Ciliates promote the transfer of the gene encoding the extended-spectrum β-lactamase CTX-M-27 between *Escherichia coli* strains

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Running title: Ciliates promote ESBL *E. coli* gene transfer

Summary

Objectives: The mechanism by which *Escherichia coli* acquires multidrug resistance genes from other bacteria in the natural environment or livestock is still unclear. The ability of ciliates to promote the transfer of genes encoding extended-spectrum β-lactamases (ESBL) between the CTX-M-27 donor and clinically isolated recipient *E. coli* strains was investigated.

Methods: Equal amounts, approximately 10^9 colony-forming units, of donor cefotaxime (CTX)-resistant *E. coli* and recipient ciprofloxacin (CPFX)-resistant *E. coli* strains were mixed together in the presence or absence of 10^5 ciliates in Page's amoeba saline for 24 h, in the presence or absence of certain drugs (cytochalasin D, cycloheximide, latrunculin B).

Results: Gene transfer frequency in the presence of ciliates was estimated at approximately 10^{-6}; but in the absence of ciliates it was approximately 10^{-10}. Protein synthesis (cycloheximide) or phagocytosis inhibitors (latrunculin B) significantly reduced the frequency of gene transfer.

Conclusions: Ciliates promote gene transfer of ESBL between *E. coli* strains, implying that the presence of ciliates may provide a significant impact on emerging multidrug resistant bacteria.

Keywords: ciliates, gene transfer, *Escherichia coli*, ESBL, CTX-M-27

Introduction

Production of extended-spectrum β-lactamases (ESBLs) by Enterobacteriaceae, specifically *Escherichia coli*, is a major concern in several countries, and is frequently implicated in human infections.[1] However, the mechanism by which *E. coli* acquires multidrug resistant genes from other bacteria in aquatic environments or livestock remains a significant challenge in the prevention and control of the emergence of multidrug resistant bacteria, leading to community- and nosocomial-acquired infections. Several studies have reported gene transfer between bacteria through protozoa present in the rumen of animals or natural environments, describing the ability of bacteria to acquire antibiotic resistance or the role of protozoa such as ciliates in promoting gene exchange in different environments.[2, 3] We have also demonstrated that protozoan *Tetrahymena* ciliates strongly promote transfer of the gene Tn*pho*A, encoding alkaline phosphatase, in *E. coli* (SM10λ *pir*$^+$ pRT733$^+$ with mobRP4) by conjugation *via* vesicle accumulation.[4] However, whether ciliates promote gene transfer of multidrug resistant genes encoding ESBLs between *E. coli* strains remains unknown. In the present study, we attempted to verify this hypothesis using a co-culture system consisting of *E. coli* strains [a cefotaxime (CTX)-resistant ESBL clinical isolate as a donor and a ciprofloxacin (CPFX)-resistant clinical isolate as a recipient] and ciliates (*Tetrahymena thermophila*) in the presence or absence of drugs, such as protein synthesis or phagocytosis inhibitors.

Methods

Among clinical isolates obtained from the Hokkaido University Hospital (Japan), ESBL

E. coli isolates were selected using a disk diffusion assay and interpretive criteria according to the method of the Clinical and Laboratory Standards Institute.[5] Four strains (designated no. 1, 2, 3 and 4) were determined to be CTX-M by polymerase chain reaction (PCR) analysis with the specific primers CTX-M-F (5'-ATG TGC AGY ACC AGT AAA G-3') and CTX-M-R (5'-GGT CAC CAG AAG GAG G-3') for detection of $bla_{\text{CTX-M}}$.[6] After sequencing of amplified products, an ESBL genotype of *E. coli* (strain no. 4) determined as CTX-M-27 was chosen as a donor for all experiments. The other CTX-M types were not tested as donors but were used as recipients for assessment of gene transfer frequency.

The experiment for assessing gene transfer frequency through ciliates was performed by a previously described method.[4] Briefly, equal amounts of colony-forming units (CFU; approximately 10^9 CFU) of both the donor ESBL *E. coli* isolated (CTX-resistant) and the same recipient *E. coli* clinical isolate (CPFX-resistant) used in a previous study,[4] were mixed with or without 10^5 *Tetrahymena thermophila* ciliates (a gift from Dr Sugai of Ibaragi University, Japan) in Page's amoeba saline (PAS) for 24 h. Bacterial concentration was adjusted using the optical density method. The viability of ciliates was confirmed *via* Trypan blue dye exclusion. To clarify whether viable donor bacteria or ciliates were needed for the gene transfer, heat-treated bacteria or ciliates prepared by treatment at 90°C for 10 min, were also used in experiments. Following incubation, the mixed culture of ciliates and bacteria were subjected to bead beating according to a previously described protocol.[4] The gene transfer frequency between CTX-resistant donor and CPFX-resistant recipient bacteria was estimated following selection with Luria-Bertani (LB) agar

containing CTX (2.5 mg/L) and CPFX (5 mg/L). Gene transfer frequency was expressed as the number of transconjugants for each recipient. The effect of drugs, such as cytochalasin D (5, 40 mg/L), cycloheximide (0.1 and 1 mg/L) or latrunculin B (5 mg/L) on the occurrence rate of transconjugants was also assessed. Cytotoxicity of drugs in ciliates with or without bacteria was minimal (data not shown). Bacteria were cultured in LB media. Ciliates were maintained in broth containing 0.75% peptone, 0.75% yeast extract and 1.5% glucose (PYG broth), as described previously.[15] The eukaryote-specific protein synthesis inhibitor cycloheximide and actin-polymerization inhibitors cytochalasin D and latrunculin B were purchased from Sigma (St Louis, MO, USA). Statistical analysis was performed with the unpaired Student's *t*-test and a *p*-value of less than 0.05 was considered significant.

Results and discussion

As shown in figure 1, the frequency of gene transfer between bacteria in the presence of ciliates increased significantly (average frequency 4.2×10^{-6}). In contrast, gene transfer frequency in the absence of ciliates was approximately 1×10^{-10}. Using transconjugant colonies cultured in LB broth, PCR with primers specific to CTX-M were used to amplify the target gene. Amplification was observed in the transconjugants and donor bacteria, but not in the recipient bacteria before mixing with ciliates, thereby demonstrating successful CTM-X-gene transfer between *E. coli* strains. Sequence alignment analysis indicated that the DNA sequence of the PCR product was identical to the CTX-M-27 sequence in GenBank (Accession number AV156925). Additionally, we also confirmed

that transfer of multidrug resistant genes occurred in three other ESBL (CTX-M) *E. coli* strains in the recipient *E. coli*. Average frequencies in the presence (absence) of ciliates were 1.7×10^{-7} (3.4×10^{-9}), 2.1×10^{-7} (1.0×10^{-10}) and 4.1×10^{-7} (1.0×10^{-10}) for strains 1, 2 and 3, respectively. These findings indicate that this gene transfer was not a unique phenomenon. In our previous study, an approximate one-log reduction in the total number of recipient bacteria was observed in cultures where ciliates were present during the 24 h incubation because of engulfment by ciliates (*T. pyriformis*), depending on the strain of the ciliate.[4] However, only a slight reduction in the total numbers of both recipient and donor in the presence of ciliates was seen (figure 2A). Taken together with the finding that the conjugation frequency between bacteria *via* ciliates occurred at 2 h (figure 2B), the affect of bacterial death due to ciliates on conjugation frequency was minimal and it is clear that the gene transfer frequency obtained is not falsely higher in the presence of ciliates than in their absence. Thus, we concluded that ciliates promote transfer of multidrug resistant genes encoding ESBLs between *E. coli* strains. The reason why the gene exchange frequency between the two *E. coli* strains without the ciliates was very low (1×10^{-10}) as compared with the previous report by using filter-mating techniques remains unknown. [7] It is possible that strain variation could be responsible for this contradiction, meanwhile it is also possible that gene exchange frequency may differ due to the fact that liquid media was used in this study as compared with solid media under the filter-mating conditions. We confirmed that the gene transfer frequency between the CTX-M-27 strain and the donor without ciliates under spotting-mating conditions on solid media was achieved at approximately 10^{-5} (data not shown).

Cycloheximide, a nonspecific eukaryotic protein synthesis inhibitor, reduced the occurrence rate of transconjugants, and the effect was concentration dependent with average frequencies of 5.4×10^{-6} (0.1 mg/L), 5.5×10^{-6} (1 mg/L), 7.2×10^{-7} (5 mg/L) (Figure 1). Latrunculin B, a phagocytosis inhibitor that binds to actin and inhibits microfilament polymerization, which is associated with limited bacterial engulfing or vesicle production, effected a similar reduction in the occurrence rate of transconjugants. The addition of cytochalasin D, an inhibitor of actin polymerization but with weak action compared to latrunculin B, had no effect on the gene transfer frequency between bacteria in ciliates. The rate using heat-treated ciliates (1×10^{-10}) was the same as that of the control culture without ciliates (1×10^{-10}), indicating that some of their cell components could not promote the gene transfer themselves. The rate using heat-treated donor bacteria (1.7×10^{-9}) was also the same as that of the control cultures without ciliates (1×10^{-10}). Thus, the results indicate that bacterial engulfing by ciliates following their accumulation in ciliate vesicles was involved in the enhanced gene transfer of ESBLs between bacteria in the presence of ciliates.

The current concern in clinical settings involves the CTX-M family of ESBLs. These enzymes have appeared in human pathogenic bacteria, leading to both community- and nosocomial-acquired infections.[8, 9] CTX-M was originally defined by the preferential cleavage of CTX versus ceftazidime (CAZ), although several recent derivatives that cleave both agents have been described.[10] In most European countries, Latin America, and East Asia including Japan, CTX-M variants have displaced TEM and SHV enzymes as the predominant β-lactamases produced by Gram-negative pathogens, especially in *E.*

coli. It has been reported that 82 CTX-M derivatives have been described (http://www.lahey.org/Studies/). Although the exact reasons and mechanisms of expansion worldwide remain unknown, the preventative measures to stop this expansion are critical in controlling infections. It is believed the most effective way to control this expansion is to determine and eliminate the site of gene delivery and receipt between human pathogenic bacteria. It is well known that ciliates are distributed in a wide range of natural environments, such as soil and water supplies, and also the digestive tract of livestock, along with numerous bacteria including human pathogens.[2, 3] Therefore, in the future, some efforts to monitor and control the number of protozoans, such as ciliates, in a particular environment like a hospital, might become absolutely essential for preventing the threats posed by multidrug resistant bacteria.

In conclusion, we have demonstrated for the first time that ciliates promote gene transfer of ESBLs (CTX-M-27) between *E. coli* strains. Further study is needed to confirm that this phenomenon occurs in a real world situation not just *in vitro*. Our results demonstrated here may provide a significant impact on the prevention and control of emerging multidrug resistant bacteria.

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Transparency declarations

None to declare.

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FIGURE LEGENDS

Figure 1. Plots of gene transfer frequency between *E. coli* strains (CTX-resistant ESBL strain no. 4 donor and CPFX-resistant clinical isolate recipient) in the presence or absence of ciliates with or without drugs. Conjugation frequency was estimated as the number of transconjugants per recipient. Data represents the averages of conjugation frequency + standard deviation. *$p < 0.05$ versus values for bacteria alone.

Figure 2. Influence of incubation time on survival of both recipient (CPFX-resistant clinical isolate recipient) and donor (CTX-resistant ESBL no. 4) *E. coli* strains (A) and gene transfer frequency between the *E. coli* strains (B) in cultures with or without ciliates. Data represents the averages of bacterial number (A) or conjugation frequency (B) + standard deviation. *$p < 0.05$ versus values for bacteria alone at each time point.

Figure 1.

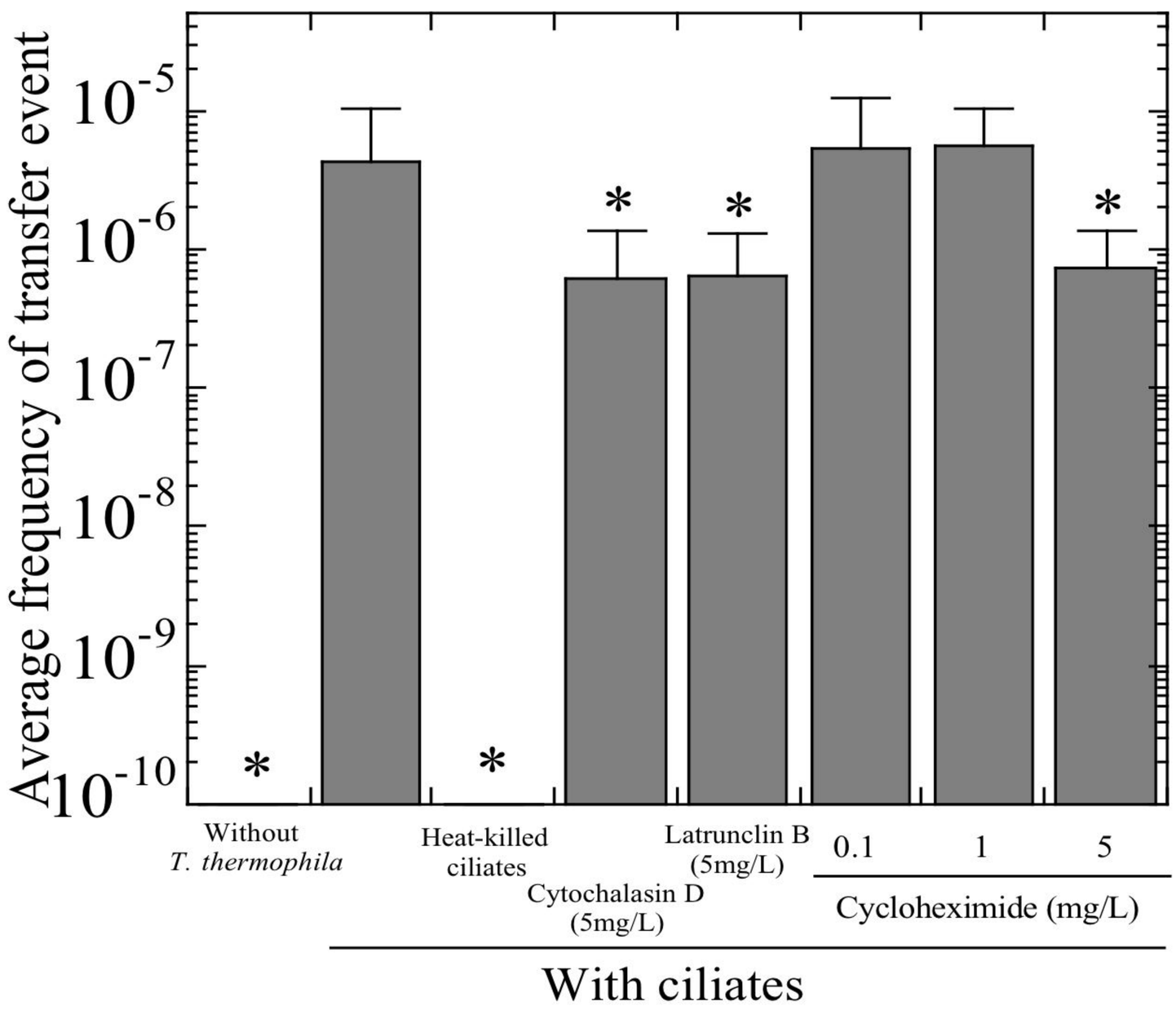
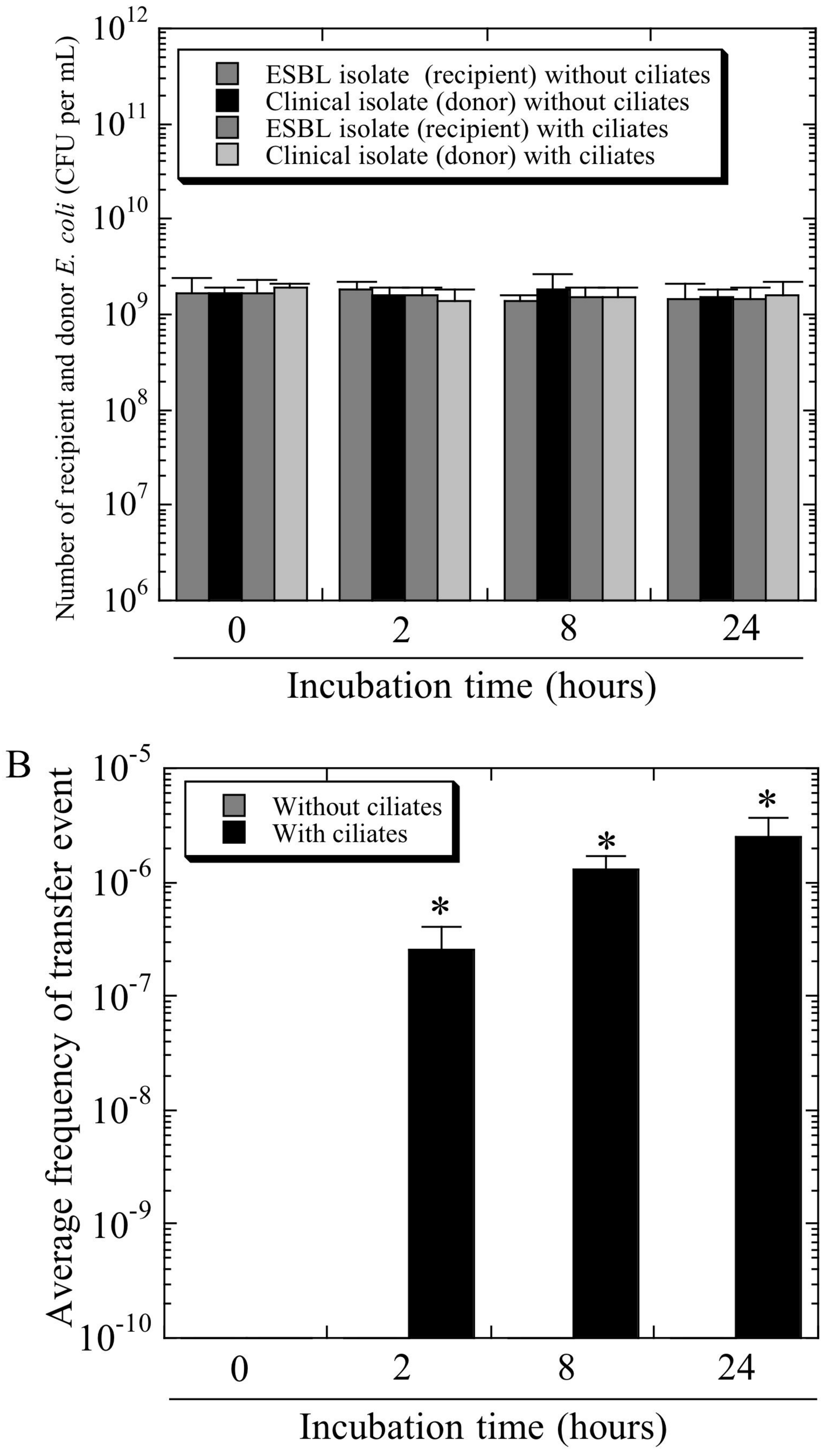

Average frequency of transfer event
10-5
10-6
10-7
10-8
10-9
10-10
*
*
*
*
Without T. thermophila
Heat-killed ciliates
Cytochalasin D (5mg/L)
Latrunclin B (5mg/L)
0.1
1
5
Cycloheximide (mg/L)
With ciliates

Figure 2


A
Number of recipient and donor E. coli (CFU per mL)
10^12
10^11
10^10
10^9
10^8
10^7
10^6
ESBL isolate (recipient) without ciliates
Clinical isolate (donor) without ciliates
ESBL isolate (recipient) with ciliates
Clinical isolate (donor) with ciliates
0
2
8
24
Incubation time (hours)
B
Average frequency of transfer event
10^-5
10^-6
10^-7
10^-8
10^-9
10^-10
Without ciliates
With ciliates
*
*
*
0
2
8
24
Incubation time (hours)