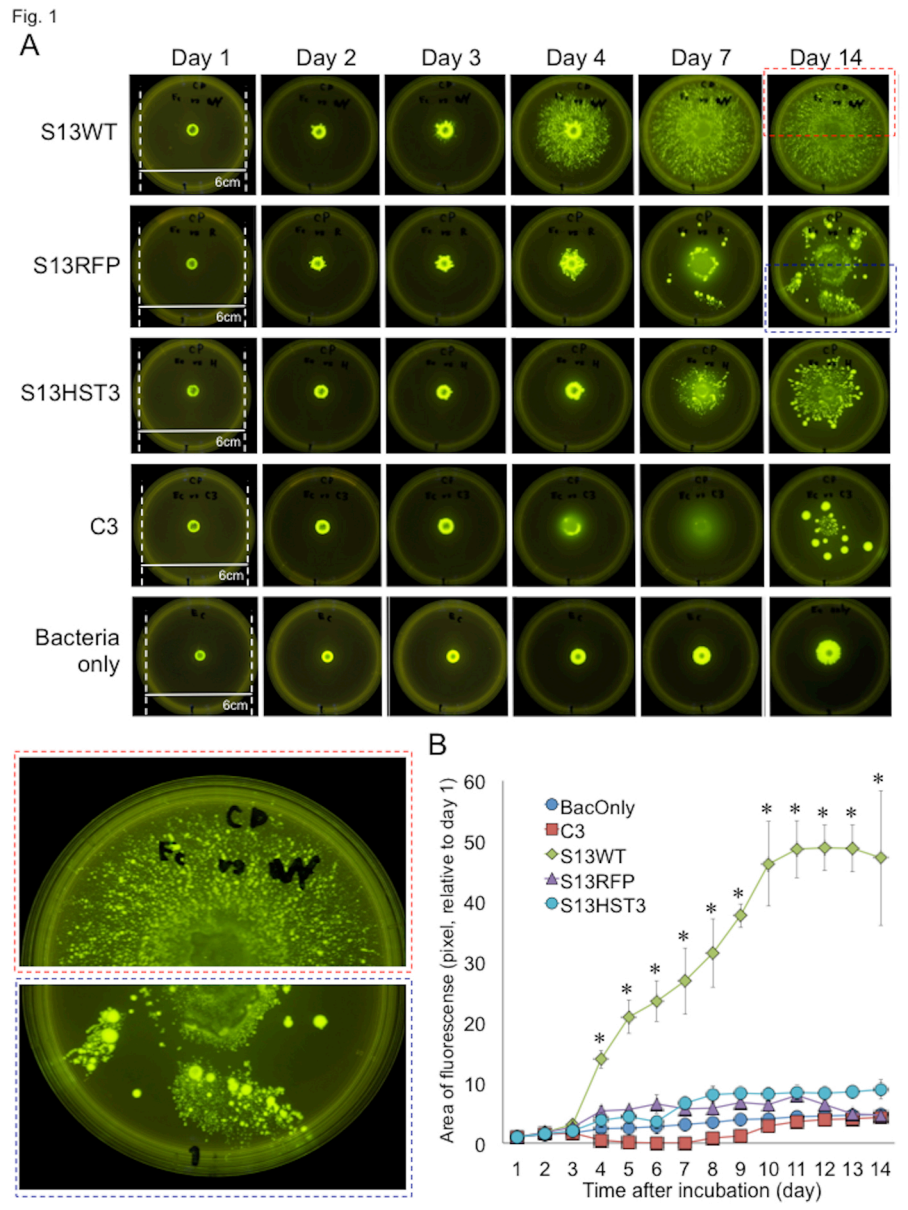
A
Day 1
Day 2
Day 3
Day 4
Day 7
Day 14
S13WT
S13RFP
S13HST3
C3
Bacteria only
6cm
B
BacOnly
C3
S13WT
S13RFP
S13HST3
Area of fluorescense (pixel, relative to day 1)
Time after incubation (day)
0
10
20
30
40
50
60
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Fig. 2

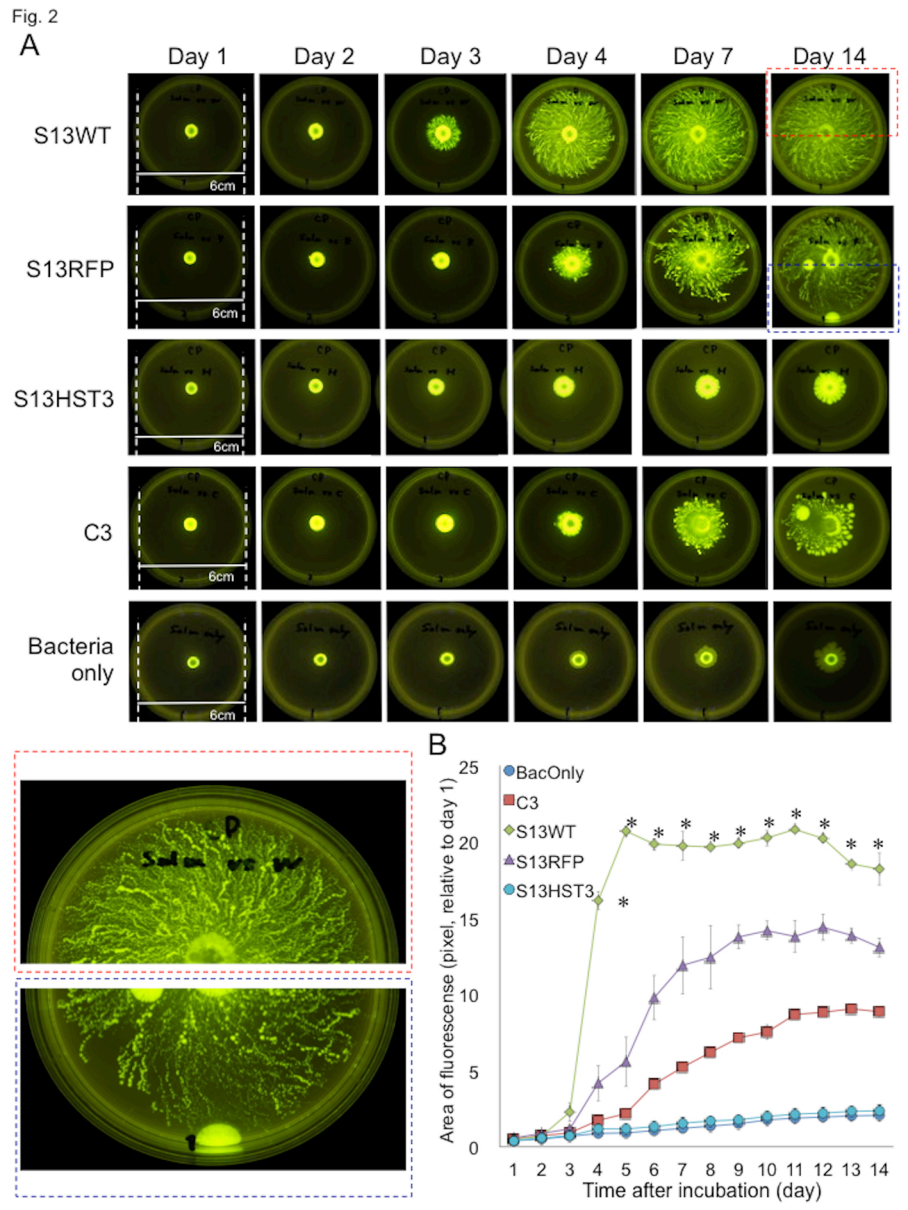
A
Day 1
Day 2
Day 3
Day 4
Day 7
Day 14
S13WT
S13RFP
S13HST3
C3
Bacteria only
6cm
B
BacOnly
C3
S13WT
S13RFP
S13HST3
Area of fluorescense (pixel, relative to day 1)
Time after incubation (day)
0
5
10
15
20
25
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Fig. 3

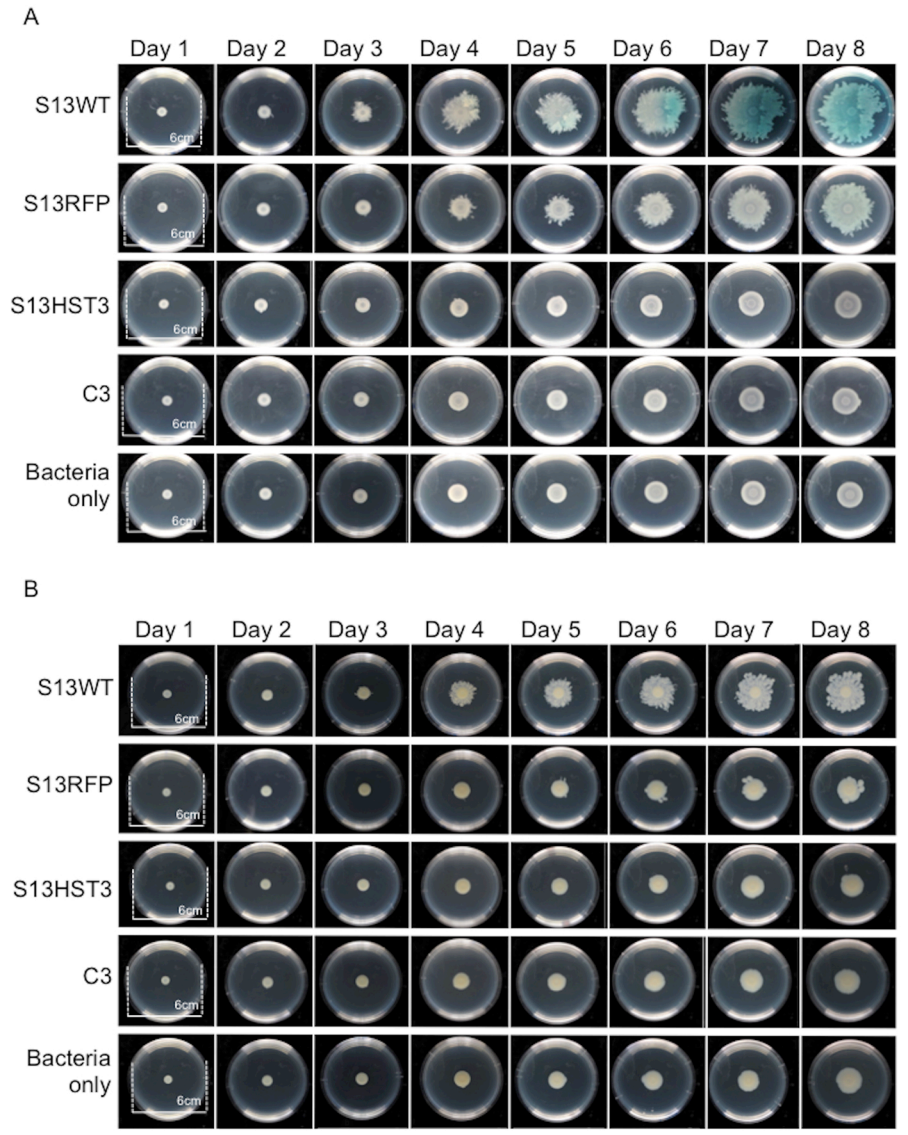
A
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
Day 8
S13WT
S13RFP
S13HST3
C3
Bacteria only
6cm
B
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
Day 8
S13WT
S13RFP
S13HST3
C3
Bacteria only
6cm

Fig. 4

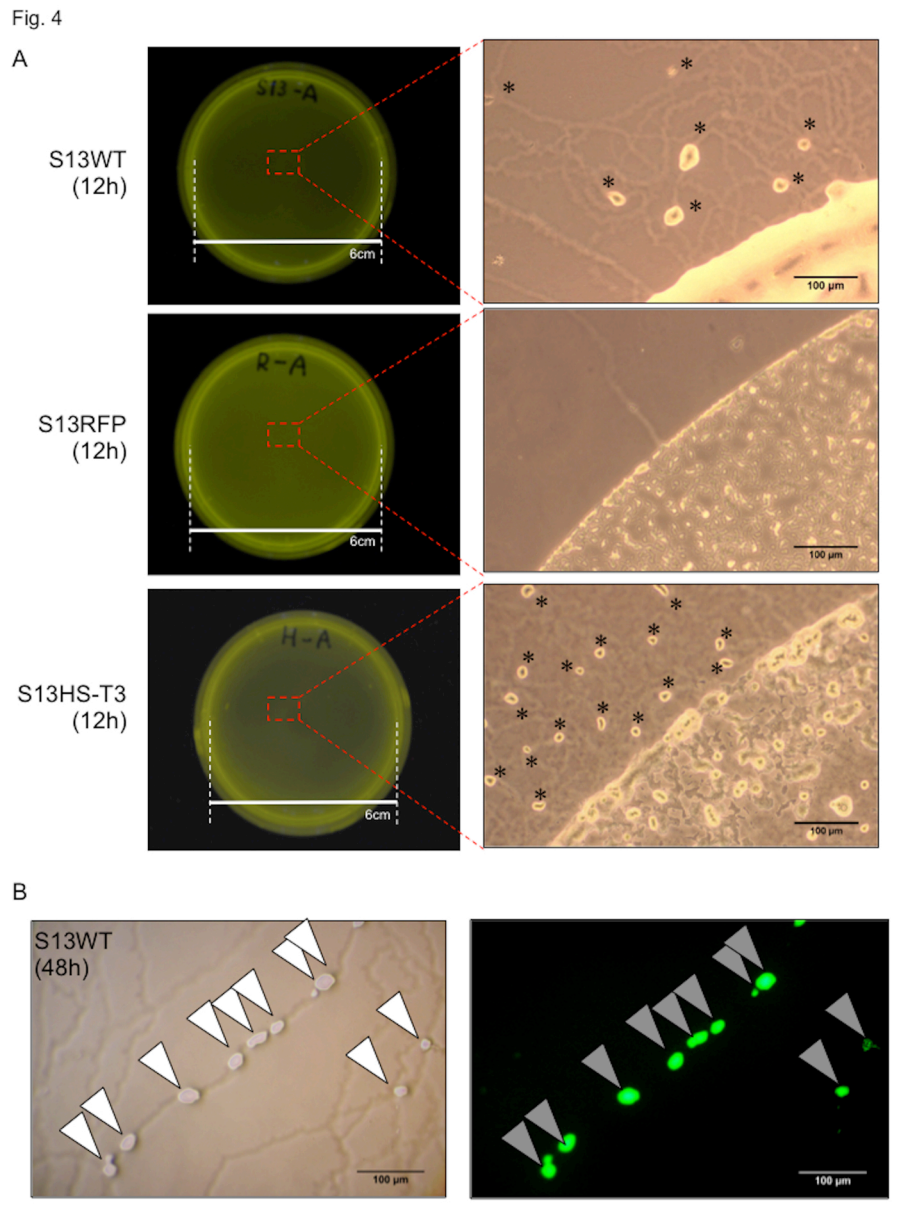
A
S13WT
(12h)
S13RFP
(12h)
S13HS-T3
(12h)
S13-A
R-A
H-A
6cm
100 μm
B
S13WT
(48h)
100 μm

Fig. 5

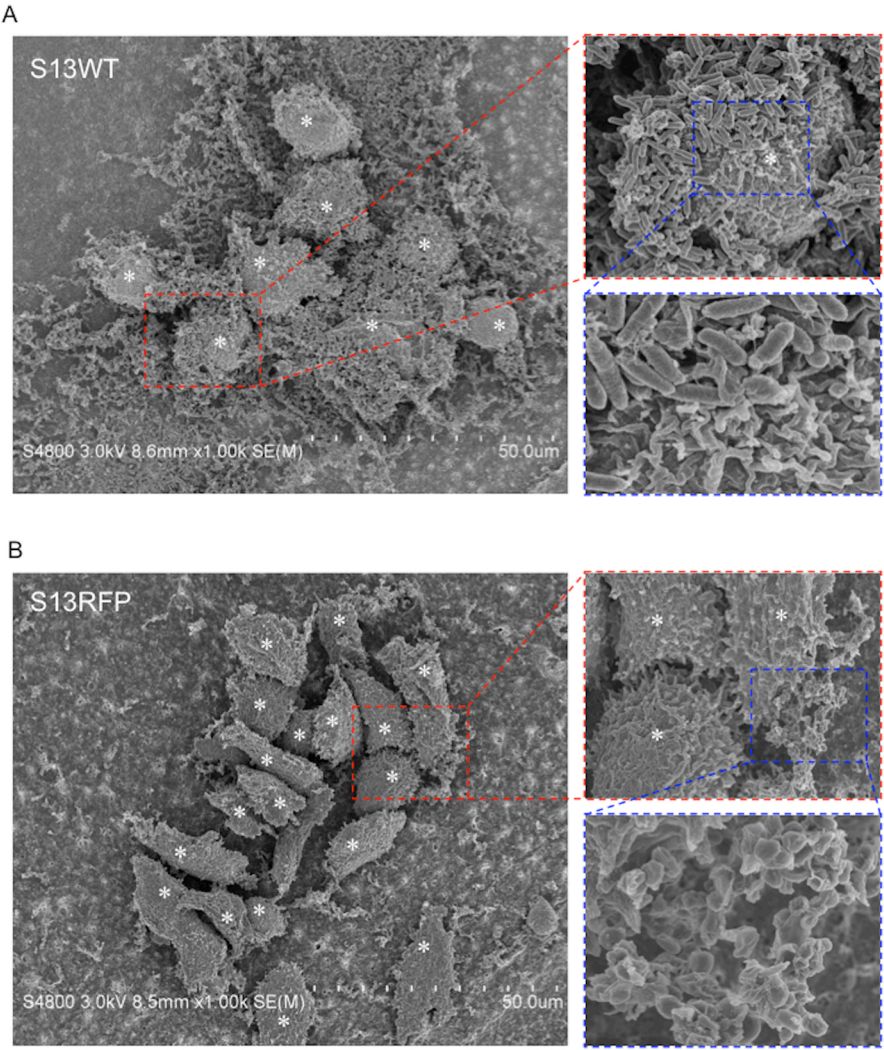
A
S13WT
S4800 3.0kV 8.6mm x1.00k SE(M)
50.0um
B
S13RFP
S4800 3.0kV 8.5mm x1.00k SE(M)
50.0um

Fig. 6