



|                  |                                                                                                                                                                                                                                                                                                                                                                                             |
|------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Title            | Acanthamoeba S13WT relies on its bacterial endosymbiont to backpack human pathogenic bacteria and resist Legionella infection on solid media                                                                                                                                                                                                                                                |
| Author(s)        | Okubo, Torahiko; Matsushita, Mizue; Nakamura, Shinji et al.                                                                                                                                                                                                                                                                                                                                 |
| Citation         | Environmental microbiology reports, 10(3), 344-354<br><a href="https://doi.org/10.1111/1758-2229.12645">https://doi.org/10.1111/1758-2229.12645</a>                                                                                                                                                                                                                                         |
| Issue Date       | 2018-06-01                                                                                                                                                                                                                                                                                                                                                                                  |
| Doc URL          | <a href="https://hdl.handle.net/2115/74841">https://hdl.handle.net/2115/74841</a>                                                                                                                                                                                                                                                                                                           |
| Rights           | This is the peer reviewed version of the following article: Environmental Microbiology Reports, 10(3), 344-354, Jun. 2018, which has been published in final form at <a href="https://doi.org/10.1111/1758-2229.12645">https://doi.org/10.1111/1758-2229.12645</a> . This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Self-Archiving. |
| Type             | journal article                                                                                                                                                                                                                                                                                                                                                                             |
| File Information | Environ Microbiol Rep_10(3)_344-544.pdf                                                                                                                                                                                                                                                                                                                                                     |



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

Torahiko Okubo<sup>1</sup>, Mizue Matsushita<sup>1</sup>, Shinji Nakamura<sup>2</sup>, Junji Matsuo<sup>1</sup>, Hiroki Nagai<sup>3</sup>,  
and Hiroyuki Yamaguchi<sup>1\*</sup>

<sup>1</sup>Department of Medical Laboratory Science, Faculty of Health Sciences, Hokkaido  
University, North-12, West-5, Kita-ku, Sapporo 060-0812, Japan

<sup>2</sup>Division of Biomedical Imaging Research, Juntendo University Graduate School of  
Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan

<sup>3</sup>Department of Microbiology, Gifu University School of Medicine, Yanagido 1-1, Gifu,  
Gifu 501-1194, Japan

Running title: Backpacking of bacteria by amoebae

\*Corresponding author:

Hiroyuki Yamaguchi

Department of Medical Laboratory Science, Faculty of Health Sciences, Hokkaido  
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](mailto:hiroyuki@med.hokudai.ac.jp)

27

28

29 E-mail addresses

30 Torahiko Okubo: [t.okubo@hs.hokudai.ac.jp](mailto:t.okubo@hs.hokudai.ac.jp)

31 Mizue Matsushita: [all-around@eis.hokudai.ac.jp](mailto:all-around@eis.hokudai.ac.jp)

32 Shinji Nakamura: [shinji-n@juntendo.ac.jp](mailto:shinji-n@juntendo.ac.jp)

33 Junji Matsuo: [matsuo@hs.hokudai.ac.jp](mailto:matsuo@hs.hokudai.ac.jp)

34 Hiroki Nagai: [hnagai@gifu-u.ac.jp](mailto:hnagai@gifu-u.ac.jp)

35 Hiroyuki Yamaguchi: [hiroyuki@med.hokudai.ac.jp](mailto:hiroyuki@med.hokudai.ac.jp)

36

37

## 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* DH5 $\alpha$ ) 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 *Protochlamydia* 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 possess 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



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

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

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

To explore the biological significance of the relationship between *Neochlamydia* and the host amoebae, we investigated the interaction between the different amoebal strains and *L. pneumophila*, an important pathogen of amoebae. Specifically, symbiotic or aposymbiotic amoebae and GFP-expressing *E. coli* were spotted into the center of a ring 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

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

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.

## Acknowledgments

This study was supported by grants-in-aid for scientific research from KAKENHI (grant number 16K15270). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors declare that no competing interests exist. We thank Chinatsu Maita for helping all the assessment, and Tamsin Sheen, PhD, from Edanz Group ([www.edanzediting.com/ac](http://www.edanzediting.com/ac)) for editing a draft of this manuscript.

## References

- Bleasdale, B., Lott, P.J., Jagannathan, A., Stevens, M.P., Birtles, R.J., Wigley, P. (2009) The *Salmonella* pathogenicity island 2-encoded type III secretion system is essential for the survival of *Salmonella enterica* serovar Typhimurium in free-living amoebae. *Appl Environ Microbiol* **75**:1793-5.
- Coers, J., Kagan, J.C., Matthews, M., Nagai, H., Zuckman, D.M., Roy, C.R. (2000) Identification of Icm protein complexes that play *distinct roles in the biogenesis of an organelle permissive for* *Legionella pneumophila* intracellular growth. *Mol Microbiol* **38**: 719-736.
- De Jonckheere, J.F. (1991) Ecology of *Acanthamoeba*. *Rev Infect Dis* **13 (Suppl. 5)**: S385–S387.
- Doyscher, D., Fieseler, L., Dons, L., Loessner, M.J., Schuppler, M. (2013) *Acanthamoeba* feature a unique backpacking strategy to trap and feed on *Listeria monocytogenes* and other motile bacteria. *Environ Microbiol* **15**:433-446.
- Ettinger, M.R., Webb, S.R., Harris, S.A., McIninch, S.P.C., Garman, G., and Brown, B.L. (2003) Distribution of free-living amoebae in James River, Virginia, USA. *Parasitol Res* **89**: 6–15.
- Everett, K.D., Bush, R.M., Andersen, A.A. (1999) Emended description of the order *Chlamydiales*, proposal of *Parachlamydiaceae* fam. nov. and *Simkaniaceae* fam. nov., each containing one monotypic genus, revised taxonomy of the family *Chlamydiaceae*, including a new genus and five new species, and standards for the identification of organisms. *Int J Syst Bacteriol* **49**:415-440.
- Fàbrega, A., Vila, J. (2013) *Salmonella enterica* serovar Typhimurium skills to succeed

- 287 in the host: virulence and regulation. *Clin Microbiol Rev* **26**:308-341.
- 288 Feng, Y., Hsiao, Y.H., Chen, H.L., Chu, C., Tang, P., Chiu, C.H. (2009) Apoptosis-like  
289 cell death induced by *Salmonella* in *Acanthamoeba rhysodes*. *Genomics* **94**:132-137.
- 290 Fritsche, T.R., Gautam, R.K., Seyedirashti, S., Bergeron, D.L., and Lindquist, T.D.  
291 (1993) Occurrence of bacterial endosymbionts in *Acanthamoeba* spp. isolated from  
292 corneal and environmental specimens and contact lenses. *J Clin Microbiol* **31**: 1122–  
293 1126.
- 294 Fukumoto, T., Matsuo, J., Okubo, T., Nakamura, S., Miyamoto, K., Oka, K., Takahashi,  
295 M., Akizawa, K., Shibuya, H., Shimizu, C., Yamaguchi, H. (2016) *Acanthamoeba*  
296 containing endosymbiotic chlamydia isolated from hospital environments and its  
297 potential role in inflammatory exacerbation. *BMC Microbiol* **16**:292.
- 298 Górnik, K., and Kuzna-Grygiel, W. (2004) Presence of virulent strains of amphizoic  
299 amoebae in swimming pools of the city of Szczecin. *Ann Agric Environ Med* **11**:  
300 233–236.
- 301 Greub, G., Raoult, D. (2004) Microorganisms resistant to free-living amoebae. *Clin*  
302 *Microbiol Rev* **17**:413-33.
- 303 Heinz, E., Kolarov, I., Kastner, C., Toenshoff, E.R., Wagner, M., and Horn, M. (2007)  
304 An *Acanthamoeba* sp. containing two phylogenetically different bacterial  
305 endosymbionts. *Environ Microbiol* **9**: 1604–1609.
- 306 Horn, M., and Wagner, M. (2004) Bacterial endosymbionts of free-living amoebae. *J*  
307 *Eukaryot Microbiol* **51**: 509–514.
- 308 Ishida K, Matsuo J, Yamamoto Y, Yamaguchi H. (2014) *Chlamydia pneumoniae* effector  
309 chlamydial outer protein N sequesters fructose bisphosphate aldolase A, providing a

benefit to bacterial growth. *BMC Microbiol* **14**:330.

Ishida, K., Sekizuka, T., Hayashida, K., Matsuo, J., Takeuchi, F., Kuroda, M., Nakamura, S., Yamazaki, T., Yoshida, M., Takahashi, K., Nagai, H., Sugimoto, C., Yamaguchi, H. (2014) Amoebal endosymbiont *Neochlamydia* genome sequence illuminates the bacterial role in the defense of the host amoebae against *Legionella pneumophila*. *PLoS One* **9**:e95166.

Khan, N.A. (2006) *Acanthamoeba*: biology and increasing importance in human health. *FEMS Microbiol Rev* **30**: 564–595.

Kilic, A., Tanyuksel, M., Sissons, J., Jayasekera, S., and Khan, N.A. (2004) Isolation of *Acanthamoeba* isolates belonging to T2, T3, T4 and T7 genotypes from environmental samples in Ankara, Turkey. *Acta Parasitologica* **49**: 246–252.

Knecht, D.A., LaFleur, R.A., Kahsai, A.W., Argueta, C.E., Beshir, A.B., Fenteany, G. (2010) Cucurbitacin I inhibits cell motility by indirectly interfering with actin dynamics. *PLoS One* **5**:e14039.

Lau, H.Y., Ashbolt, N.J. (2009) The role of biofilms and protozoa in *Legionella* pathogenesis: implications for drinking water. *J Appl Microbiol* **107**:368-78.

Lessard, J.L. (1988) Two monoclonal antibodies to actin: one muscle selective and one generally reactive. *Cell Motil Cytoskeleton* **10**: 349-62.

Lorenzo-Morales, J., Ortega-Rivas, A., Foronda, P., Martinez, E., and Valladares, B. (2005a) Isolation and identification of pathogenic *Acanthamoeba* strains in Tenerife, Canary Islands, Spain from water sources. *Parasitol Res* **95**: 273–277.

Marciano-Cabral, F., and Cabral, G. (2003) *Acanthamoeba* spp. as agents of disease in humans. *Clin Microbiol Rev* **16**: 273–307.

- 333 Matsuo, J., Hayashi, Y., Nakamura, S., Sato, M., Mizutani, Y., Asaka, M., Yamaguchi, H.  
 334 (2008) Novel *Parachlamydia acanthamoebae* quantification method based on  
 335 coculture with amoebae. *Appl Environ Microbiol* **74**:6397-404.
- 336 Matsuo, J., Kawaguchi, K., Nakamura, S., Hayashi, Y., Yoshida, M., Takahashi, K.,  
 337 Mizutani, Y., Yao, T., Yamaguchi, H. (2010) Survival and transfer ability of  
 338 phylogenetically diverse bacterial endosymbionts in environmental *Acanthamoeba*  
 339 isolates. *Environ Microbiol Rep* **2**:524-33.
- 340 Nakamura, S., Matsuo, J., Hayashi, Y., Kawaguchi, K., Yoshida, M., Takahashi, K.,  
 341 Mizutani, Y., Yao, T., Yamaguchi, H. (2010) Endosymbiotic bacterium  
 342 *Protochlamydia* can survive in *acanthamoebae* following encystation. *Environ*  
 343 *Microbiol Rep* **2**:611-8.
- 344 Nellen, W., Gallwitz, D. (1982) Actin genes and actin messenger RNA in  
 345 *Acanthamoeba castellanii*. Nucleotide sequence of the split actin gene I. *J Mol Biol*  
 346 **159**: 1-18.
- 347 Okude, M., Matsuo, J., Nakamura, S., Kawaguchi, K., Hayashi, Y., Sakai, H., Yoshida,  
 348 M., Takahashi, K., Yamaguchi, H. (2012) Environmental chlamydiae alter the growth  
 349 speed and motility of host *acanthamoebae*. *Microbes Environ* **27**:423-9.
- 350 Rao, C., Benhabib, H., Ensminger, A.W. (2013) Phylogenetic reconstruction of the  
 351 *Legionella pneumophila* Philadelphia-1 laboratory strains through comparative  
 352 genomics. *PLoS One* **8**: e64129.
- 353 Rivera, F., Roy-Ocotla, G., Rosas, I., Ramirez, E., Bonilla, P., and Lares, F. (1987)  
 354 Amoebae isolated from the atmosphere of Mexico City and environs. *Environ Res*  
 355 **42**: 149–154.



- 356 Sampo, A., Matsuo, J., Yamane, C., Yagita, K., Nakamura, S., Shouji, N., Hayashi, Y.,  
 357 Yamazaki, T., Yoshida, M., Kobayashi, M., Ishida, K., Yamaguchi, H. (2014)  
 358 High-temperature adapted primitive *Protochlamydia* found in *Acanthamoeba*  
 359 isolated from a hot spring can grow in immortalized human epithelial HEp-2 cells.  
 360 *Environ Microbiol* **16**:486-97.
- 361 Sawyer, T.K. (1989) Free-living pathogenic and nonpathogenic amoebae in Maryland  
 362 soils. *Appl Environ Microbiol* **55**: 1074–1077.
- 363 Schmitz-Esser, S., Toenshoff, E.R., Haider, S., Heinz, E., Hoenninger, V.M., Wagner, M.,  
 364 and Horn, M. (2008) Diversity of bacterial endosymbionts of environmental  
 365 *Acanthamoeba* isolates. *Appl Environ Microbiol* **74**: 5822–5831.
- 366 Schroeder, J.M., Booton, G.C., Hay, J., Niszl, I.A., Seal, D.V., Markus, M.B., Fuerst,  
 367 P.A., Byers, T.J. (2001) Use of subgenic 18S ribosomal DNA PCR and sequencing  
 368 for genus and genotype identification of acanthamoebae from humans with keratitis  
 369 and from sewage sludge. *J Clin Microbiol* **39**: 1903-11.
- 370 Schuster, F.L., and Visvesvara, G.S. (2004) Free-living amoebae as opportunistic and  
 371 non-opportunistic pathogens of humans and animals. *Int J Parasitol* **34**: 1001–1027.
- 372 Scidmore, M.A. (2011) Recent advances in *Chlamydia* subversion of host cytoskeletal  
 373 and membrane trafficking pathways. *Microbes Infect* **13**:527-35.
- 374 Tezcan-Merdol, D., Ljungström, M., Winiecka-Krusnell, J., Linder, E., Engstrand, L.,  
 375 Rhen, M. (2004) Uptake and replication of *Salmonella enterica* in *Acanthamoeba*  
 376 *rhysodes*. *Appl Environ Microbiol* **70**:3706-14
- 377 Thomas, V., Casson, N., and Greub, G. (2006) *Criblamydia sequanensis*, a new  
 378 intracellular *Chlamydiales* isolated from Seine river water using amoebal co-culture.

*Environ Microbiol* **8**: 2125–2135.

Tsvetkova, N., Schild, M., Panaiotov, S., Kurdova-Mintcheva, R., Gottstein, B., Walochnik, J., et al. (2004) The identification of free-living environmental isolates of amoebae from Bulgaria. *Parasitol Res* **92**: 405–413.

Van Haastert, P.J. (2011) Amoeboid cells use protrusions for walking, gliding and swimming. *PLoS One* **6**:e27532. \*27. Fukui, Y., Uyeda, T.Q., Kitayama, C., Inoué, S. (2000) How well can an amoeba climb? *Proc Natl Acad Sci USA* **97**:10020-5.

Visvesvara, G.S., Moura, H., and Schuster, F.L. (2007) Pathogenic and opportunistic free-living amoebae: *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri*, and *Sappinia diploidea*. *FEMS Immunol Med Microbiol* **50**: 1–26.

Westendorf, C., Negrete, J.Jr., Bae, A.J., Sandmann, R., Bodenschatz, E., Beta, C. (2103) Actin cytoskeleton of chemotactic amoebae operates close to the onset of oscillations. *Proc Natl Acad Sci USA* **110**:3853-8.

Yamaguchi, H., Matsuo, J., Yamazaki, T., Ishida, K., Yagita, K. (2015) Draft genome sequence of high-temperature-adapted *Protochlamydia* sp. HS-T3, an amoebal endosymbiotic bacterium found in *Acanthamoeba* isolated from a hot spring in Japan. *Genome Announc* **3**: e01507-14.

## Legends to figures

**Fig. 1.** Movement of amoebae carrying GFP-expressing *E. coli* DH5 $\alpha$  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 strain. 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  $\mu$ g/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* DH5 $\alpha$  (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

agar for 1 day, at 37°C. Agar were supplemented with chloramphenicol (10 µg/mL) to maintain the plasmid. Amoebae ( $1 \times 10^5$  cells, counted using a cell counting chamber) were mixed with GFP-expressing *E. coli* (approximately  $1 \times 10^7$  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 LB (diameter, 6 cm) agar plates. The plates were incubated at 30°C in a moist environment (about 100% humidity) for up to 14 days, and amoebal walking was then visualized using the fluorescent bacterial signals as a tracer under a transilluminator.

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

**Fig. 2.** Movement of amoebae carrying GFP-expressing *Salmonella* Enteritidis YH0815 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 *A. castellanii* C3 (ATCC 50739). Enlarged images of S13WT (dashed square with red color) and S13RFP (dashed square with blue color) amoebal tracks. See the legend for Fig. 1. **B.** Changes in the fluorescence of areas corresponding to GFP-expressing *Salmonella* Enteritidis. The bacterial strain was genetically modified to carry a GFP-expressing plasmid (pBBR122 encoding *gfp*) for ease of visualization. See the legend for Fig. 1. Data are expressed as average pixel values  $\pm$  SD. Comparisons among groups were performed using a multiple comparison test for parametric analysis using the Bonferroni/Dunn method. Asterisks indicate a *p*-value  $< 0.05$  (considered significant) vs. other values at the same time point.

**Fig. 3.** Movement of amoebae carrying other bacteria (**A.** *P. aeruginosa* ATCC 27853; **B.** *S. maltophilia* DA185) on LB agar plates. Changes in fluorescence patterns up to 8 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 *A. castellanii* C3 (ATCC 50739). See the legend for Fig. 1.

**Fig. 4.** Amoebal tracks on LB agar plates in the first 48 h post-simultaneous inoculation with GFP-expressing *E. coli*. **A.** Microscopic observation of the amoebal tracks at 12 h 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,  $\times 100$ . **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,  $\times 100$ .

**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<sup>-</sup>/T4BSS<sup>+</sup>/Tra<sup>+</sup>) (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 ( $1 \times 10^5$  cells, counted using a cell counting chamber) were mixed with GFP-expressing *E. coli* DH5 $\alpha$  (approximately  $1 \times 10^7$  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  $\mu$ g/mL). A 20- $\mu$ L 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* DH5 $\alpha$  (**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.

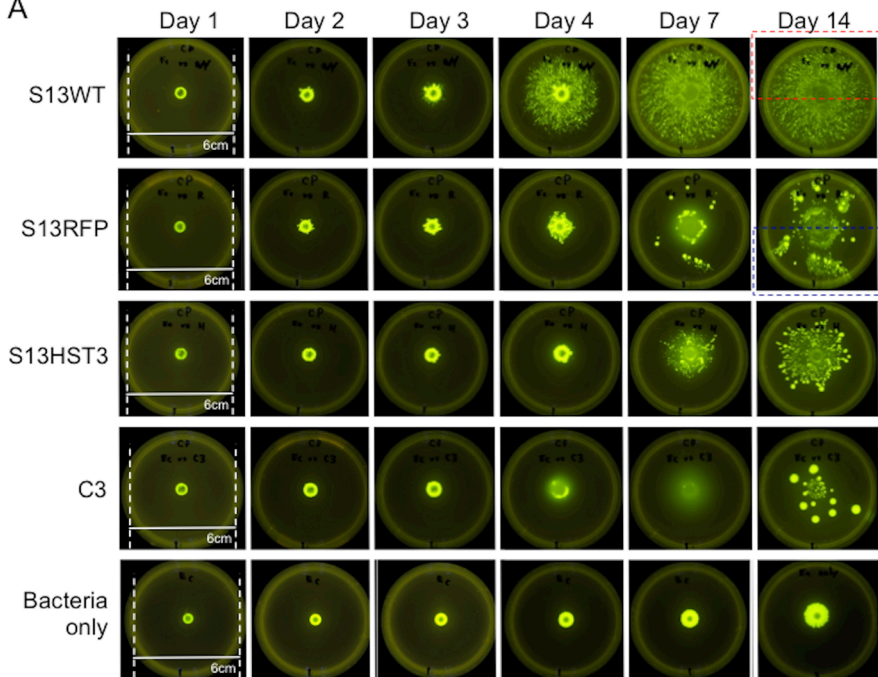
**Supplementary Fig. 3 (Fig. S3)** Time course showing the movement of amoebae on the surface of B-CYE agar plates for a prolonged period. Amoebae were spotted with GFP-expressing *E. coli* into the center of a ring of mCherry-expressing *L. pneumophila* Lp01. S13WT; symbiotic amoeba strain S13WT harboring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



aprosymbiotic 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



B

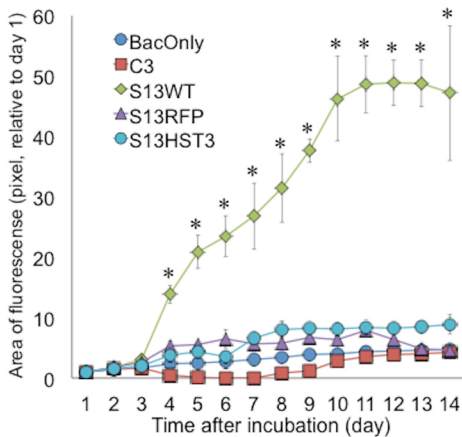
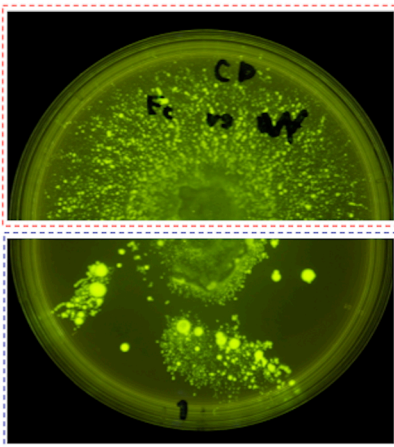
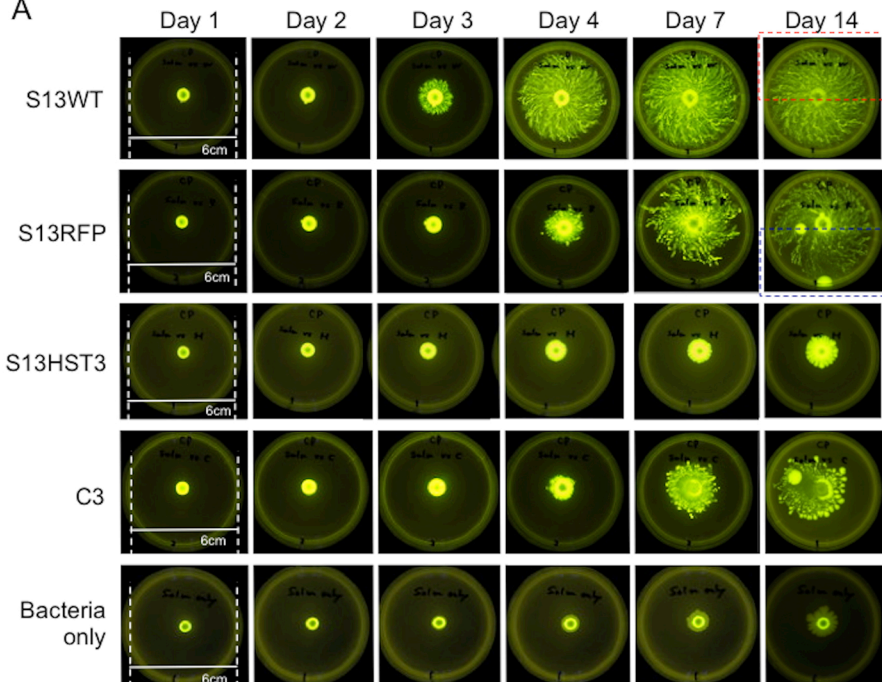


Fig. 2

A



B

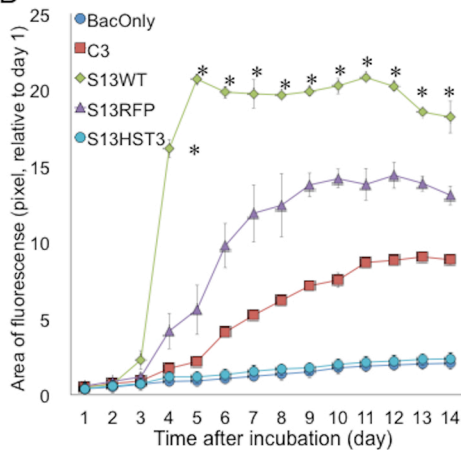
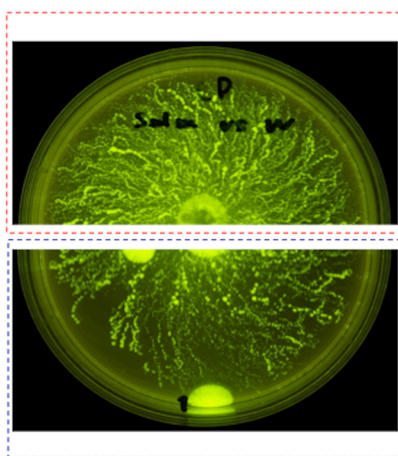
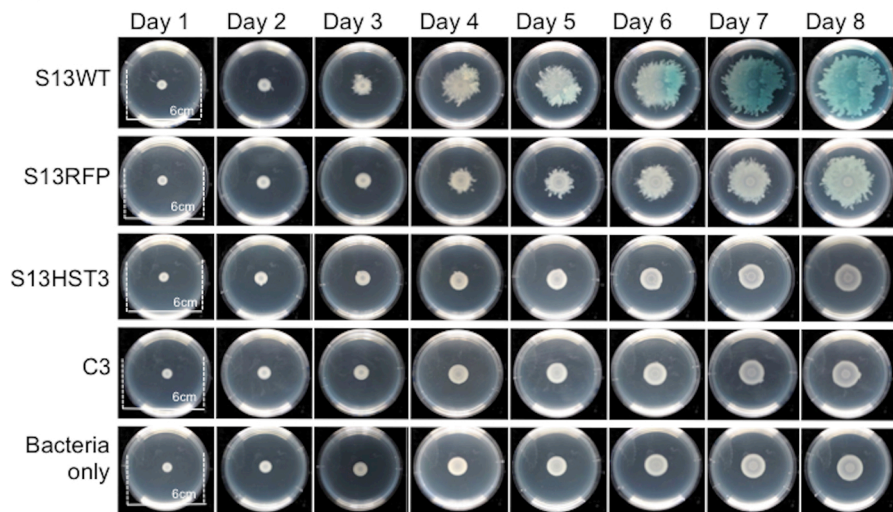


Fig. 3

A



B

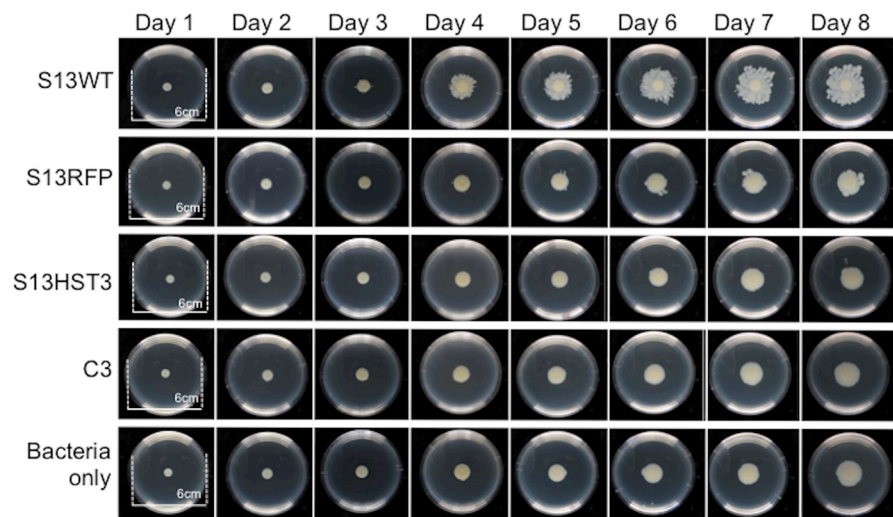
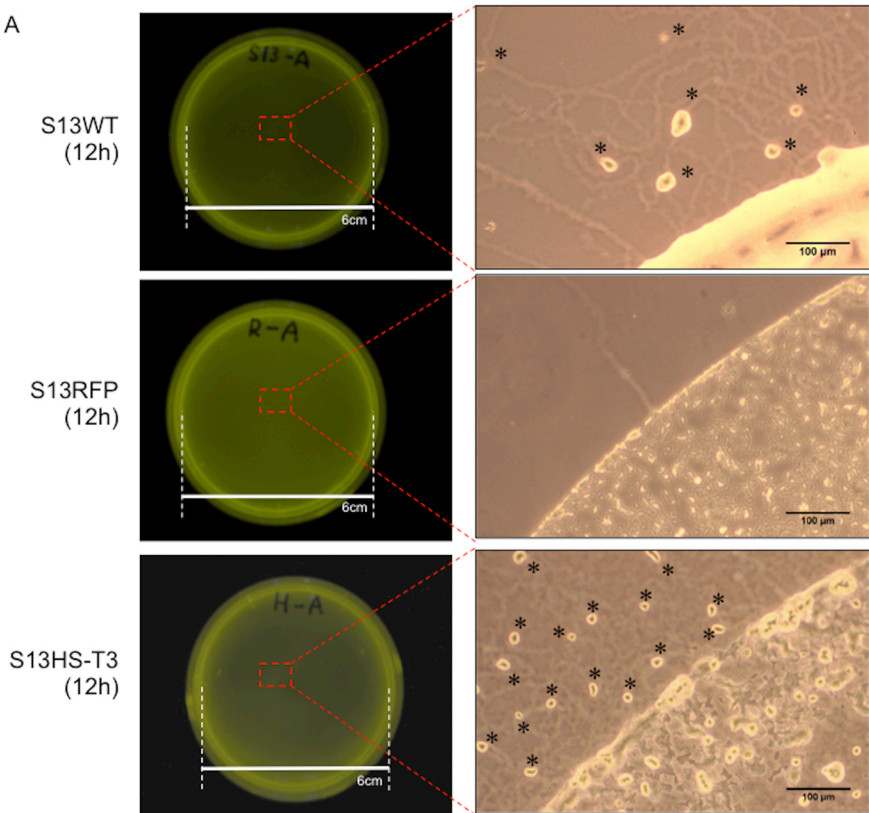


Fig. 4

A



B

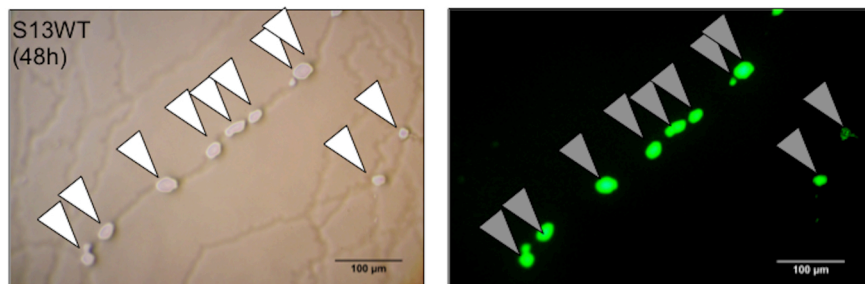
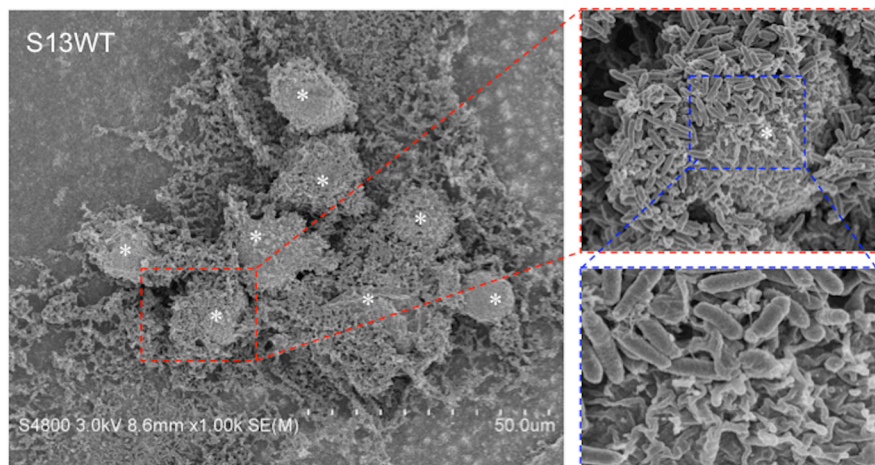




Fig. 5

A



B

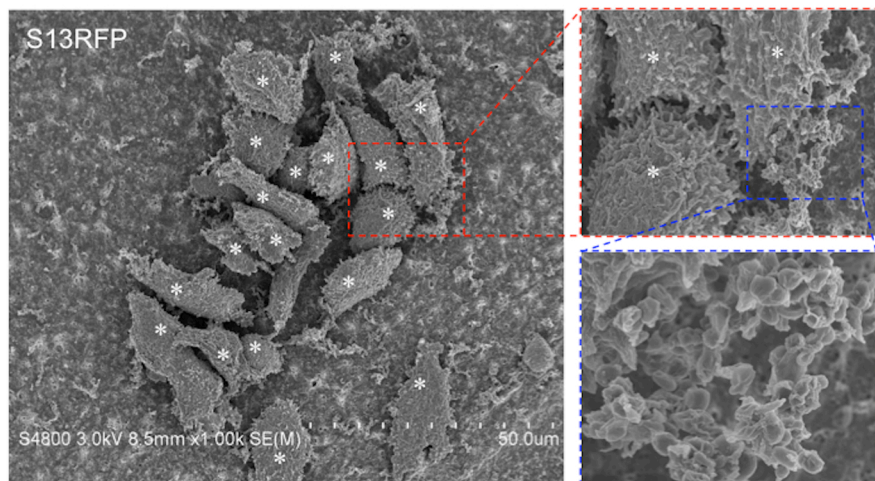


Fig. 6

