Tetrahymena promotes interactive transfer of carbapenemase gene encoded in plasmid between fecal *Escherichia coli* and environmental *Aeromonas caviae*

Running title: Ciliates promote bacterial gene transfer

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ABSTRACT

Ciliates (*Tetrahymena*) can facilitate plasmid transfer among *Escherichia coli* or from *E. coli* to *Salmonella* Enteritidis via vesicle accumulation. In this study, we assessed whether ciliates promote the interactive transfer of plasmids encoding $bla_{\text{IMP-1}}$ between fecal *E. coli* and environmental *Aeromonas caviae*. Both bacteria were mixed with or without ciliates and incubated overnight at 30°C. The frequency of plasmid-acquired bacteria was estimated by colony counts using an agar plate containing ceftazidim (CAZ) followed by minimum inhibitory concentration (MIC) assessment. Cultures containing ciliates interactively transferred the plasmid between *E. coli* and *Aeromonas* with a frequency of 10^{-4} to 10^{-5}. All plasmid-acquired bacteria showed a MIC against CAZ of >128μg/ml, and the plasmid transfer was confirmed by polymerase chain reaction (PCR) amplification of the $bla_{\text{IMP-1}}$ gene. Fluorescent observation showed both bacteria accumulated in the same vesicle and that transwell sequestering significantly decreased the transfer frequency. Although ciliates preferentially ingested *E. coli* rather than *A. caviae*, both bacteria were co-localized into same vesicles of ciliates, indicating that their meeting was associated with the gene transfer. Thus, ciliates interactively promote

plasmid transfer between *E. coli* and *A. caviae*. The results of this study will facilitate control of the spread of multiple-antibiotic resistant bacteria.

Keywords: *Aeromonas caviae*; ciliates; gene transfer; *Escherichia coli*; one health

INTRODUCTION

There is currently a pandemic of multidrug-resistant (MDR) *Enterobacteriaceae* producing extended spectrum β-lactamase (ESBL), such as carbapenemase encoded by plasmids such as bla_{NDM} or bla_{IMP}, which resist carbapenem, and their continuing spread is a growing global concern that is becoming an immeasurable threat to hospitals and other healthcare-associated facilities (1-5). Additionally, lack of comprehensive management protocols encompassing livestock, circulating food, public hygiene practices, and monitoring of natural environments has resulted in failure to control dissemination of ESBL-producing bacteria to or from humans through fecal waste, contaminated water, and processed meat products (6-9). Therefore, a "One Health" approach requiring the combined efforts of physicians, veterinarians, epidemiologists, public health workers and urban planners worldwide has been proposed to control the emergence of MDR bacteria (10).

Ciliate protozoa (*Tetrahymena*) ubiquitously inhabit animal rumens or natural environments such as rivers and ponds in most of the world (11-13). The organisms are at the top of the complex-food chain in the microbial community, where they act as grazers

71 that feed on bacteria (14). Moreover, it has been reported that ciliates facilitate cell-to-cell
72 contact among bacteria captured in their vesicles by expelling vesicles with
73 undigested-live bacteria to outside environments, thereby contributing to gene transfer
74 (15, 16). Indeed, we previously demonstrated that ciliates strongly promote transfer of the
75 Tn*phoA*, which encodes alkaline phosphatase, in *Escherichia coli* (SM10 λ *pir*$^{+}$ pRT733^{+}
76 with mobRP4) to a clinical isolate of *E. coli* via vesicle accumulation (17). We also found
77 that ciliates can facilitate the transfer of plasmids encoding $bla_{\text{NDM-5}}$ between *E. coli*
78 strains or from *E. coli* to *Salmonella* Enteritidis (18, 19). These findings suggest that
79 ciliates support effective plasmid transfer among bacteria in natural environments and are
80 involved in their circulation between humans and natural environments. Alternatively, we
81 speculated that ciliates can be a hotspot in natural environments, facilitating bacterial
82 plasmid transfer from human pathogens to environmental bacteria, responsible for
83 spreading plasmid into the environments, presumably coming the plasmid back to human
84 pathogenic bacteria. Additionally, *Aeromonas caviae* could play a role as both the
85 recipient and donor of plasmids to human pathogenic bacteria because they are
86 environmental bacteria that inhabit rivers and coastal areas as a fish pathogen, and are

therefore closely connected to human life. (20). However, it is not yet known if ciliates can promote the transfer of plasmids between fecal *E. coli* and environmental organisms such as *Aeromonas caviae*.

In this study, we assessed whether ciliates promoted the interactive transfer of plasmids encoding bla_{IMP-1} between fecal *E. coli* and environmental *A. caviae*. We present here the first evidence of the interactive transfer of this plasmid between *E. coli* and *A. caviae* in two distinct *Tetrahymena*.

MATERIALS AND METHODS

Ciliates and bacteria used for this study

Table 1 shows a list of the traits and sources of ciliates and bacteria used in this study. Two distinct ciliate protozoa, *Tetrahymena thermophila* (Tth; kindly provided by Dr. Sugai of Ibaraki University, Japan) and *Tetrahymena* sp. (Tsp; gifted from Dr. Tukii of Hosei University, Japan) were used in this study. Ciliates were maintained in peptone-yeast extract glucose broth (PYG) containing 0.75% peptone (Difco), 0.75% yeast extract (Difco), and 1.5% glucose (Wako) at 30°C as previously described (21). *Citrobacter*

freundii carrying a plasmid encoding bla_{IMP-1} was originally isolated from Hokkaido University Hospital and *Aeromonas caviae* no. 86 was kindly gifted from Professor Y. Tamura, Rakuno Gakuen University. Other bacteria (*E. coli* J53, *E. coli* DH5α, *E. coli* ATCC 29213, and *Staphylococcus aureus* ATCC 25922) were purchased from the American Type Culture Collection (ATCC). *E. coli* TC170328 strain carrying the plasmid encoding bla_{IMP-1} was experimentally established by the broth-mating method with *E. coli* J53 and *C. freundii* as previously described (22). Moreover, green fluorescent protein (GFP)-expressing *E. coli* DH5α was constructed according to a previous study (23), then used for imaging analysis (see below).

Mixed culture and assessment of gene transfer frequency

The frequency of bacterial gene transfer through protozoa was assessed as previously described (17). Briefly, equal amounts (approximately 10^9 colony-forming units [CFU]) of both donor [ceftazidime (CAZ)-resistant] (0.5 ml) and recipient bacteria (0.5 ml) were mixed with or without 10^6 cells of ciliates (1.0 ml) in Page's amoeba saline (PAS) (24), then incubated for 24 h at 30°C. In addition, an experiment with a transwell (0.4 μm pore

size) was conducted to separate ciliates from donor and recipient bacteria. After incubation, the solution was spread on Trypticase-soy agar (Nissui Pharmaceutical, Japan) containing 50 μg/ml of CAZ and 50 μg/ml of irgasan (Sigma-Aldrich) for *A. caviae*-derived plasmid-acquired bacteria or Luria-Bertani (LB) agar (Nacalai tesque) containing 50 μg/ml of CAZ and 100 μg/ml of sodium azide (Wako) for *E. coli* J53-derived plasmid-acquired bacteria. The remaining solution was used for DNA extraction for polymerase chain reaction (PCR). The gene transfer frequency was calculated by the following equation: frequency of transfer events = the number of plasmid-acquired bacterial colonies/total colony numbers (donor plus recipient).

DNA extraction and PCR

DNA was extracted from a single-colony culture of the bacteria (donor, recipient and plasmid-acquired bacteria) by boiling as previously described (17), after which 2 μl of boiling template was used for PCR with primers against $bla_{\text{IMP-1}}$ (25). The replicon type of the $bla_{\text{IMP-1}}$-encoding plasmid was also determined by PCR (26).

Imaging

To confirm co-localization of *E. coli* and *A. caviae* in ciliate vesicles, 24-h-mixed cultures comprising ciliates (*T. thermophile* or *T. pyriformis*), GFP-expressing *E. coli* DH5α (fluorescence color: green,) (22) and vital-stained *A. caviae* no. 86 (fluorescence color: red) were fixed with 4% formalin and then analyzed by fluorescence microscopy (BioZero, Keyence). Vital staining of bacteria was performed using a PKH-26 labeling kit (PKH-26GL, Sigma) according to the manufacturer's protocols.

Plasmid-acquired bacterial determination

Bacterial identification was accomplished using a Matrix Assisted Laser Desorption/Ionization (MALDI) Biotyper system (Bruker Daltonics) according to the manufacturer's instructions.

Antimicrobial susceptibility test

The minimum inhibitory concentration (MIC) of CAZ against donors, recipients, and plasmid-acquired bacteria in this study was determined by the agar-dilution method (27).

151 *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 were used as quality control strains.

152

153 ## Grazing assay

154 Briefly, equal amounts (approximately 10^9 colony-forming units [CFU]) of *E. coli* DH5α
155 (5 ml PAS) or *A. caviae* no. 86 (5 ml PAS)) were mixed with or without 10^6 cells of
156 ciliates, Tth or Tsp (10 ml PAS), then incubated for 24 h at 30°C. Samples were
157 subsequently collected at 0, 1, 2, 4, 8, and 24 h after incubation, and colony numbers of
158 each bacteria were estimated by distinct colony color (*E. coli*, blue: *A. caviae*, white) on
159 Colimark agar plates (Eiken). Grazing rates were expressed as decreasing bacterial rate
160 (%) at 24 h compared to starting bacterial number (100%) immediately after incubation.

161

162 ## Meeting frequency

163 Amounts (approximately 10^8 colony-forming units [CFU]) of GFP-expressing *E. coli*
164 DH5α and vital stained *A. caviae* no. 86 (See above) were mixed with 10^5 cells of ciliates,
165 Tth or Tsp in 1 ml of PAS, then incubated for 1 h at 30°C. Following incubation, mixed
166 solutions were fixed with 4% formalin and then analyzed to estimate the meeting

frequency by fluorescence microscopy (BioZero, Keyence), which was determined by counting the number of vesicles (green, *E. coli* alone; red, *A. caviae* alone; yellow, both) formed into ciliates. Microscopic fields were randomly selected and at least 100 ciliate cells were counted.

Statistical analysis

All experiments were repeated at least three times. Statistical analysis was conducted using the Mann-Whitney U-test. A p value <0.05 was considered to be significant.

RESULTS

Transfer frequency of the plasmid encoding bla_{IMP-1} between *E. coli* and *A. caviae* in cultures with ciliates

First, we assessed if ciliates were associated with increased transfer of a plasmid encoding bla_{IMP-1} of *E. coli* TC17032828 (Donor) to *A. caviae* no. 86 (recipient) (Fig. 1A, left). In contrast to the absence of ciliates (1.0×10^{-7}), the gene transfer frequency was

183 increased in the presence of ciliates to 1.0×10^{-5} (Tth) [$p<0.05$ vs. ciliate(-)] or 1.0×10^{-4}
184 (Tsp) [$p<0.05$ vs. ciliate(-)] (Fig. 1B, left). Successful gene transfer was also confirmed
185 by PCR amplification with primers specific to $bla_{\text{IMP-1}}$ (Fig. 1C, left). Next, we assessed
186 whether ciliates facilitated reverse transfer of the *A. caviae* no. 86^{r} plasmid to *E. coli* J 53
187 (Fig. 1A, right). As expected, the gene transfer frequency was significantly increased in
188 the presence of ciliates with a frequency of 1.0×10^{-4} (Tth) [$p<0.05$ vs. ciliate(-)] or
189 1.0×10^{-4} (Tsp) [$p<0.05$ vs. ciliate(-)] (Fig. 1B, right). Successful plasmid transfer was also
190 confirmed by PCR amplification with primers specific to $bla_{\text{IMP-1}}$ (Fig. 1C, right).
191 Moreover, assessment of MICs revealed that plasmid-acquired bacteria (*E. coli*
192 TC17032828 and *A. caviae* no. 86^{r}) were resistant to CAZ, similar to those of *C. freundii*
193 carrying the plasmid encoding $bla_{\text{IMP-1}}$, an origin of the plasmid (Table 2). These results
194 also confirmed that representative colonies of plasmid-acquired bacteria were correctly
195 identified to the species level using the MALDI Biotyper system (Supporting Information
196 Fig S1). Overall, the results of this study indicate that cultures with ciliates could
197 interactively prompt the transfer of plasmids encoding $bla_{\text{IMP-1}}$ between *E. coli* and *A.*
198 *caviae*. In addition, PCR-based replicon typing showed that the $bla_{\text{IMP-1}}$-encoding

plasmid was incA/C type.

Co-localization of *E. coli* and *A. caviae* in ciliate vesicles, and the influence of separating bacteria from ciliates on gene transfer frequency

We evaluated whether the accumulation of two different kinds of bacteria [GFP-expressing *E. coli* DH5α (green) and vital-stained *A. caviae* no. 86^r carrying a plasmid encoding bla_{IMP-1} with PKH-26 (red)] could occur in the same ciliate vesicle. Co-localization of the two different bacteria in the same ciliate vesicle was observed, regardless of ciliate strains, suggesting that the gene transfer events effectively occurred through bacterial accumulation in the ciliate vesicle (Fig. 2). Interestingly, some vesicles packed with both bacteria were expelled from ciliates, after which these bacteria were released to the outside of disrupted vesicles, implying circulation of bacteria via ciliates in culture (Fig. 2, bottom right panel). To confirm this hypothesis, we estimated the gene transfer frequency of *A. caviae* no. 86^r carrying a plasmid encoding bla_{IMP-1} to *E. coli* J53 when ciliates were segregated from these bacteria with a transwell membrane. Segregation significantly diminished the gene transfer frequency when compared to

215 cultures without the transwell, regardless of ciliate strains (Fig. 3).

216

217 **Grazing rate and meeting frequency showing both ciliates favor *E. coli***

218 **rather than *A. caviae* for digesting their bacteria**

219 To determine why the gene transfer frequency from *E. coli* (donor) to *A. caviae*

220 (recipient) was inferior to that from *A. caviae* (donor) to *E. coli* (recipient), we estimated

221 the grazing rate (decreasing bacterial number) with meeting frequency of both ciliates on

222 digesting their bacteria. As a result, grazing rates of Tth and Tsp at 24 h after incubation

223 with *E. coli* were 29.1±48.2% and 1.7±1.5%, respectively (Fig. 4A). Meanwhile, the rates

224 of Tth and Tsp with *A. caviae* were 171.6±277.6% and 51.5±26.1%, respectively (Fig.

225 4B). The results showed both ciliates preferred to ingest *E. coli* rather than *A. caviae*. To

226 confirm this, we assessed the meeting frequency of the bacteria inside the vesicles of

227 ciliates. As expected, *E. coli* alone was more frequently seen in the vesicles than *A. caviae*,

228 regardless of ciliate strains used for this study [Fig. 5A (green, *E. coli*; red, *A. caviae*;

229 yellow, both together) and B (white bar)]. Moreover, both bacteria were found to

230 co-localize into the same vesicles of ciliates (Fig. 5B, see black bars), indicating that their

meeting prompted gene transfer.

DISCUSSION

Multidrug-resistant *Enterobacteriaceae* producing ESBL such as carbapenemase encoded by bla_{IMP} or bla_{NDM}, which are resistant to carbapenem, pose a severe threat to hospitals and other healthcare facilities (1-5). Therefore, it is necessary to develop comprehensive management measures encompassing livestock, circulating food, public hygiene practices, and monitoring of natural environments to control the emergence of these bacteria (6-10). In this study, we demonstrated interactive transfer of the plasmid encoding bla_{IMP-1} between *E. coli* and *A. caviae* in ciliates, indicating a pathway responsible for plasmid transfer among bacteria underlying the circulation of MDR bacteria.

Enterobacteriaceae producing carbapenemase encoded by bla_{IMP-1} have been widely detected in patients and hospital facilities, as well as livestock and aquatic environments, including sewers and rivers, which has led to circulation of these bacteria between

247 humans and the environment (28-31). *E. coli* is a representative fecal bacterium
248 belonging to *Enterobacteriaceae* that can widely adapt to a variety of mammalian bodies,
249 including animal or human intestines. These bacteria contain plasmids that encode drug
250 resistant genes, resulting in the bacterial constant release to natural environments such as
251 rivers or soil. In fact, many studies have revealed the actual release of these bacteria such
252 as *Salmonella* or *E. coli* into natural environments (32-35). Moreover, *Aeromonas* spp.
253 including *A. caviae* comprise a representative natural environmental bacterium widely
254 distributed in coastal areas and rivers as a fish pathogen, as well as in many areas in which
255 people live (36, 37), that serve as a potential reservoir of the plasmid. Therefore, these
256 two bacteria and $bla_{\text{IMP-1}}$ as a factor gene were selected as the donor and recipient to
257 assess gene transfer via *Tetrahymena* cells.

258 The gene transfer frequency between *E. coli* and *A. caviae* was significantly higher in
259 the presence of ciliates. However, when compared with the transfer from *E. coli*
260 TC170328 to *A. caviae* no. 86, ciliates weakly promoted the transfer of the plasmid from
261 *A. caviae* no. 86^{r} to *E. coli* JR53. Because both ciliates favored *E. coli* rather than *A.*
262 *caviae*, the preferential ingestion of *E. coli* rather than *A. caviae* in both ciliates is rigidly

involved in the difference in gene transfer frequency depending on the direction of plasmid transfer between *E. coli* and *A. caviae*. Moreover, sequence analysis revealed that the plasmid was assigned to a type with incA/C, which can effectively mobilize into the *Enterobacteriaceae* family (37).

Imaging analysis revealed co-localization of *E. coli* and *A. caviae* in ciliate vesicles. Because of unsuccessful establishment of fluorescence-expressing bacteria, live labeling of *A. caviae* was conducted by staining with PKH-26, a hydrophobic fluorescence dye. After staining, the bacteria were still viable, indicating that the staining protocol had minimal effects on bacterial survival. After mixed-culture, ciliates that ingested the live-stained *A. caviae* became red. Spread of the dye to the ciliate's cytoplasm revealed obvious ingestion of *A. caviae*. Interestingly, ciliates expelled pellets containing bacteria, which subsequently released the bacteria to the culture, further increasing the frequency of encounter with other environmental bacteria. These findings are concordant with the results of previous studies (15, 16). Meanwhile, because of an increase in the pellet amounts expelled by ciliate (Tsp), it is likely to be changed depending on the ciliate's strain, presumably indicating the presence of optimal sets with bacteria and ciliate strains

279 on accelerating gene transfer frequency. Because several studies have shown that living
280 bacteria such as *Legionella* and non-pathogenic *E. coli* packaged into multi-membrane
281 vesicles can be released outside of protozoa such as *Tetrahymena* or *Acanthamoeba*, such
282 bacterial expulsion may involve the inability to digest bacteria responsible for host
283 cellular protection (38-40). Therefore, in this study, we defined the pellets as being
284 actively expelled, rather than excreted, from *Tetrahymena*.

285 Segregation using a transwell membrane significantly diminished gene transfer
286 frequency, regardless of ciliate strains. Overall, these findings and those presented above
287 indicated that transfer occurred via a series of processes comprising attachment, digestion
288 and accumulation of both bacteria into ciliates. It should be noted that an increasing trend
289 of the baseline of gene transfer frequency was observed the cultures with transwell,
290 although this was not significant. It is well known that filter-associated mating between
291 bacteria is very effective (41, 42); therefore, it is possible that bacterial accumulation on
292 the pore membrane slightly promoted gene transfer frequency.

293 In conclusion, we demonstrated that ciliates promote transfer of plasmids between *E.*
294 *coli* and *A. caviae*. While further study is needed to clarify these results using other

bacteria, the information presented herein will be useful to control of the spread of multiple-antibiotic resistant bacteria.

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DISCLOSURE

The authors declare that they have no conflicts of interest associated with this work. Moreover, the funder had no role in the design, data collection, interpretation, or submission of this study.

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Figure legends

Figure 1 Gene transfer frequency of *E. coli* and *A. caviae* containing the plasmid encoding $bla_{\text{IMP-1}}$ in cultures with ciliates. **A.** Direction of plasmid transfer between *E. coli* and *A. caviae*. A1 and A2 show the direction of plasmid transfer form *E. coli* to *A. caviae* and from *A. caviae* to *E. coli*, respectively. **B.** Plots show frequency of transfer of a plasmid encoding $bla_{\text{IMP-1}}$ from *E. coli* TC170328 (Donor) to *A. caviae* no. 86 (Recipient) (B1) and from *A. caviae* no. 86^{r} back to *E. coli* J 53 (B2). Gene transfer frequency was estimated as the number of plasmid-acquired bacteria for each recipient (see text). Data shown are the averages of gene transfer frequency ± standard deviations. Black and white boxes show the range from the mean to 75% and 25% values, respectively. *, $p< 0.05$ versus values for control without ciliates. **C.** Representative images showing the results of agarose gel electrophoresis of the $bla_{\text{IMP-1}}$ PCR products amplified from the recipient (R) and plasmid-acquired bacteria (Pa). C1 and C2 show the direction of plasmid transfer form *E. coli* to *A. caviae* and from *A. caviae* to *E. coli*, respectively. Pa1–6 show the PCR results of representative plasmid-acquired bacterial colonies. N, negative control

473 [DNAase-free water (sigma)]. M, DNA ladder marker.

474

475 **Figure 2** Co-localization of *E. coli* DH5α and *A. caviae* no. 86^r in ciliate vesicles. Upper
476 and lower sections of each of the four panels show mixed cultures of both bacteria with
477 the ciliates, *T. thermophile* (Tth) and *T. pyriformis* (Tsp), respectively. Green indicates
478 GFP-expressing *E. coli* DH5α, while red shows live stained *A. caviae* no. 86^r. Squares
479 surrounded by dashed lines are enlargements of the same image (lower right panel). Scale,
480 50 μm.

481

482 **Figure 3** Influence of separating bacteria (*A. caviae* no. 86^r and *E. coli* J53) from ciliates
483 with a transwell on gene transfer frequency. Upper images show the direction of plasmid
484 transfer and images of the experiment. Plots show the gene transfer frequency of *A.*
485 *caviae* no. 86^r (donor) containing a plasmid encoding bla_{IMP-1} to *E. coli* J53 (recipient).
486 Gene transfer frequency was estimated as the number of plasmid-acquired bacteria for
487 each recipient (see the text). Data represent the average gene transfer frequency ±
488 standard deviation. Black and white boxes show the range from the mean to 75% and

489 25% values, respectively. *, $p<0.05$ versus values for control without ciliates of each
490 experiment.
491
492 **Figure 4** Grazing rate of both ciliates when incubated with *E. coli* and *A. caviae*. The
493 rates were monitored for 24 h after incubation as described in the methods. **A.** Grazing
494 rate of Tth with *E. coli* and *A. caviae*. **B.** Grazing rate of Tsp with *E. coli* and *A. caviae*.
495 Tth, *T. thermophile*. Tsp, *Tetrahymena*. Ec DH5α, *E. coli* DH5 α . Ae 86, A. caviae no. 86.
496 *, $p< 0.05$ versus values for control without ciliates at each of the time points.
497
498 **Figure 5** Meeting frequency of *E. coli* and *A. caviae* inside vesicles of both ciliates. The
499 frequency was estimated at 1 h after incubation as described in the methods. **A.**
500 Co-localization of these bacteria inside both ciliates. Green, GFP-expressing *E. coli* DH5
501 α . Red, vital stained *A. caviae* no. 86 with PKH-26 dye. Yellow, both bacteria
502 co-localized inside the vesicles of ciliates. Magnification, ×400. **B.** Assessing the
503 frequency of *E. coli* and *A. caviae* inside vesicles of both ciliates.
504

505 ## Supporting Information

506 **Figure S1**. Representative plasmid-acquired bacteria (*E. coli* TC170328 and *A. caviae* no.

507 86) of colonies identified to the species level using the MALDI Biotyper system.

508

List of Abbreviations:

ATCC, American Type Culture Collection; CAZ, ceftazidime; CFU, colony-forming units; DNA, deoxyribonucleic acid; ESBL, extended spectrum β-lactamase; GFP, green fluorescent protein; LB, Luria-Bertani; MALDI, matrix assisted laser desorption/ionization; MDR, multidrug-resistant; MIC, minimum inhibitory concentration; PAS, Page's amoeba saline; PCR, polymerase chain reaction; PYG, peptone-yeast extract glucose

Fig. 1

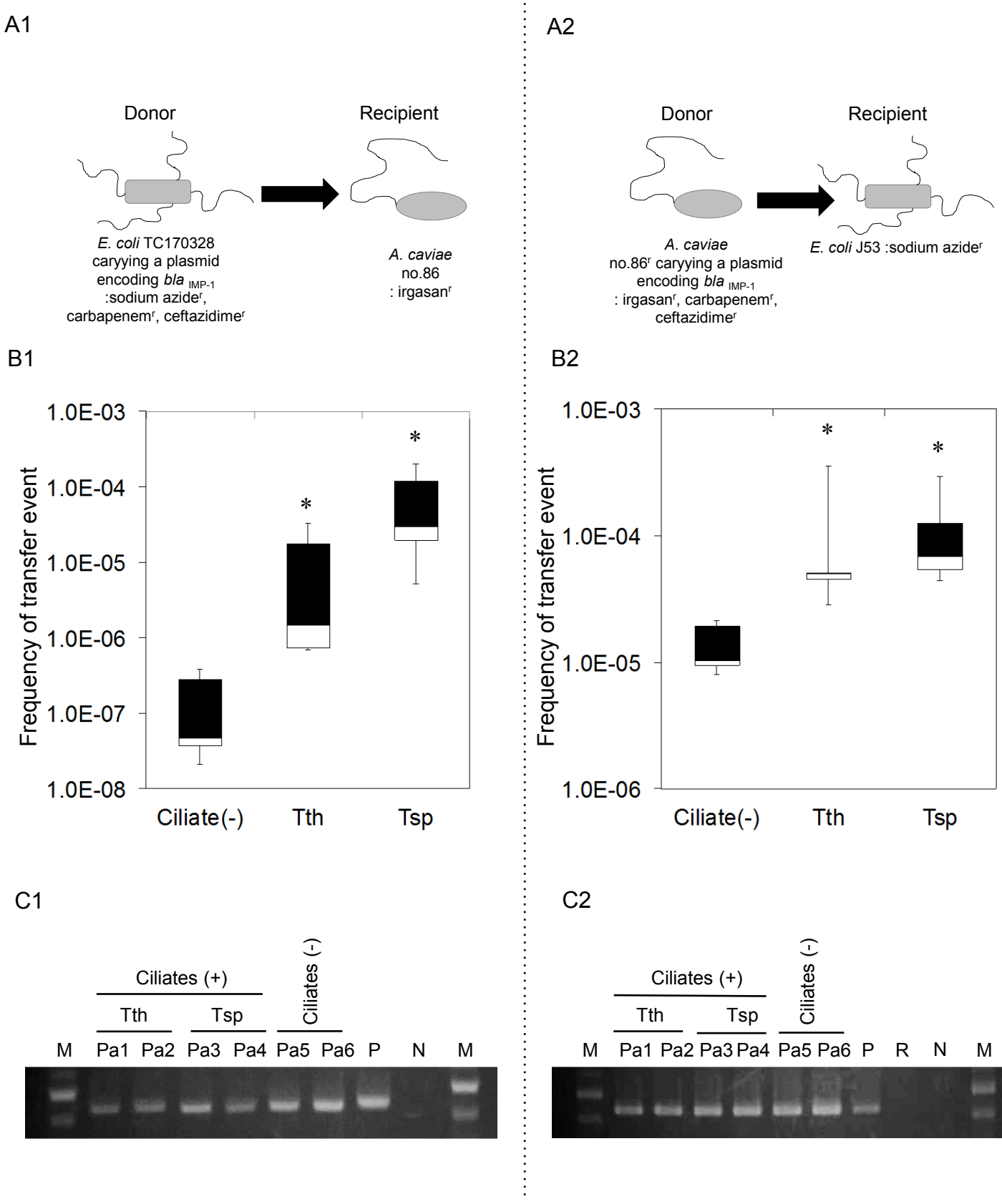
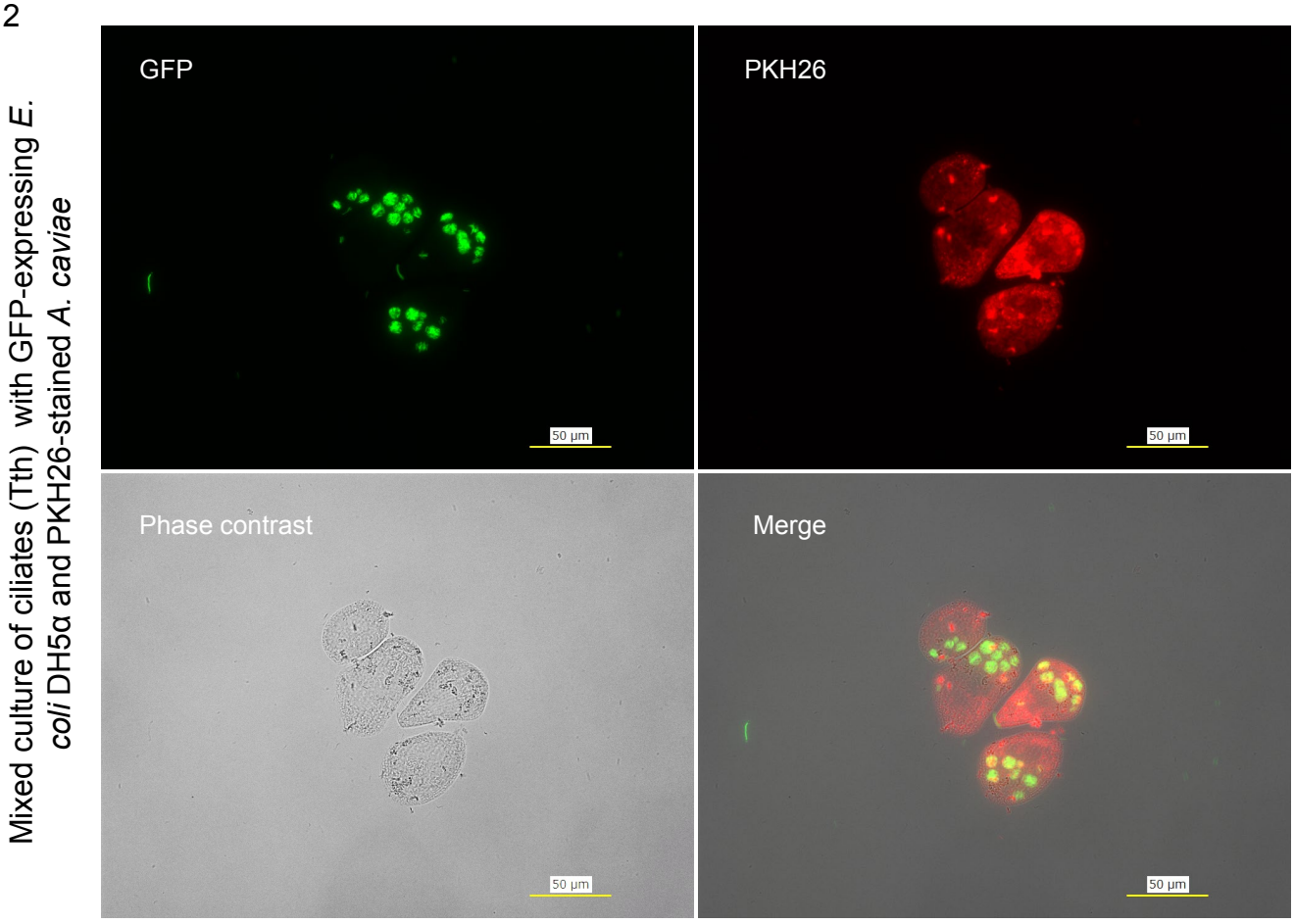

A1
Donor
Recipient
E. coli TC170328 caryying a plasmid encoding bla IMP-1 :sodium azider, carbapenemr, ceftazidimer
A. caviae no.86 : irgasanr
A2
Donor
Recipient
A. caviae no.86r caryying a plasmid encoding bla IMP-1 : irgasanr, carbapenemr, ceftazidimer
E. coli J53 :sodium azider
B1
Frequency of transfer event
1.0E-03
1.0E-04
1.0E-05
1.0E-06
1.0E-07
1.0E-08
Ciliate(-)
Tth
Tsp
B2
Frequency of transfer event
1.0E-03
1.0E-04
1.0E-05
1.0E-06
Ciliate(-)
Tth
Tsp
C1
Ciliates (+)
Tth
Tsp
Ciliates (-)
M Pa1 Pa2 Pa3 Pa4 Pa5 Pa6 P N M
C2
Ciliates (+)
Tth
Tsp
Ciliates (-)
M Pa1 Pa2 Pa3 Pa4 Pa5 Pa6 P R N M

Fig. 2


Mixed culture of ciliates (Tth) with GFP-expressing E. coli DH5α and PKH26-stained A. caviae
GFP
PKH26
Phase contrast
Merge
50 µm

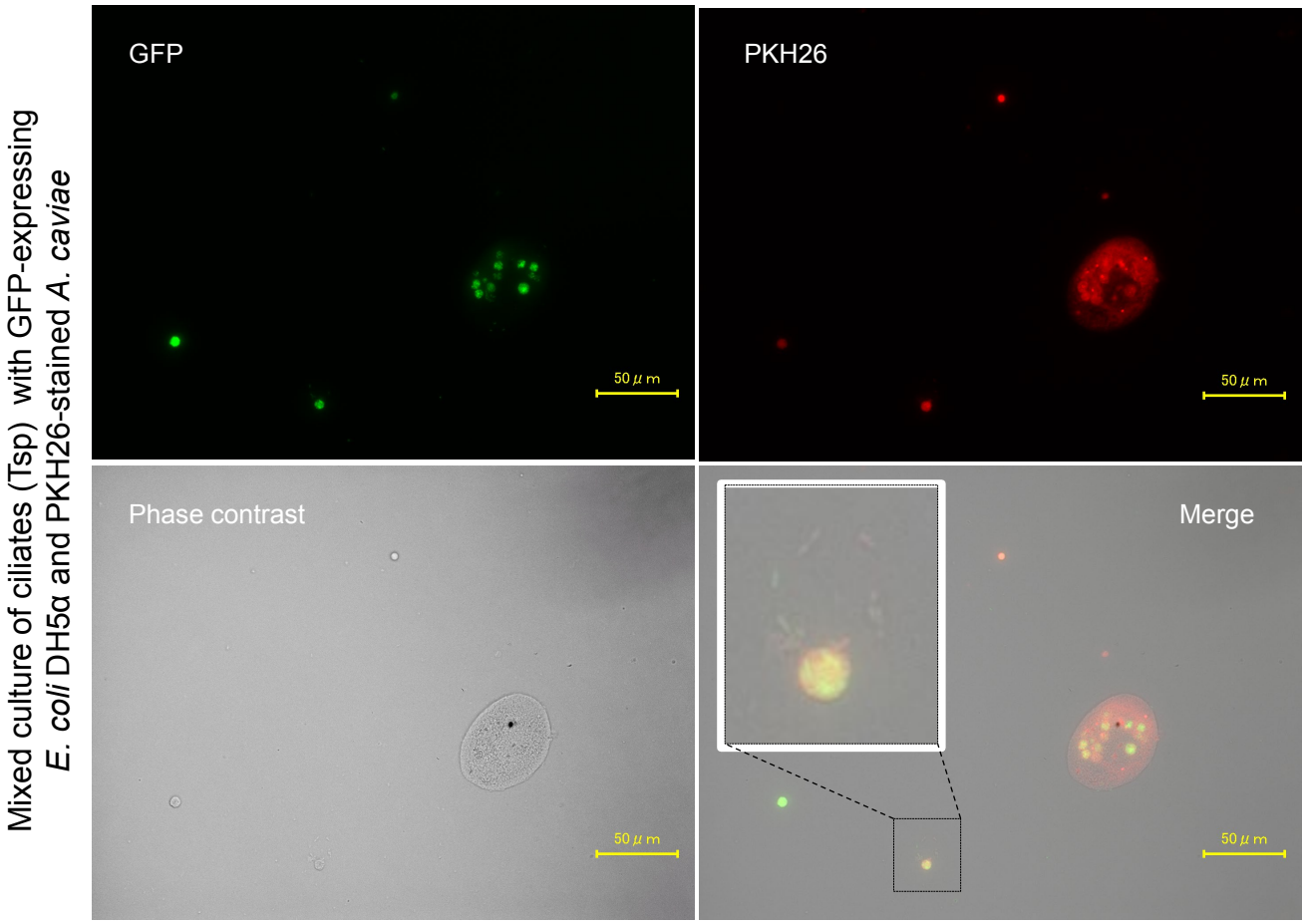

Mixed culture of ciliates (Tsp) with GFP-expressing E. coli DH5α and PKH26-stained A. caviae
GFP
PKH26
Phase contrast
Merge
50 µm

Fig. 3

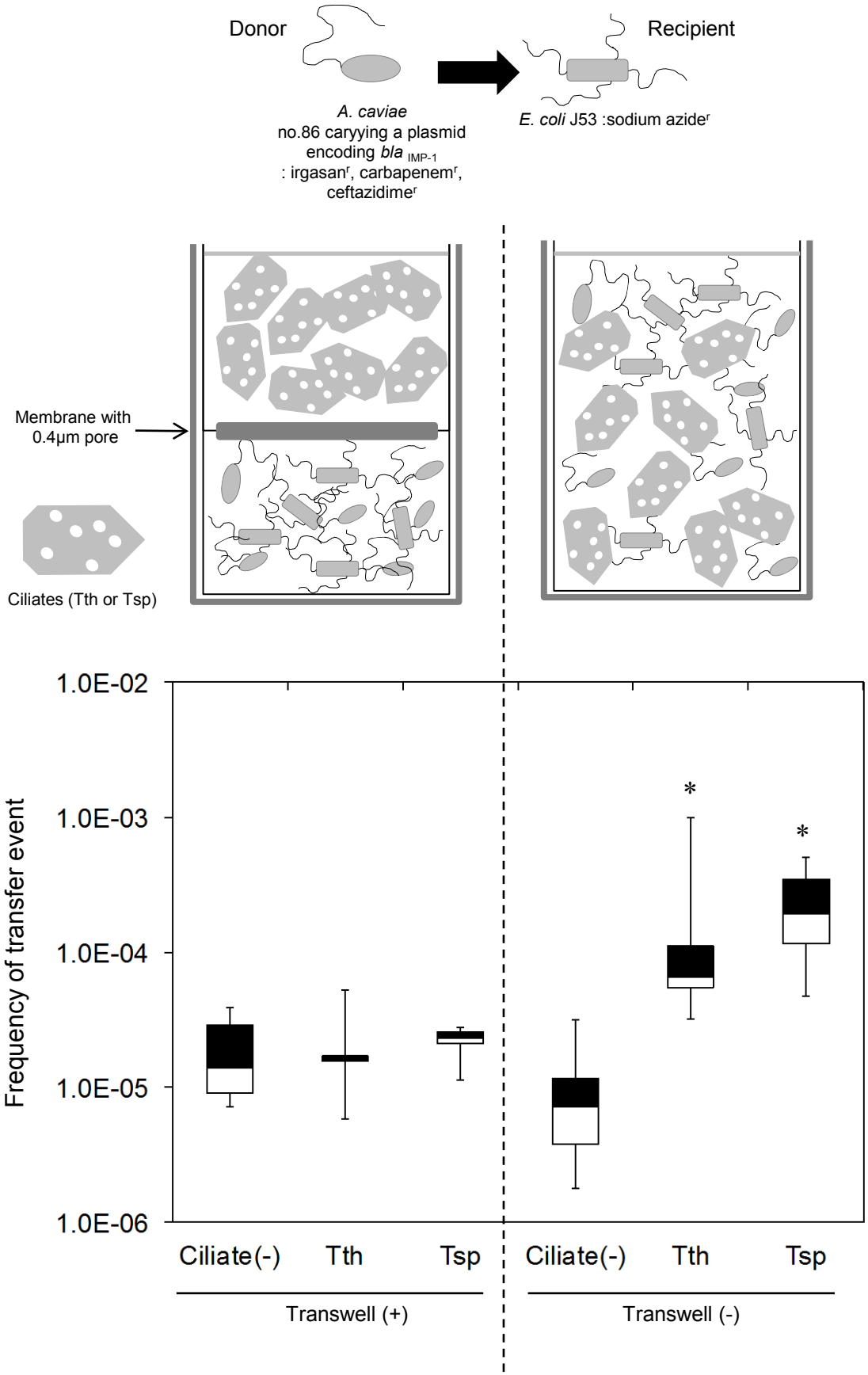
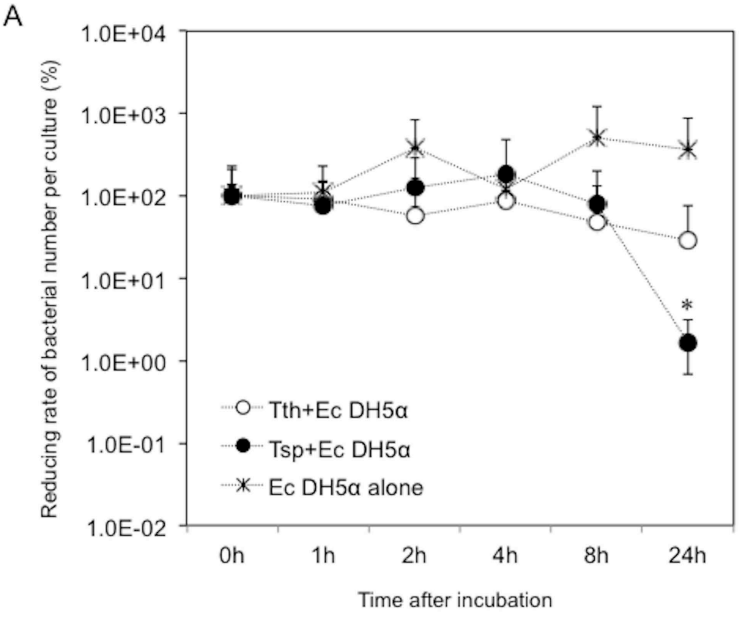

Donor
Recipient
A. caviae
no.86 caryying a plasmid
encoding bla IMP-1
: irgasanr, carbapenemr,
ceftazidimer
E. coli J53 :sodium azider
Membrane with
0.4μm pore
Ciliates (Tth or Tsp)
Frequency of transfer event
1.0E-02
1.0E-03
1.0E-04
1.0E-05
1.0E-06
*
*
Ciliate(-)
Tth
Tsp
Ciliate(-)
Tth
Tsp
Transwell (+)
Transwell (-)

Fig. 4


A
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
1.0E-01
1.0E-02
Reducing rate of bacterial number per culture (%)
Tth+Ec DH5α
Tsp+Ec DH5α
Ec DH5α alone
*
0h
1h
2h
4h
8h
24h
Time after incubation

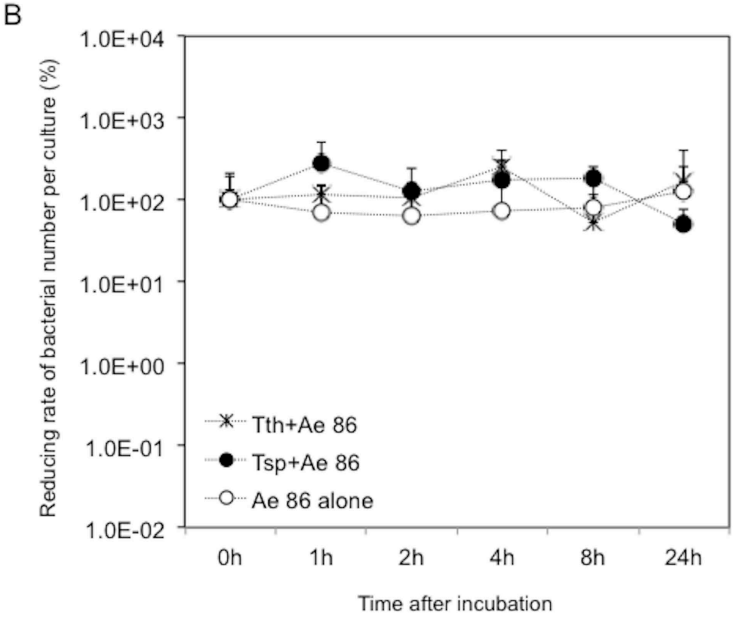
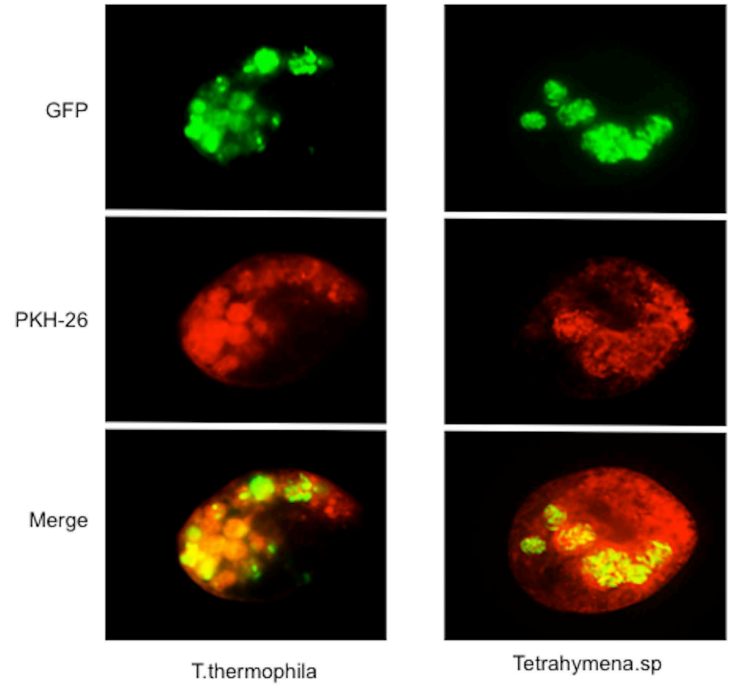

B
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
1.0E-01
1.0E-02
Reducing rate of bacterial number per culture (%)
Tth+Ae 86
Tsp+Ae 86
Ae 86 alone
0h
1h
2h
4h
8h
24h
Time after incubation

Fig. 5

A


GFP
PKH-26
Merge
T.thermophila
Tetrahymena.sp

B

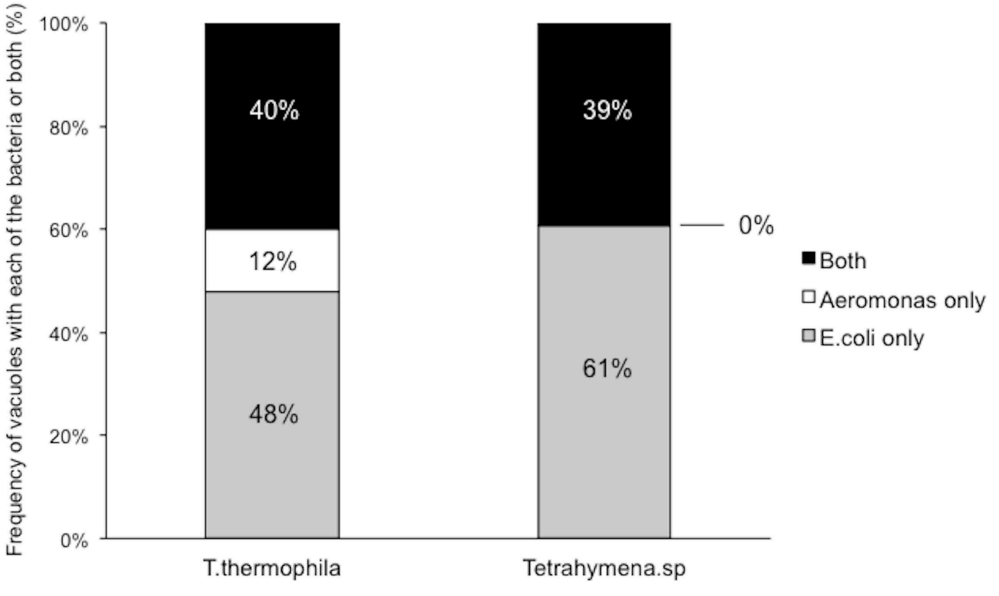

Frequency of vacuoles with each of the bacteria or both (%)
100%
80%
60%
40%
20%
0%
40%
12%
48%
39%
0%
61%
T.thermophila
Tetrahymena.sp
Both
Aeromonas only
E.coli only

Table 1 Bacteria and protozoa used for this study

Bacteria and protozc	Purpose	Characteristics or sources
Protozoa		
Ciliates		
T. thermophila (Tth)	Host cells / Imaging	Gifted from Dr. Sugai of Ibaragi University, Japan
Tetrahymena sp. (Tsp)	Host cells / Imaging	Gifted from Dr. Tukii of Hosei University, Japan
Bacteria		
C. freundii	Donor (original)	Clinical isolate, carrying a plasmid encoding *bla* $_{IMP-1}$:carbapenem^r, ceftazidime^r
E. coli J53	Recipient	Sodium azide^r, purchased from ATCC
E. coli TC170328	Donor	Carrying a plasmid encoding *bla* $_{IMP-1}$:sodium azide^r, carbapenem^r, ceftazidime^r
		*Established by mating with *C. freundii* caryying a plasmid encoding *bla* $_{IMP-1}$
A. caviae no.86	Recipient /Imaging	Environmental isolate (river), irgasan^{r,} gifted from Dr. Tamura of Rakno University, Japan
E. coli DH5α	Imaging	For expressing GFP
E. coli ATCC 29213	Control for MIC	purchased from ATCC
S. aureus ATCC 25922	Control for MIC	purchased from ATCC

Table 2 MICs of bacteria against CAZ

Bacteria	Classification	MIC against CAZ (μg/ml)
C. freundii	Donor (original)	**>128**
E. coli TC170328	Transconjugant/Donor	**>128**
A. caviae no.86	Recipient	1
A. caviae no.86^{r}	Transconjugant/Donor	**>128**
E. coli J53	Recipient	0.5
E. coli ATCC 29213	Control for MIC	0.5
S. aureus ATCC 25922	Control for MIC	16