# Ciliates rapidly enhance frequency of conjugation between *Escherichia coli* strains through bacterial accumulation in vesicles

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*Running title*: Ciliates promote bacterial conjugation

**Abstract**

The mechanism underlying bacterial conjugation through protozoa was investigated. Kanamycin-resistant *Escherichia coli* SM10λ$^{+}$ carrying pRT733 with Tn*phoA* was used as donor bacteria and introduced by conjugation into ciprofloxacin-resistant *E. coli* clinical isolate recipient bacteria. Equal amounts of donor and recipient bacteria were mixed together in the presence or absence of protozoa (ciliates, free-living amoebae, myxamoebae) in Page's amoeba saline for 24 h. Transconjugants were selected with Luria Broth agar containing kanamycin and ciprofloxacin. The frequency of conjugation was estimated as the number of transconjugants for each recipient. Conjugation frequency in the presence of ciliates was estimated to be approximately $10^{-6}$, but in the absence of ciliates, or in the presence of other protozoa, it was approximately $10^{-8}$. Conjugation also occurred in culture of ciliates at least 2 h after incubation. Successful conjugation was confirmed by polymerase chain reaction. Addition of cycloheximide or latrunculin B resulted in suppression of conjugation. Heat killing the ciliates or bacteria had no effect on conjugation frequency. Co-localization of green fluorescent protein-expressing *E. coli* and PKH67-vital stained *E. coli* was observed in the same ciliate vesicles, suggesting that both donor and recipient bacteria had accumulated in the same vesicle. In this study, the conjugation frequency of bacteria was found to be significantly higher in vesicles purified from ciliates than those in culture suspension. We conclude that ciliates rapidly enhance the conjugation of *E. coli* strains through bacterial accumulation in vesicles.

*Keywords*: protozoa; ciliates; conjugation; *Escherichia coli*

## 1. Introduction

The microbial community in natural environments, such as soil, pond water, or animal rumen, comprises a diverse mixture of bacteria and eukaryotic organisms, such as protozoa and fungi, interacting via complex metabolic pathways to ensure survival under meager nutrient conditions (Russell et al., 2001; Tyson et al., 2004). Bacteria can accommodate to a wide range of environmental conditions or acquire virulence by virtue of high-frequency gene exchange (Denamur et al., 2000; Ochman et al., 2000). Among the gene exchange strategies (conjugation, transduction, and transformation), conjugation, or induction of horizontal gene transfer, enables bacteria to access the genetic diversity of other members of the population or of separately evolving organisms most effectively (Dröge et al., 1998; Heinemann et al., 1998; Loewe et al., 2003; Nielsen et al., 1998; Smith et al., 2000). As bacteria continue to evolve, they undergo mutations, such as point mutations, deletions, insertions, and transpositions, leading to acquisition of novel virulence factors or to emergence of multidrug resistance (Baquero et al., 2009). Although gene transfer in natural environments has long been considered to mediate the evolution of bacteria, few studies have addressed questions regarding sites supporting effective gene transfer among bacteria.

Protozoa, such as amoebae, flagellates, and ciliates, are key eukaryotic organisms feeding on bacteria, thereby delivering organic matter to natural environments through digestion of bacteria (Mitchell et al., 2003; Novarino et al., 1997). However, many bacteria engulfed into the vesicles of protozoa, such as those of free-living amoebae, are not digested, and survive after being expelled to the outside environment (Abd et al., 2007; Adékambi et al., 2006; Akya et al., 2009; Barker et al., 1994, Berker et al., 1998; Bouyer et al., 2007; Brandl et al., 2005; Gourabathini et al., 2008; Greub et al., 2004; King et al., 1998; La Scola et al., 2001; McCuddin

et al., 2006; Licht et al., 1999). This phenomenon is universal to the microbial community, creating an opportunity for cell-to-cell contact among bacteria accumulating in the vesicles of protozoa, and possibly contributing to gene transfer by conjugation among packed bacteria in these vesicles. Several studies have reported gene transfer between bacteria through protozoa present in animal rumen or natural environments, describing the ability of bacteria to acquire antibiotic resistance or the role of protozoa in promoting gene exchange in different environments (McCuddin et al., 2006; Jonecova et al., 1994; Licht et al., 1999; Schlimme et al., 1997). However, a detailed understanding of the mechanisms underlying bacterial gene transfer through protozoa still remains to be elucidated. To test the hypothesis that protozoa can enhance bacterial gene transfer through bacterial accumulation in their vesicles**,** we performed experiments with the mixed-culture system consisting of *Escherichia coli* [kanamycin (KM)-resistant *E. coli* (plasmid resistance) as donor and ciprofloxacin (CPFX)-resistant *E. coli* (genomic resistance) as recipient] and protozoa (ciliates: *Tetrahymena*; free-living amoebae: *Acanthamoeba*; myxamoebae: *Dictyostelium*). We also examined bacterial gene transfer in the mixed culture in the presence or absence of several types of drugs, such as inhibitors of protein synthesis or phagocytosis. The aims of the present study were to determine: (i) the effect of protozoa on horizontal gene transfer frequency, (ii) the effect of drugs on horizontal gene transfer frequency through ciliates, and (iii) the localization of bacteria in ciliates after engulfing**.**

## 2. Materials and Methods

### *2. 1. Drugs*

Cycloheximide, a eukaryote-specific protein synthesis inhibitor, and cytochalasin D and latrunculin B, two actin polymerization inhibitors, were purchased from Sigma. Latrunculin B, a phagocytosis inhibitor binding to actin and inhibiting microfilament polymerization, is associated with limited bacterial engulfing or vesicle production (Sugita et al., 2009; Zackroff et al., 2002). Cytochalasin D is a weak inhibitor of actin polymerization compared with latrunculin B (Coué et al., 1987). KM (Sigma) and CPFX (Sigma) were used for selection of transconjugants at final concentrations of 50 μg/ml and 5 μg/ml, respectively. Fluorescence-labeled-polystyrene (FITC-labeled) beads (diameter 2.0 μm, excitation 470 nm, emission 505 nm), used to determine the phagocytic activity of ciliates, were also purchased from Sigma.

### *2. 2. Bacteria and protozoa*

The bacteria and protozoa used in this study are listed in Table 1. Bacteria were statically cultured in Luria Broth (LB), composed of 1% NaCl (Wako), 1% peptone (Difco), and 0.5% yeast extract (Difco), at 37°C. Protozoa were also statically maintained in peptone-yeast extract glucose broth, composed of 0.75% peptone (Difco), 0.75% yeast extract (Difco)**,** and 1.5% glucose (Wako), at 30°C as described previously (Matsuo et al., 2008). *E. coli* strain SM10λ *pir*$^{+}$ carrying pRT733 plasmid, a well-characterized laboratory strain used mainly as donor bacteria for gene transfer experiments (Taylor et al., 1989), was used in this study to mediate gene transfer by conjugation.

*2. 3. Mixed culture system and assessment of conjugation frequency*

Frequency of bacterial conjugation through protozoa was estimated by the following method. Briefly, equal amounts (approximately $10^9$ colony-forming units [CFU]) of both donor *E. coli* (KM-resistant) (0.5 ml) and recipient *E. coli* clinical isolate (CPFX-resistant) (0.5 ml) were mixed together with or without $10^3$-$10^6$ protozoan cells (1.0 ml) in Page's amoeba saline (composition per liter: $Na_2HPO_4$ 0.142 g, $KH_2PO_4$ 0.136 g, NaCl 0.12 g, $MgSO_4.7H_2O$, $CaCl_2.6H_2O$ 0.4 mg, pH 6.8)(PAS; Page, 1988) (Fig. 1). The mixed solution was centrifuged at 2,500 × g for 10 min at 4°C. The pellets were resuspended in 0.5 ml PAS and then incubated for 24 h at 15°C, 30°C, or 37°C under normal atmosphere; three different temperatures were tested as the phagocytic activity of the protozoa varied with culture temperature. The bacterial concentration was adjusted by using the optical density method, and the protozoan concentration was determined by using the trypan blue dye exclusion modified method [trypan blue solution (Sigma) containing 0.6% ethanol stains for viable protozoa]. Heat-killed ciliates or heat-killed bacteria, prepared by treatment at 90°C for 10 min, were also used. A mixed culture (100 μl) of ciliates and/or bacteria was collected after 0, 2, 8, and 24 h of incubation, and subjected to bead beating with glass beads (diameter 0.5-0.7 μm) for the CFU assay (assessment of transconjugants) or DNA extraction for PCR (confirmation of gene transfer). Bead beating with glass beads was performed as follows. Glass beads (0.4 g) were placed into the mixed solution (100 μl) and then vortexed for 3 min at room temperature. After centrifugation at 200 × g for 1 min at 4°C, the supernatants were used to assess the rate of transconjugant formation by the CFU assay and DNA extraction. The protozoa were completely destroyed by vortexing for 3 min, which was confirmed by microscopic observation. The frequency of conjugation between the KM-resistant donor and CPFX-resistant recipient

bacteria was estimated following selection with LB agar containing KM and CPFX. Conjugation frequency was estimated as the number of transconjugants for each recipient. The effect of drugs [cytochalasin D (5, 40μg/ml), cycloheximide (0.1, 1μg/ml), or latrunculin B (5μg/ml)] on rate of transconjugant formation was also assessed. The cytotoxicity of these drugs in protozoa, with or without bacteria, was found to be minimal (data not shown).

*2. 4. DNA extraction and polymerase chain reaction (PCR)*

Extraction of DNA from a single colony was performed by the following method. A single colony derived from donor, recipient, or a transconjugant was grown in LB broth overnight at 37 °C. The bacterial culture solution (1 ml) was centrifuged at 5,000 × g for 5 min. The pellets were collected and resuspended in 500 μl DNase-free water (Sigma) and then boiled for 5 min. Boiled template (2 μl) was used for PCR with primers against *phoA* (Forward 5'-TGA ATG AAC TGC AGG ACG AG-3'; Reverse 5'-ATA CTT TCT CGG CAG GAG CA-3') and against bacterial *16S rRNA* (Forward 5'-AGA GTT TGA TYM TGG CTC AG-3'; Reverse 5'-CAK AAA GGA GGT CC-3'[Y, C or T; K, G or T]) (Widjojoatmodjo et al., 1994). Reactions were carried out in 25 μl reaction buffer (each dNTP, 200 μmol/l; 1 × commercial reaction buffer) containing DNA polymerase [0.625 U *Taq* DNA polymerase (England Biolabs)]. The PCR cycle consisted of 10 min of denaturation at 94°C followed by 35 cycles each of 30 s of denaturation at 94°C; 30 s of annealing at 55°C for *phoA*, 52°C for bacterial *16S rRNA*; and 45 s of extension at 72°C. The product size was 171 bp for *phoA* and 212 bp for *rRNA*. The primers for amplification of *phoA* were designed from GeneBank cDNA sequences (accession number U25548) by using the website program Primer 3 (http://frodo.wi.mit.edu/). The amplified products were separated on 2.0% agarose gel and visualized by ethidium bromide staining. The

presence of amplified target genes in positive specimens (donor and transconjugant) was confirmed by direct oligonucleotide sequencing of the PCR products (Macrogen, Seoul, Korea).

*2. 5. Assessment of phagocytic activity*

Phagocytic activity of ciliates with FITC-labeled beads was assessed by the following method. Ciliates were statically cultured in PAS with the FITC beads at 30°C for up to 24 h. Culture solutions were collected for assessment of phagocytic activity after 0, 0.5, 1, 2, 8, and 24 h of incubation. Percentage of ciliates containing beads for each specimen was estimated by observing three to five randomly selected fields containing more than 200 cells under a fluorescence microscope.

*2. 6. Assessment of bacterial localization and frequency of conjugation in ciliate vesicles*

Two experiments were performed under the same mixed culture conditions as described above to confirm the presence of transconjugants in ciliate vesicles under a confocal fluorescence microscope and to assess the frequency of conjugation in purified ciliate vesicles. To confirm co-localization of donor and recipient bacteria in individual ciliate vesicles, 24-h–mixed cultures consisting of ciliates (*T. pyriformis*), green fluorescent protein (GFP)-expressing *E. coli* (provided in transformation kit, see below) (fluorescence color: green), and vital-stained CPFX-resistant *E. coli* (fluorescence color: red) were used. Following fixing of bacteria with 0.5% formalin, bacterial localization in ciliates was determined by using confocal fluorescence microscopy. Fluorescence vital staining of bacteria was performed with the PKH-67 labeling kit (PKH67GL, Sigma) (Wallace et al., 2008) and GFP expression was

performed with the Biotechnology Explorer ™ kit (pGLO™ Bacterial Transformation Kit 166-0003EDU, Bio-Rad) (Hanahan, 1983) according to the manufacturers' protocols. To confirm an increase in conjugation frequency in vesicles, ciliate vesicles were purified from the mixed-culture system (*T. pyriformis*) with bead beating for 3 min, followed by density gradient centrifugation with Histopaque (Sigma) according to the method described previously (Pinherio et al., 2007). As mentioned above, the complete destruction of ciliates by bead beating was confirmed by microscopic observation.

*2. 7. Statistical analysis*

All experiments were repeated at least three times, and statistical analysis was performed with the unpaired Student's *t*-test. A *p* value of less than 0.05 was considered significant.

## 3. Results and discussion

### *3. 1. Ciliates rapidly enhance the frequency of conjugation of* E. coli *strains*

The bacterial prey rate of protozoa varies depending on type of protozoa (Bott, 2007; Clarholm et al., 2007) and is thought to be directly associated with the frequency of bacterial conjugation. Therefore, to assess which type of protozoa could be associated with enhanced gene transfer between bacteria, three different types of protozoa, including *Tetrahymena* (ciliates), *Acanthamoeba* (free-living amoebae), and *Dictyostelium* (myxamoebae), were selected for this study. As shown in Fig. 2A, the rate of transconjugant formation with both strains of ciliates differed slightly; the frequency for *T. pyriformis* was $1.9 \times 10^{-6}$ ($p < 0.05$ vs. conjugation frequency in absence of protozoa at 37°C) and the frequency for *T. thermophila* was $1.2 \times 10^{-7}$ (not significant) in the presence of $10^5$ cells at 37°C. However, the frequency for *T. thermophila* ($1.0 \times 10^{-6}$) ($p < 0.05$ vs. conjugation frequency in absence of protozoa at 30°C) was the same as for *T. pyriformis* ($2.2 \times 10^{-6}$) ($p < 0.05$ vs. conjugation frequency in absence of protozoa at 30°C) in the presence of $10^5$ ciliates at 30°C. In the absence of protozoa, conjugation frequency reached only $10^{-7}$ (Fig. 2A). Representative pictures of transconjugant colonies are shown in Fig. 2B. Successful conjugation was confirmed by PCR with primers specific to *phoA* (Fig. 2C). Thus, these results indicate that ciliates, but not other protozoa, promote plasmid transfer between *E. coli* strains.

The conjugation frequency varied significantly according to the initial number of ciliates (optimal ciliate density; $10^5$ cells /ml) and culture temperature (optimal culture temperature; 30°C) used. However, neither *Acanthamoeba* nor *Dictyostelium* resulted in enhanced conjugation frequency, regardless of number of ciliates and culture temperature (Fig. 2B). It is well known that the bacterial prey rate of ciliates, which ranges from 200 to 5,000 bacteria per

hour, is very high compared with that of free-living amoebae or myxamoebae (Bott, 1995; Clarholm et al., 2007) and that it is maximal at culture temperatures ranging from 30°C to 37°C (Fields et al., 1998; Suhr-Jessen et al., 1979). These findings on bacterial prey of protozoa indicate that optimal conditions are critical for the effective gene transfer between bacteria in the presence of protozoa. However, it is possible that stress induced by the higher level of ciliate predation may increase adaptive mechanisms of bacteria, such as gene transfer or pilus formation, to generate a biofilm that is resistant to attack by ciliates.

To confirm the high prey rate of ciliates, we assessed changes in the uptake of FITC-labeled beads by ciliates in the mixed culture system at 30°C and changes in frequency of conjugation between bacteria over time. Transconjugants rapidly formed in both types of ciliates, after 2 h of incubation (Fig. 3A). In cultures with only *T. pyriformis*, a rapid reduction in the number of bacteria was observed within of 24 h of incubation, but minimal changes in conjugation frequency were seen (Fig. 3B). To confirm the early engulfment and accumulation of bacteria in ciliates, ciliate cultures were inoculated with FITC-labeled beads and accumulation of beads in ciliates was monitored by using fluorescence microscopy (Fig. 3C and D). Beads were found to accumulate in ciliates very early, at least 30 min after incubation (Fig. 3C and D), and changes in uptake of FITC-labeled beads by ciliates were observed coincident with changes in conjugation frequency, with maximal bacterial conjugation frequency observed after 2 h of incubation with ciliates. A significant difference in the bead uptake rate of different ciliate strains was observed after 8 h of incubation. This difference has been postulated by other researchers to arise from the different optimal growth temperatures of the various ciliate strains used (Fields et al., 1998; Suhr-Jessen et al., 1979). Thus, these findings suggest that rapid engulfing of large amounts of bacteria into ciliate vesicles is a prerequisite to effective

conjugation between the enclosed bacteria.

*3. 2. Effect of drugs on frequency of conjugation of* E. coli *strains in the presence of ciliates*

First, we confirmed by fluorescence microscopy that addition of cycloheximide or latrunculin B, but not cytochalasin D, to the cultures inhibited the accumulation of FITC-labeled beads in the vesicles of protozoa, indicating that these two drugs suppressed the bacterial engulfing and vesicle production by ciliates (Fig. 4A). To assess the mechanism underlying plasmid transfer between *E. coli* strains in the presence of ciliates, the effect of drugs (cycloheximide, cytochalasin D, latrunculin B) was assessed in the mixed culture system at 30°C. Cycloheximide led to a reduction in the rate of transconjugant formation in a concentration dependent manner (Fig. 4B). Latrunculin B produced a similar reduction in the rate of transconjugant formation. However, cytochalasin D had no effect on the conjugation frequency between bacteria in ciliates. The conjugation rate using heat-killed ciliates or heat-killed donor bacteria was the same as that of the control culture without ciliates. Addition of DMSO (vehicle control of cytochalasin D) to the mixed culture had no effect on the conjugation frequency. Thus, these results indicate that bacterial engulfing or vesicle production by ciliates is involved in the enhanced conjugation between bacteria in the presence of ciliates.

As the heat-killed *E. coli* strain SM10λ strain, which is known to be mediate gene transfer by conjugation (Taylor et al., 1989), completely lost the ability for gene transfer to recipient bacteria in the presence of ciliates in this study, bacterial gene transfer through ciliates was surmised to be induced by bacterial conjugation.

*3. 3. Accumulation of bacteria in ciliate vesicles and frequency of conjugation in vesicles purified from ciliates*

Another experiment was performed to determine whether the accumulation of two different strains of *E. coli* [GFP-expressing *E. coli* (green) and vital-stained CPFX-resistant *E. coli* (red)] can occur in the same ciliate vesicle. Co-localization of these two different bacterial strains in the same ciliate vesicle was observed, suggesting that efficient transconjugation occurs through bacterial accumulation in the ciliate vesicle (Fig. 5A). To confirm this hypothesis, ciliate vesicles containing bacteria were purified, and the conjugation frequency in these purified vesicles was compared with that in the culture supernatant. As expected, the frequency of conjugation between bacteria in the purified vesicles was higher than that in the supernatant (Fig. 5B). However, the frequency of conjugation in vesicles was higher than that in the supernatant only during the first 2 h of the experiment, with no significant difference observed at 8 h and 24 h, showing that conjugation occurred rapidly following bacterial accumulation in the vesicles. Although the mechanism underlying the high frequency of conjugation in the vesicles during the first 2 h remains elusive at this time, the results indicate that bacteria passing through ciliates remain viable and that transconjugants rapidly accumulate in the supernatant following expulsion of the bacteria-containing vesicles by the ciliates. These results therefore imply that both vesicles expelled by ciliates and vesicles within ciliates play a critical role in the survival of human pathogens in natural environments through dynamic gene exchange. In fact, several studies have already reported that bacteria, such as *E. coli* O157:H7 (Gourgbathini et al., 2008), *S. enterica* (Brandl et al., 2005), and *L. pneumophila* (Fields et al., 1984), are capable of surviving and replicating inside ciliate vesicles or expelled vesicles, indicating the potential of ciliates distributed in a wide range of natural environments to maintain bacterial viability in

harsh environments. The finding reported by Jones (1999) that a strain of *E. coli* O157 survived for at least 60 days in soil at 25°C and for at least 100 days at 4°C also shows the potential of vesicles contained by ciliates inhabiting in natural environments, such as soil, as “hot spots” for gene transfer, helping bacteria adapt to harsh environments.

In conclusion, we have demonstrated that ciliates, but not other protozoa, rapidly enhanced the frequency of conjugation of *E. coli* strains through bacterial accumulation in ciliate vesicles. Elucidating the mechanism underlying bacterial conjugation in ciliate vesicles might enhance our understanding regarding the site of bacterial conjugation in natural environments, with important implications for biosafety considerations.

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**Legends to figures**

**Fig. 1.** Brief outline of mixed culture system and assessment of frequency of bacterial conjugation. See the text. PAS, Page's amoeba saline (Page, 1988).

**Fig. 2.** Effect of protozoa on the frequency of conjugation of *E. coli* strains.

(A) Plots of frequency of conjugation between *E. coli* strains in the presence or absence of protozoa (*Tetrahymena*, *Acanthamoeba*, and *Dictyostelium*). Conjugation frequency was estimated as the number of transconjugants for each recipient. See the inset of Fig. 1. Data represent averages of conjugation frequency + standard deviations. *, $p< 0.05$ versus values for bacteria alone. (B) Representative images of transconjugant (upper) and recipient (bottom) colonies formed on plates at 30°C. (C) Representative results of agarose gel electrophoresis of the *phoA* PCR products amplified from donor, recipient, or transconjugant obtained through ciliates D, donor; R, recipient; C, transconjugant; N, negative control [DNase-free water (Sigma) instead of DNA template was used for PCR].

**Fig. 3.** Influence of incubation time on plasmid transfer in bacteria cultured with ciliates.

(A) Frequency of conjugation between the *E. coli* strains in the presence or absence of ciliates at each incubation time point at 30°C. Conjugation frequency was estimated as the number of transconjugants for each recipient. See the inset of Fig. 1. Data represent averages of conjugation frequency + standard deviations. *, $p< 0.05$ versus values for bacteria alone at the each time point. (B) Survival of bacteria in cultures despite engulfing and digestion by ciliates. Data represent averages of colony-forming units (CFU) + standard deviations. *, $p< 0.05$ versus

values for bacteria alone at the each time point. (C) Representative images of ciliates engulfing FITC-labeled beads at several incubation time points. (D) The kinetics depict changes in the bead uptake rate in ciliates. Data represent the averages of FITC-labeled bead uptake rate ± standard deviations.

**Fig. 4.** Effect of drugs (cycloheximide, cytochalasin D, or latrunculin B) or heated-killed bacteria on frequency of conjugation between *E. coli* strains in the presence of ciliates.

(A) Effect of the drugs on the FITC-labeled bead uptake rate of ciliates. Percentage of ciliates containing beads for each specimen was estimated after 2 h of addition of the drugs by observing three to five randomly selected fields containing more than 200 cells under a fluorescence microscope. Data represent averages of FITC-labeled bead uptake rate ± standard deviations. *, $p< 0.05$ versus values for ciliates suspended in PAS with FITC-labeled beads in the absence of drugs. (B) Frequency of conjugation in the mixed culture system at 30°C was estimated as the number of transconjugants for each recipient. See the inset of Fig. 1. Data represent averages of conjugation frequency ± standard deviations. DMSO (dimethyl sulfoxide) (Sigma) was used for vehicle control of cytochalasin D. *, $p< 0.05$ versus values for bacteria alone mixed with donor and recipient.

**Fig. 5.** Accumulation of bacteria in ciliate vesicles and formation of transconjugants in vesicles purified from ciliates.

(A) Representative fluorescence image showing a ciliate vesicle engulfing bacteria mixed with green fluorescent protein (GFP)-expressing *E. coli* (green) and vital-stained CPFX-resistant *E. coli* (red) at 8 h for up to 24 h of incubation in the mixed culture system at 30°C. Magnification,

1,000×. (B) Conjugation frequency between *E. coli* strains in the purified vesicles of ciliates at each incubation time point in the mixed culture system at 30°C. Conjugation frequency was estimated as the number of transconjugants for each recipient. See the inset of Fig. 1. Data represent the averages of conjugation frequency ± standard deviations. *, $p < 0.05$ versus values taken immediately after incubation.

Fig. 1

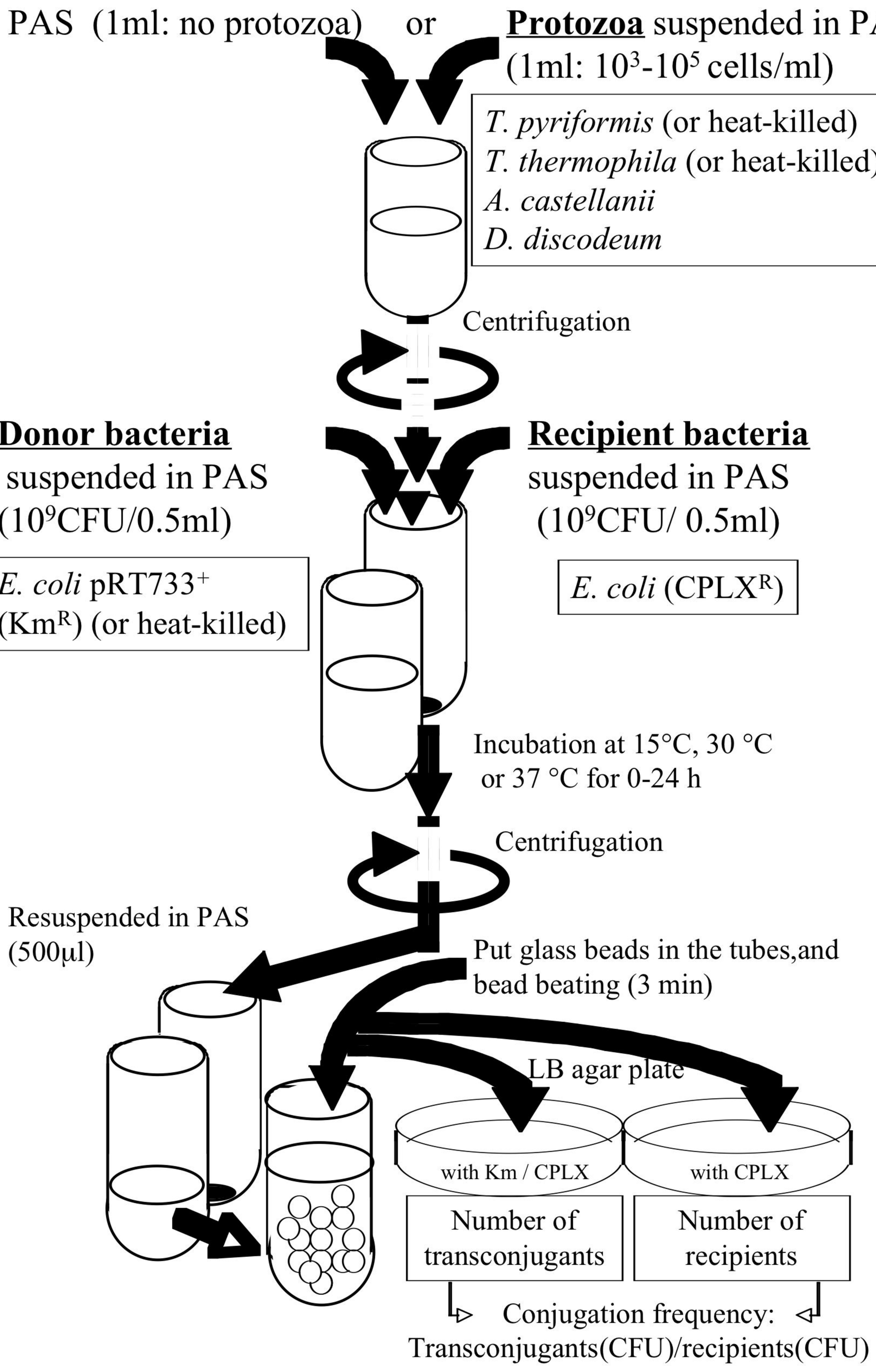
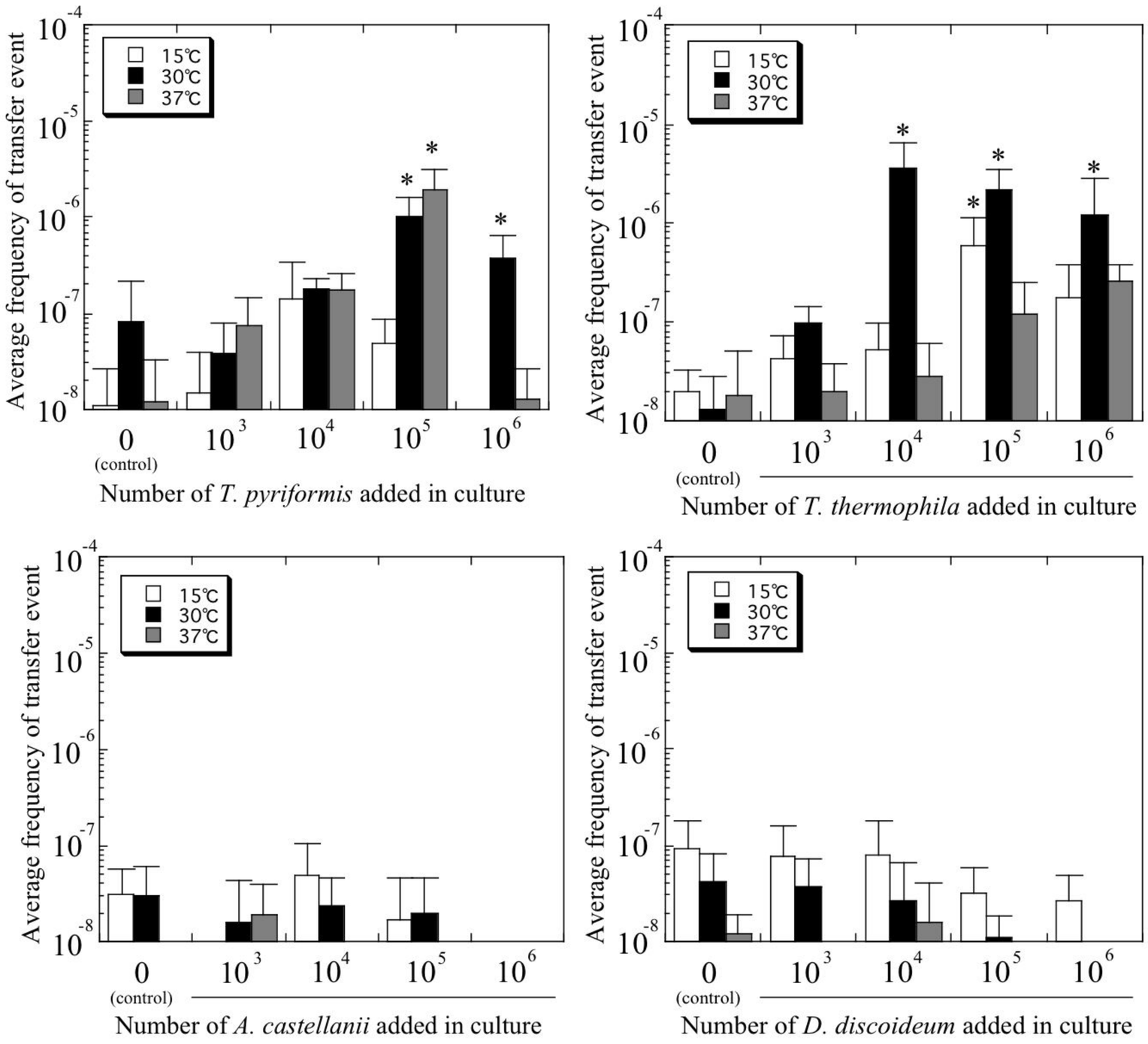
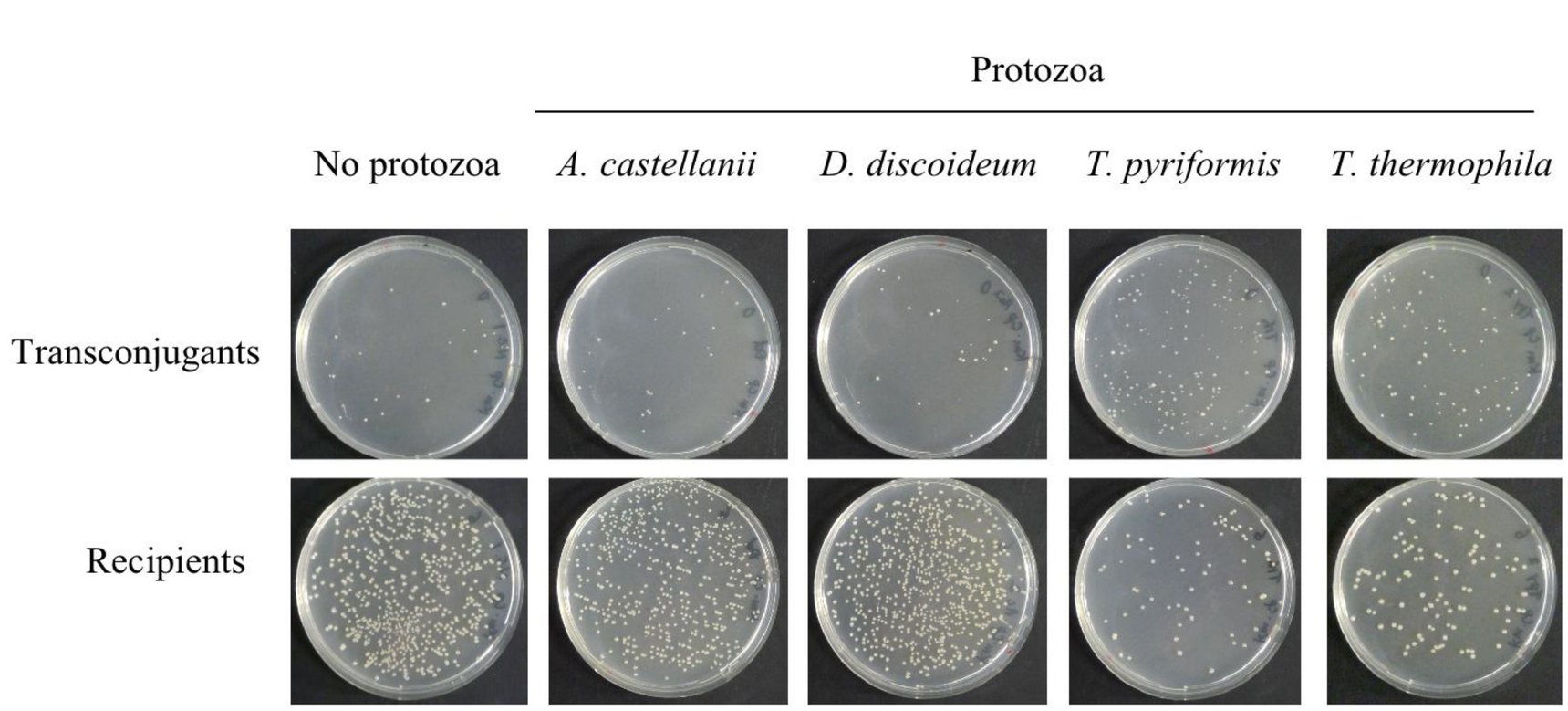

PAS (1ml: no protozoa)
or
**Protozoa** suspended in PAS
(1ml: $10^3$-$10^5$ cells/ml)
*T. pyriformis* (or heat-killed)
*T. thermophila* (or heat-killed)
*A. castellanii*
*D. discodeum*
Centrifugation
**Donor bacteria**
suspended in PAS
($10^9$CFU/0.5ml)
*E. coli* pRT733$^+$
($Km^R$) (or heat-killed)
**Recipient bacteria**
suspended in PAS
($10^9$CFU/ 0.5ml)
*E. coli* ($CPLX^R$)
Incubation at 15°C, 30 °C
or 37 °C for 0-24 h
Centrifugation
Resuspended in PAS
(500μl)
Put glass beads in the tubes,and
bead beating (3 min)
LB agar plate
with Km / CPLX
with CPLX
Number of
transconjugants
Number of
recipients
Conjugation frequency:
Transconjugants(CFU)/recipients(CFU)

Fig.2

A


Average frequency of transfer event
15℃
30℃
37℃
0 (control)
Number of T. pyriformis added in culture
Number of T. thermophila added in culture
Number of A. castellanii added in culture
Number of D. discoideum added in culture

B


Protozoa
No protozoa
A. castellanii
D. discoideum
T. pyriformis
T. thermophila
Transconjugants
Recipients

C

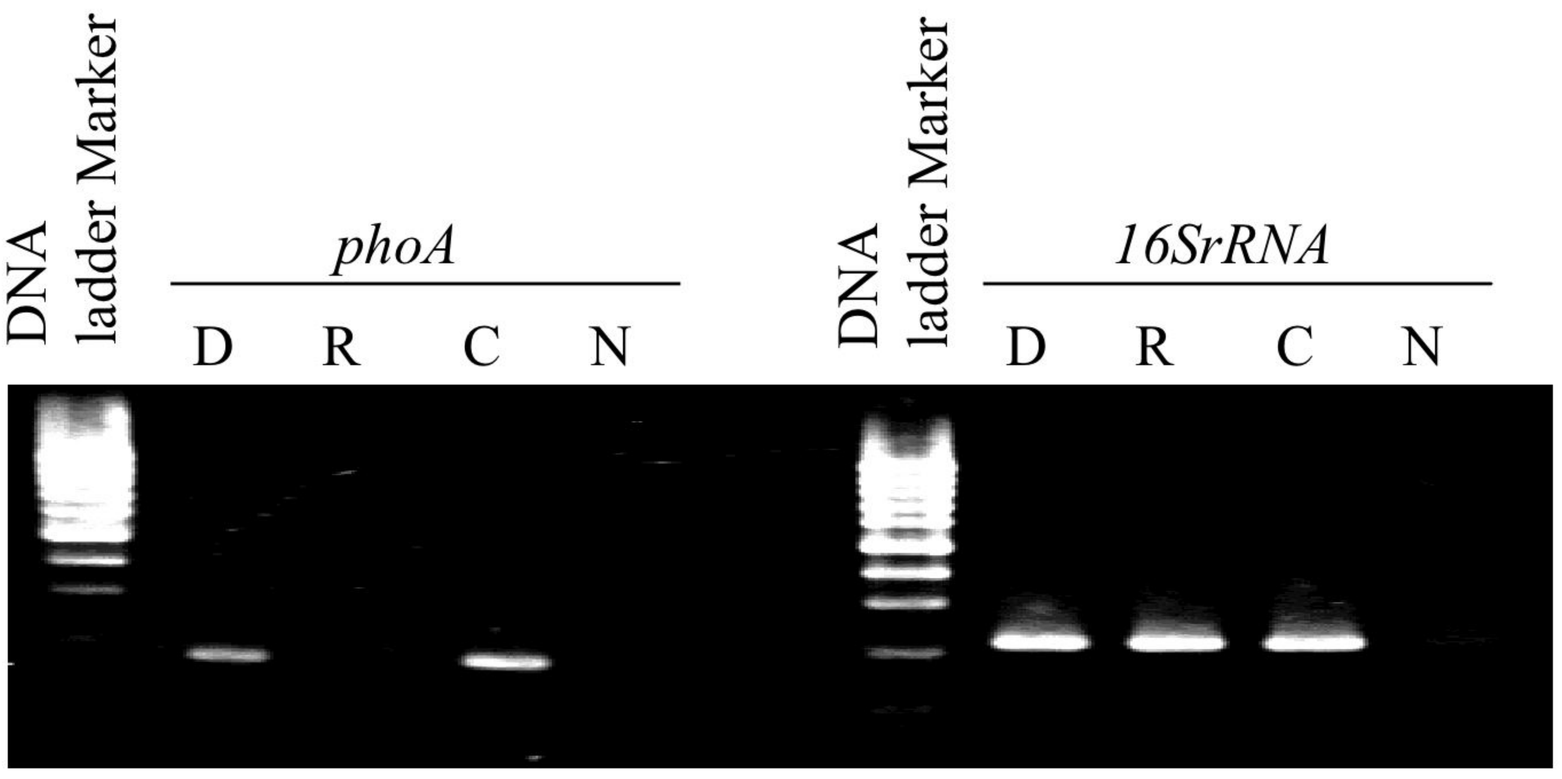
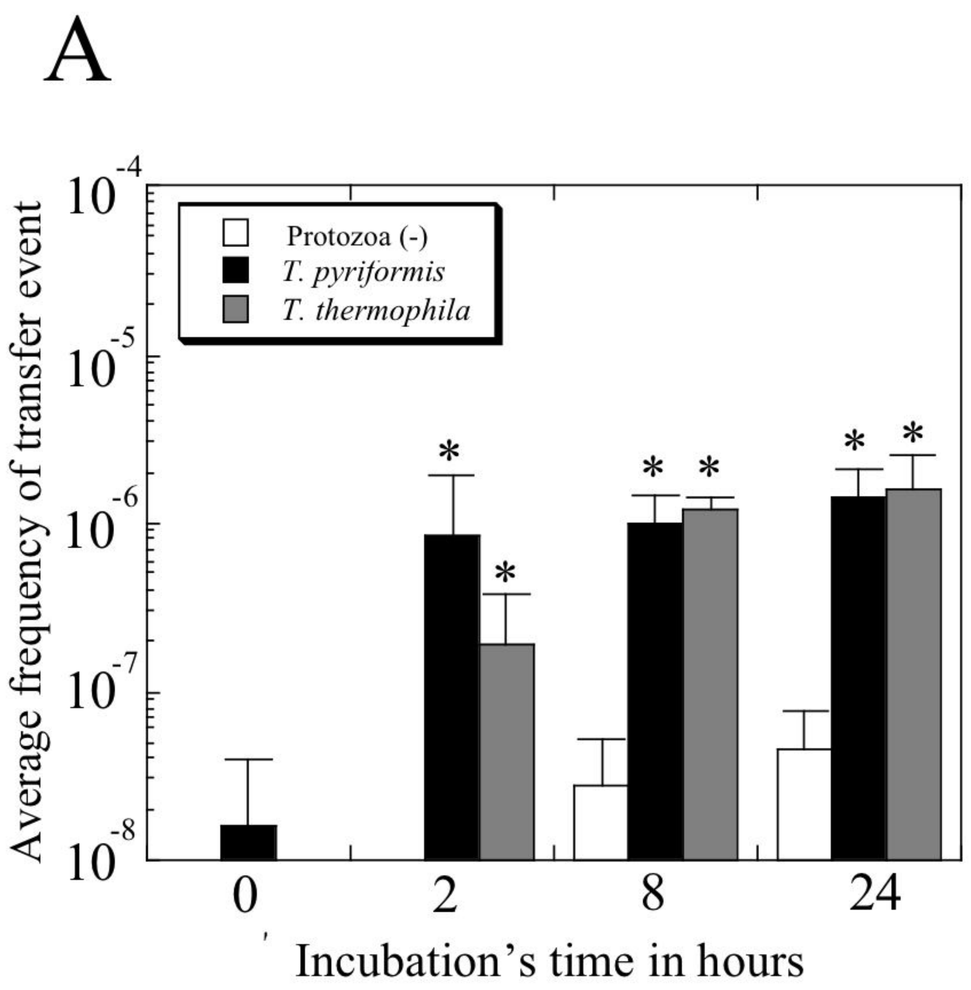
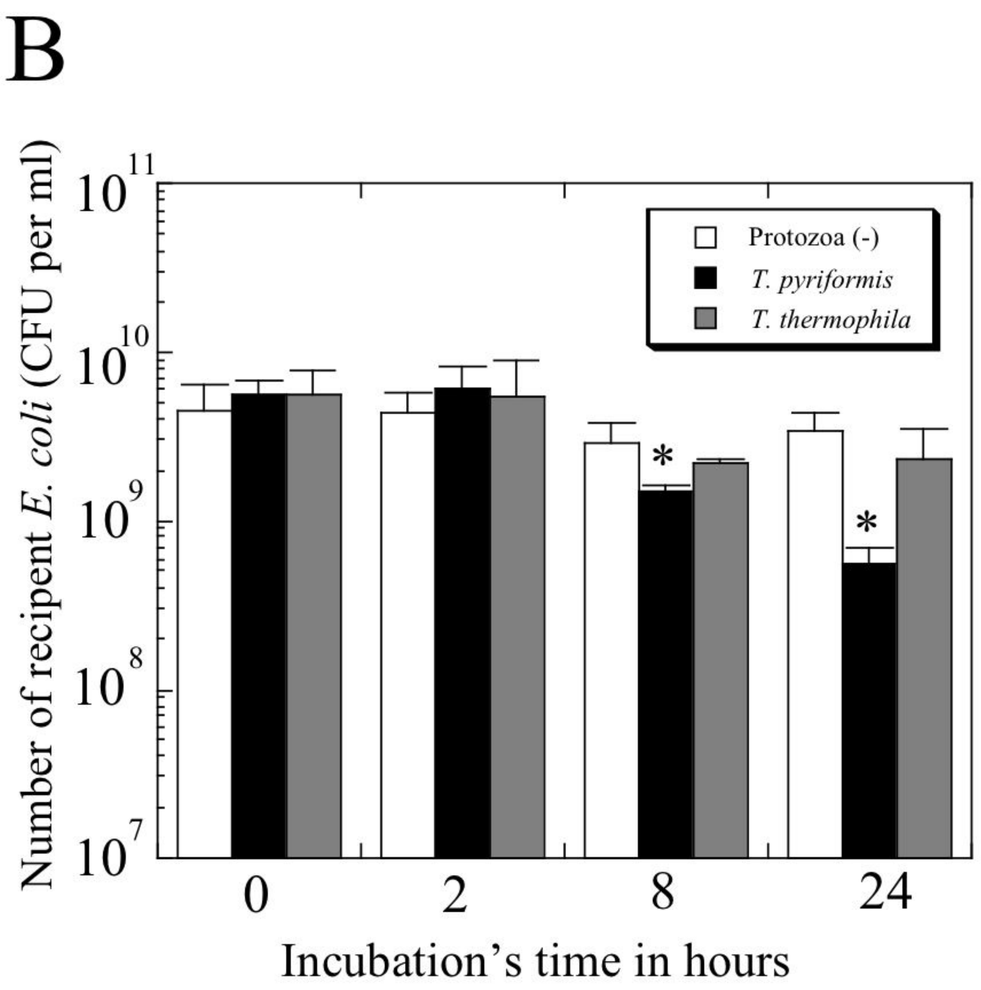
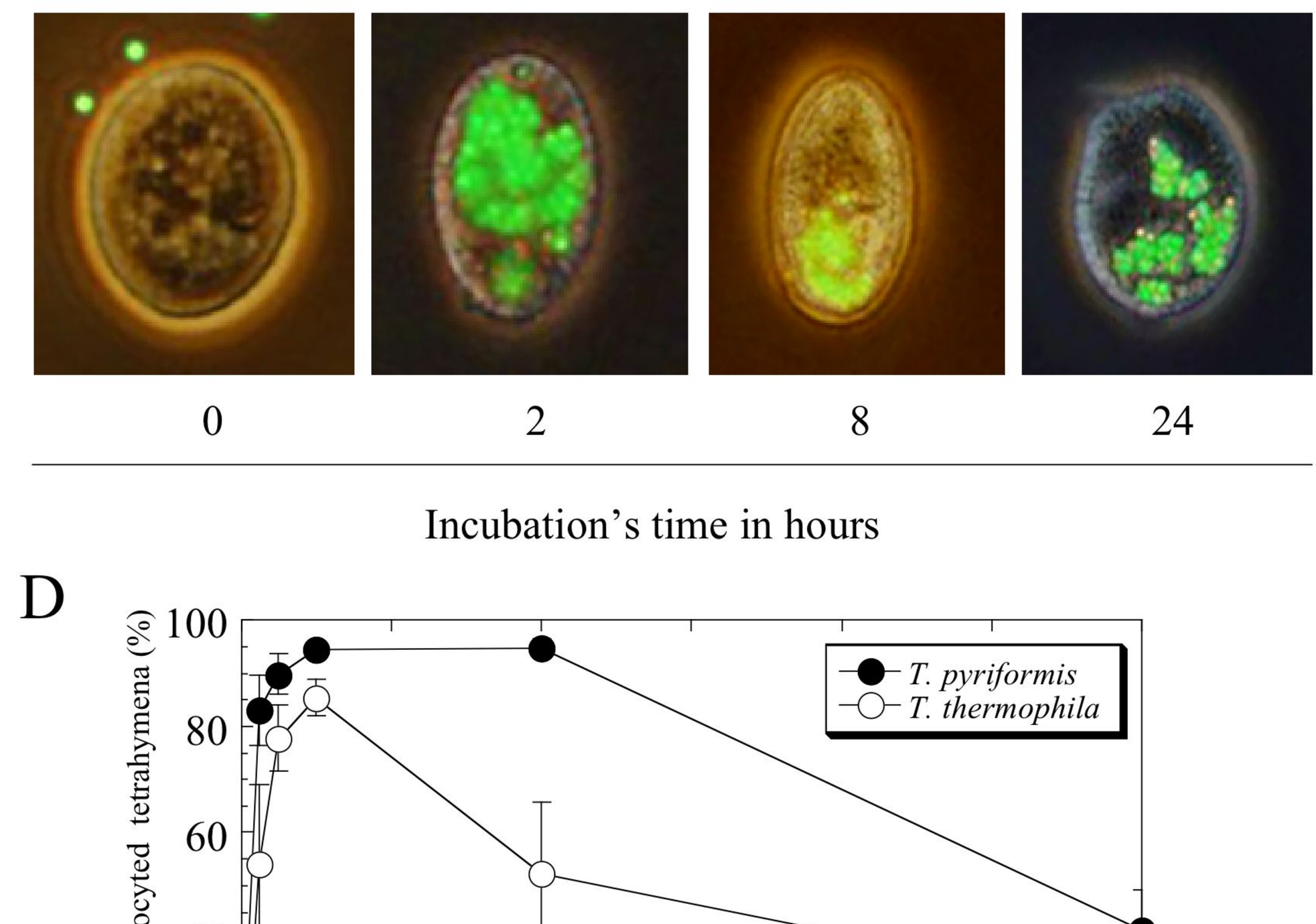

DNA ladder Marker
phoA
16SrRNA
D
R
C
N

Fig. 3


A
Protozoa (-)
T. pyriformis
T. thermophila
Average frequency of transfer event
10-4
10-5
10-6
10-7
10-8
0
2
8
24
Incubation's time in hours

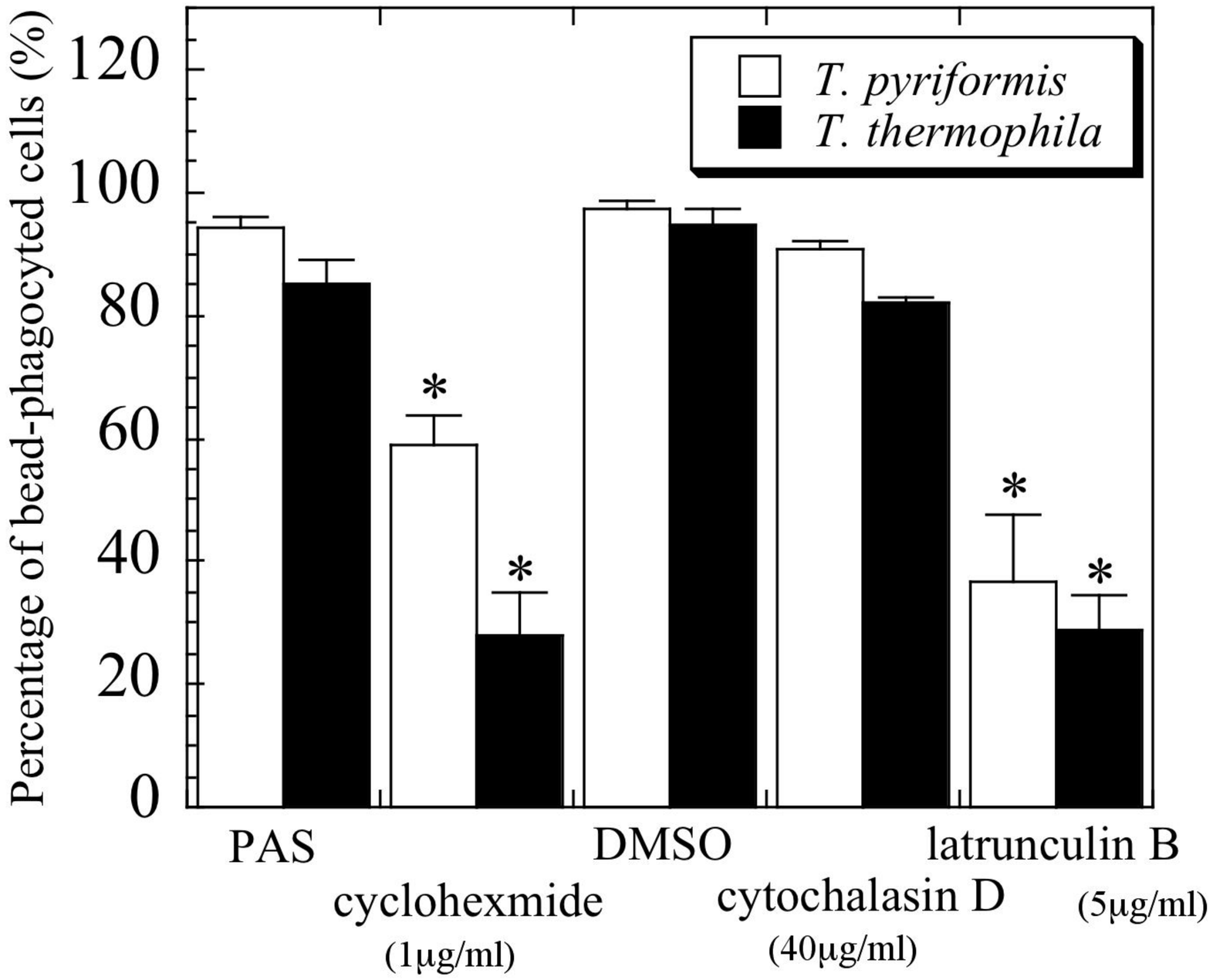

B
Protozoa (-)
T. pyriformis
T. thermophila
Number of recipient E. coli (CFU per ml)
10^11
10^10
10^9
10^8
10^7
0
2
8
24
Incubation's time in hours


C
0
2
8
24
Incubation's time in hours

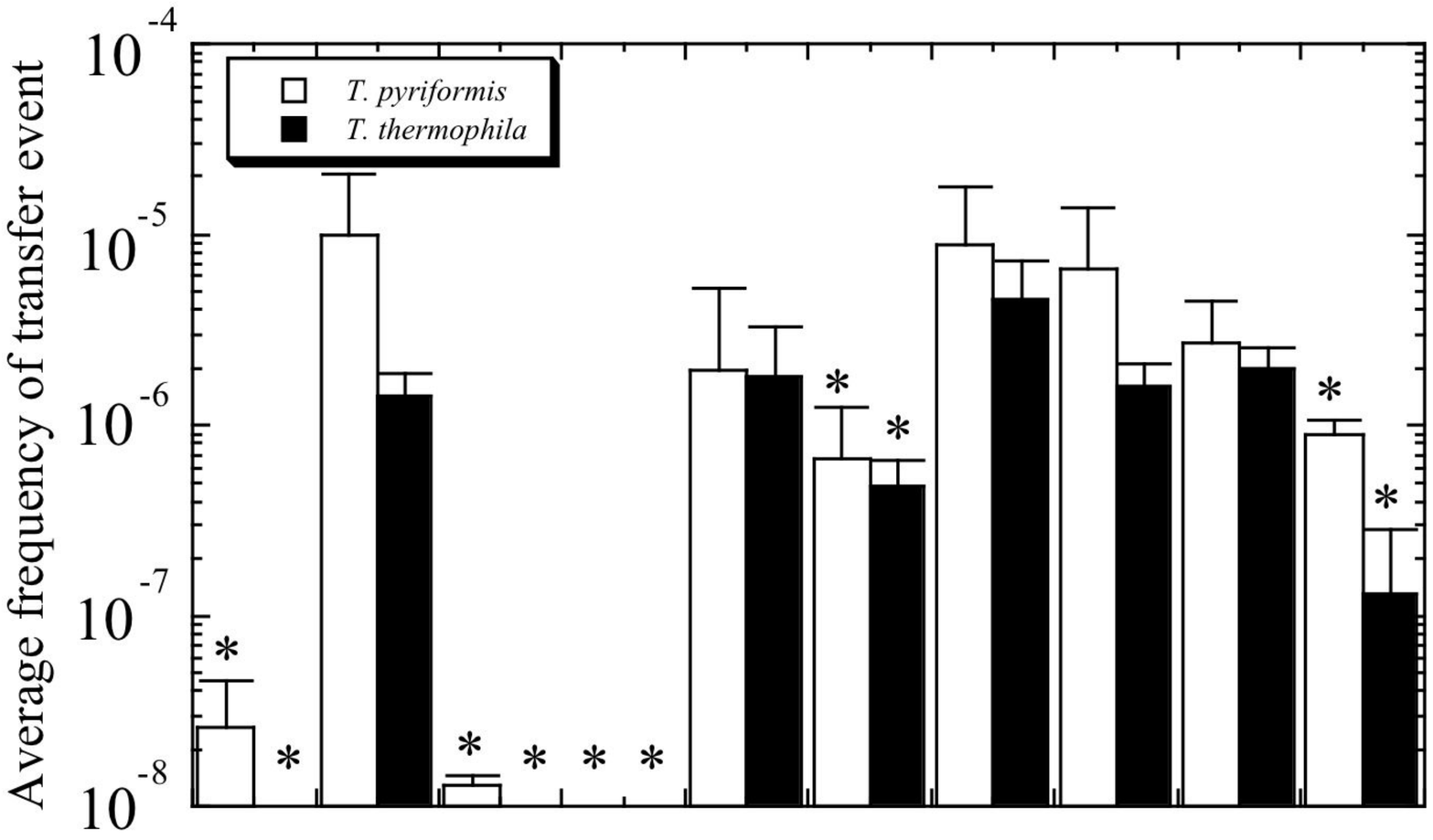

D
T. pyriformis
T. thermophila
FITC-beads phagocyted tetrahymena (%)
100
80
60
40
20
0
0
4
8
12
16
20
24
Incubation's time in hours

Fig. 4

A




B

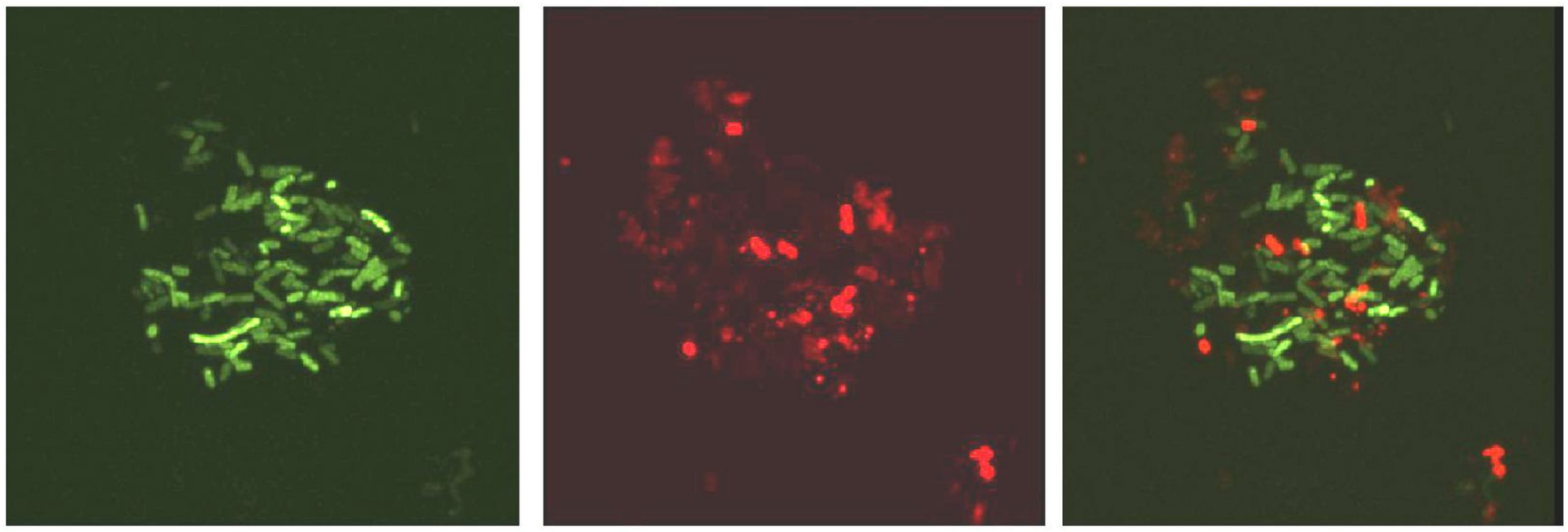



| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Protozoa | - | + | + Heat killed | + | + | + | + | + | + | + |
| Donor (D) /Recipent ( R) | +/+ | +/+ | +/+ | +/+ D: Heat killed | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ |
| Cycloheximide ( μg/ml) | - | - | - | - | + 0.1 | + 1 | - | - | - | - |
| Cytochalasin D ( μg/ml) | - | - | - | - | - | - | - DMSO | + 5 | + 40 | - |
| Latrunculin B ( μg/ml) | - | - | - | - | - | - | - | - | - | + 5 |

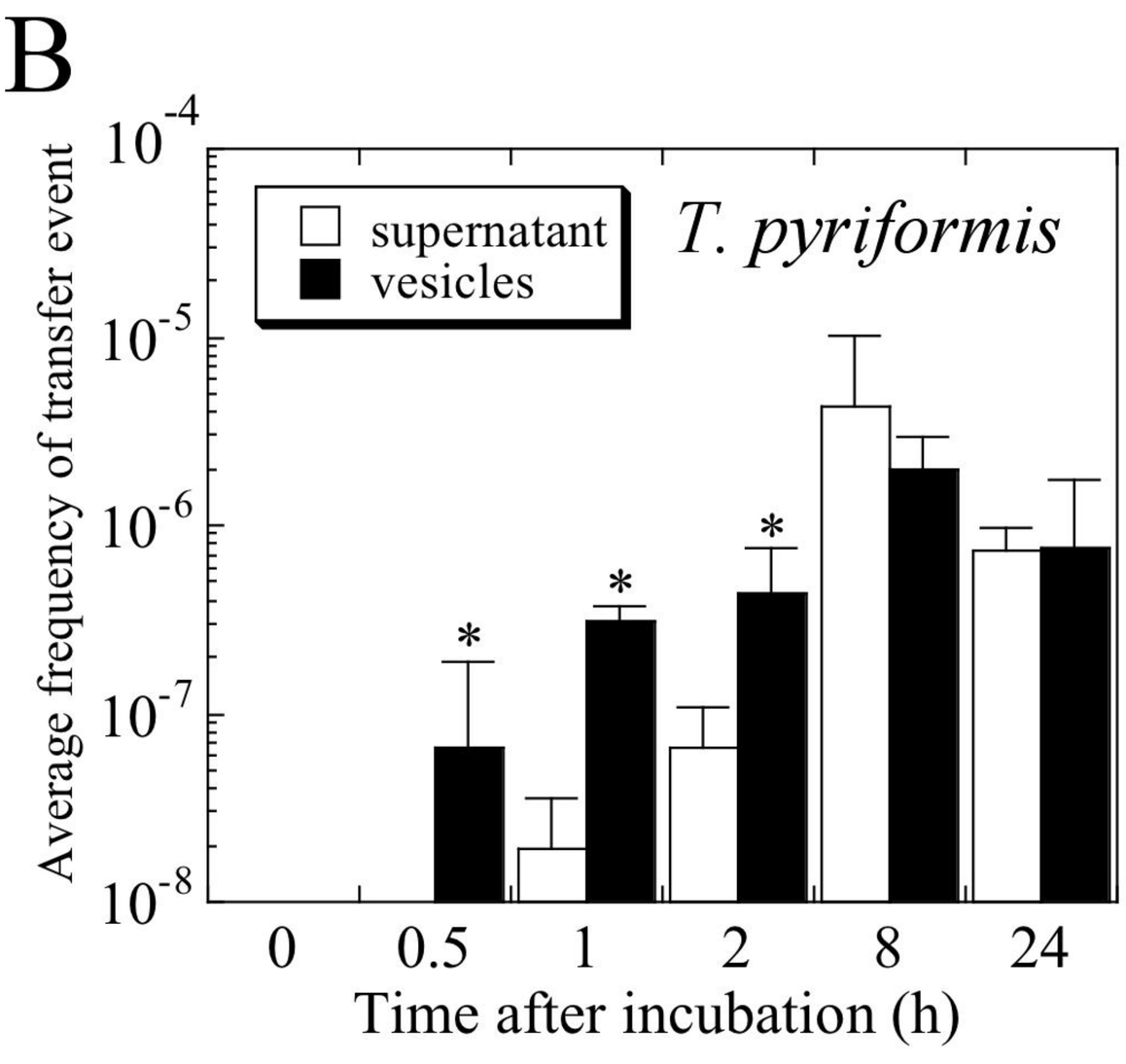
Negative control  Positive control  Vehicle control

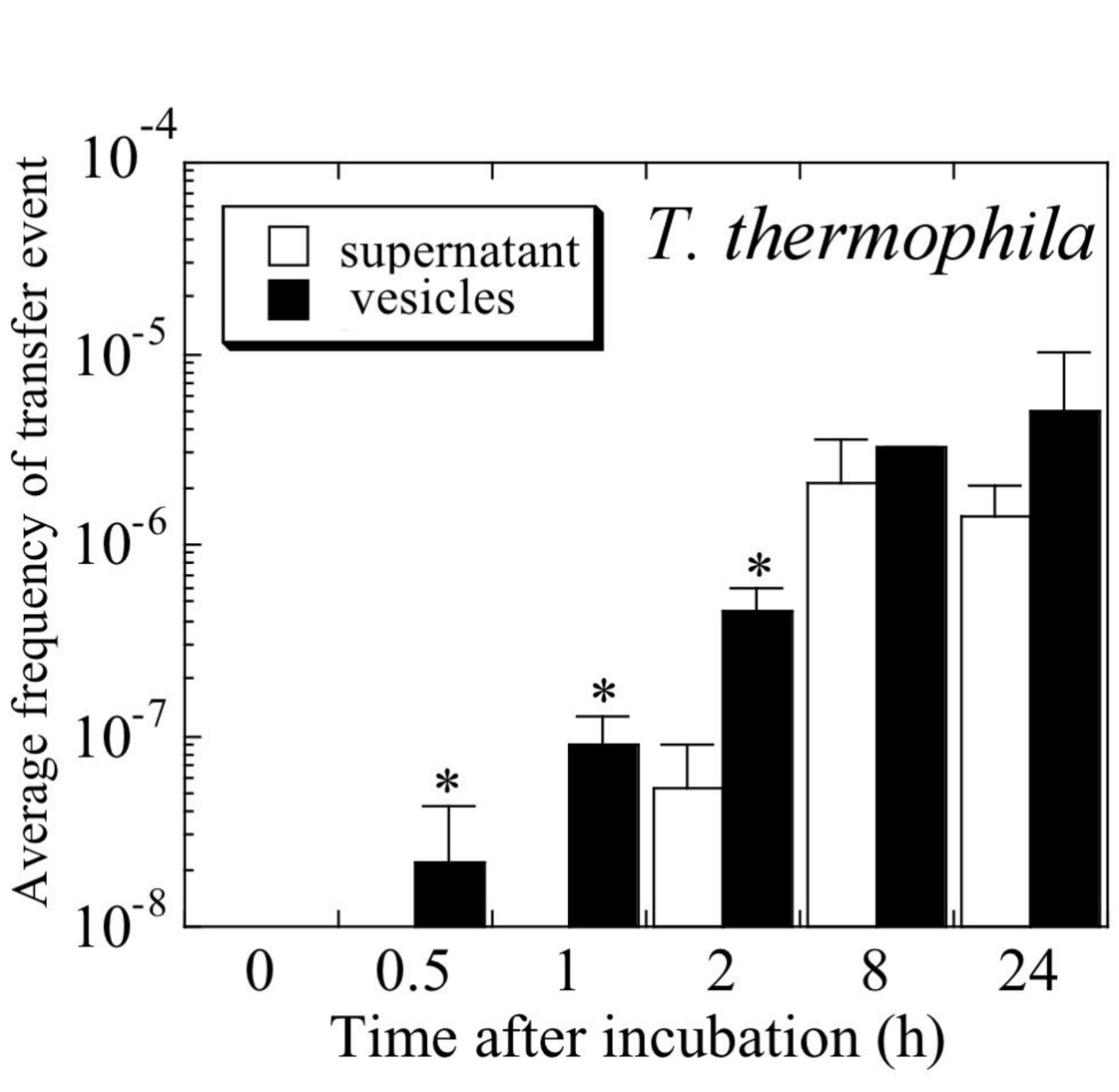
Fig. 5

A


Green
(GFP-expressing *E. coli*)
Red
(vital stained *E. coli*)
Merge

B


supernatant
vesicles
*T. pyriformis*
Average frequency of transfer event
$10^{-4}$
$10^{-5}$
$10^{-6}$
$10^{-7}$
$10^{-8}$
0
0.5
1
2
8
24
Time after incubation (h)
*


supernatant
vesicles
*T. thermophila*
Average frequency of transfer event
$10^{-4}$
$10^{-5}$
$10^{-6}$
$10^{-7}$
$10^{-8}$
0
0.5
1
2
8
24
Time after incubation (h)
*