Identification of Host Factors Regulating *Francisella* Infection and Development of Infection Model

フランシセラ感染を抑制する宿主因子の探索とモデル構築

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March, 2023

Preface

To elucidate the infection and virulence mechanisms in bacterial infections, many studies have been conducted on bacterial factors, and various virulence factors have been identified. However, host factors have not been studied as much as bacterial factors. In addition, the hosts models with significant symptoms such as human cell models and mouse infection models have been considered as important models, and little attention has paid to the natural host that shows subclinical infection. In order to identify important host factors in bacterial infection and to construct a model of infection in natural hosts, I conducted research on the following two themes.

Chapter 1: Identification of host factors in *Francisella* infection.

Chapter 2: Development of protist infection model using *Paramecium.*

Francisella tularensis are Gram-negative intracellular bacteria [1]. Some intracellular bacteria avoid or utilize phagocytosis of host cells, and their infection were suppressed by suppression of cell phagocytosis and the host's innate immune response [2, 3, 4, 5, 6]. However, it is not completely clear what kind of the host factors are involved in *F. tularensis* infection. In chapter 1, I identified host factors

which regulated *Francisella* infection through the screening of inhibitor library and analysis of the inhibitor, Cucurbitacin I.

Paramecium spp. single-celled and free-living protists, are general ciliates found in freshwaters, such as ponds, lakes, and rivers. They are well studied in various fields as ideal model organisms since they are easy to deal with [7, 8, 9]. Our previous study has been indicated that *Paramecium* is useful as a protist infection model such as *Legionella pneumophila* or *F. tularensis* [10, 11, 12, 13]. However, *Paramecium* is classified by morphology and Syngen [8, 9, 14, 15, 16, 17, 18]. Therefore, the classification requires knowledge and skill and a more simplified classification method is required as a model. In chapter II, I investigated polymerase chain reaction (PCR) method for *Paramecium* classification. Moreover, I actually explored the possibility of using *Paramecium* as an infection model for *Francisella*.

Chapter 1 Identification of host factors in *Francisella* infection

Introduction

F. tularensis are Gram-negative intracellular bacteria and *F. tularensis* caused Tularemia of zoonosis. The severity of Tularemia depends on subspecies and the route, dose of infection. *F. tularensis* identified into 4 subspecies; *F. tularensis* subsp. *tularensis* (Type A) and *F. tularensis* subsp. *holarctica* (Type B), *F. tularensis* subsp. *mediasiatica*, *F. tularensis* subsp. *novicida* (*F. novicida*). Type A and Type B have been reported to be pathogenic to humans [1]. *F. novicida* is closely related to TypeA and is also a commensal intracellular pathogen that replicates in macrophages [19] *F. novicida* has low pathogenicity in humans but is thought to have considerable homology with *F. tularensis* and serves as a practical surrogate [20].

The establishment of *F. tularensis* infection rely on host's factors [2, 3]. *Franciscella* are ingested through the pseudopodial loop of macrophages and incorporated into spacious vacuoles with endosomal markers [21, 22]. The organism then escapes from the phagosomal membrane and replicates in the

cytoplasm [23]. In the late stages of infection, the replicated *Francisella* re-enter the autophagosome. There, they acquire amino acids from degraded proteins and replicate [24]. Cytoplasmic bacteria with defective or damaged replication are trapped by *Francisella* containing vacuoles (FCVs), which are lysosome-associated membrane protein 1 (LAMP-1) positive autophagosomes, and are degraded by the ubiquitin-SQSTM1-LC3 pathway [25, 26].

Cucurbitacin I is a triterpenoid compound derived from the fruit extract of Cucurbitaceae family plants, such as cucumber. It inhibits Janus tyrosine kinase 2 (JAK2) phosphorylation resulted in suppresses levels of tyrosine phosphorylated Signal Transducer and Activator of Transcription (STAT3); Cucurbitacin I inhibits JAK2/ STAT3 pathway [27, 28]. The JAK2/STAT3 pathway is involved in various biological processes such as immunity, cell division, cell death, and tumor formation [29]. The JAK2/STAT3 pathway is activated by the binding of hormones such as prolactin, growth factors such as epidermal growth factor (EGF) and cytokines such as the interleukin-6 (IL-6) family to the extracellular domain of specific intracellular receptors (RTKs). Especially, JAK2 mediates signaling through several cytokine receptors, including IL-6 and IFN-γ. The interaction

between ligands and receptors results in dimerization of the receptor subunits. Close proximity of JAK2s non-covalently bound to the intracellular domain of the receptor leads to autophosphorylation of JAK2s and activation of their kinase property [30, 31]. When the JAK2 protein is phosphorylated, tyrosine residues in the intracellular domain of the receptor are phosphorylated by its activated kinase domain, creating a docking site for STAT3 within SH2 domains of the receptor. This allows cytoplasmic STAT3 protein to bind to the phosphorylated tyrosine residues on the receptor. STAT3 is then phosphorylated by JAK2, dimerizes, dissociates from the receptor, moves to the nucleus. The dimer of phosphorylated STAT3 binds to specific DNA sequences, inducing transcription of target genes, such as Cyclin D1, cMyc, Bclx1, bcl-2, MCL-1, and P53 leading to cell proliferation, differentiation, apoptosis, and immune regulation. In addition, JAK2/STAT3 signaling can interact with other pathways including MAPK/ERK and PI3K/ACT/mTOR signaling pathways to activate specific cellular responses [29, 30, 31, 32, 33]. In chapter 1, to identify the host factors important for *Francisella* infection, I screened 361 inhibitors and inhibitors that affected *F. novicida* infection were selected. As the result, I focused on Cucurbitacin I, an inhibitor of

JAK2/STAT3 pathway and investigated the effect of JAK2/STAT3 pathway on *F. novicida* infection.

Materials and Methods

All experiments were conducted in compliance with the institutional biosecurity guidelines and were approved by Yamaguchi University.

Cell and culture medium

J774.1 cell was the cell line like mouse monocyte-macrophage and cultured at 37°C under 5% CO_2 in Roswell Park Memorial Institute (RPMI) 1640 medium (Thermo Fisher Scientific K.K, Tokyo, Japan) supplied with 10% Fetal bovine serum (FBS) (Thermo Fisher Scientific K.K).

Bacteria strain and culture condition

Francisella tularensis subspecies *novicida* ATCC15482 strain (*F. novicida*) was cultured aerobically at 37°C with Brain heart infusion broth (Becton and Dickinson company, USA) suppled with L-cysteine (Fujifilm, Tokyo, Japan) (BHIc), BHIc plates containing 1.5% Agar (Fujifilm, Osaka, Japan) [34] or chemically defined medium (CDM) [35]. Green Fluorescent Protein (GFP)-expressing *F. novicida* was cultured with BHIc containing 2.5 μg/ml chloramphenicol [36].

Escherichia coli ATCC15482 strain was cultured aerobically at 37°C in a Luria-Bertani medium (LB) (LB broth miller, nacalai tesque, Kyoto, Japan), LB plates containing 1.5% Agar. Bacterial concentrations were adjusted by optical density (OD, λ=595 nm).

Screening

Inhibitor Kit for screening was provided from Molecular Profiling Committee, Grant-in-Aid for Transformative Research Areas "Advanced Animal Model Support (AdAMS)" from The Ministry of Education, Culture, Sports, Science and Technology, Japan (JSPS KAKENHI Grant Number JP 22H04922). The 361 inhibitors contained in the Inhibitor Kit were dissolved in Dimethyl sulfoxide (DMSO). Table 1 listed all inhibitors used in this screening.

J774.1 cells (25×10^4 cells/ml) were seeded at 100 µl/well in 96 well plate and cultured overnight. The culture medium was changed to medium containing each inhibitor at final concentration of 1 or 10 µM with one exception (No. 144 at 1 or 0.1 µM), and incubated for 2hours. After the treatment, the cells were infected with GFP-expressing *F. novicida* for 24h. Then, cells were washed three times with phosphate-buffered saline (PBS), and the fluorescence intensity was measured

using Multilabel reader ARVO X4 system (PerkinElmer, Massachusetts, USA). The intensity was also confirmed by fluorescence microscopic observation using IX71 (Olympus Corporation, Tokyo, Japan).

Inhibitors

Paclitaxel (Fujifilm), SB218078 (Merck, Darmstadt, German), Cucurbitacin I (Merck) and STAT3 inhibitor Stattic (Merck) were dissolved in DMSO (Fujifilm) at 2 mM concentration and diluted to 200 µM, 20 µM and 2 µM. The same amount of the inhibitors added to culture medium (Inhibitor 0.5 µl/ Medium 100 µl) at final concentrations of 10, 1, 0.1 and 0.01 µM.

Inhibitor treatment before infection

Treatment of inhibitors and infection were carried out according to the previous report with slight modification [37, 38, 39]. Briefly, J774.1 cells (25×10^4 cells/ml) were seeded at 100 µl/well in 96 well plate or 500 µl/well in 24 well plate and cultured overnight. The cells were treated with indicated concentration of Cucurbitacin I, Stattic, or DMSO control for 2h. After the treatment, the cells were infected with *F. novicida* at multiplicity of infection (MOI) = 1. The plates were centrifuged for 10minutes at 300×g and incubated for 1h at 37°C. The cells were

cultured in culture medium containing inhibitors and 50 μg/ml of gentamycin for 1h to kill extracellular bacteria. Then, cells were washed three times with PBS and cultured in medium containing inhibitors at 37°C.

Inhibitor treatment after infection

Treatment of inhibitors and infection were carried out according to the previous report with slight modification [37, 38, 39]. Briefly, J774.1 cells (25×10^4 cells/ml) were seeded at 100 μl/well in 96 well plate or 500 μl/well in 24 well plate and cultured overnight. The cells were infected with *F. novicida* at MOI = 1. The plates were centrifuged for 10min at 300×g and incubated for 1h at 37°C. The cells were cultured in culture medium containing inhibitors and 50 μg/ml of gentamycin for 1h to kill extracellular bacteria. Then, cells were washed three times with PBS. The cells were cultured with culture medium containing indicated concentration of Cucurbitacin I, Stattic, or DMSO control for 12h.

Colony forming units (CFU)

J774.1 cells (25×10^4 cells/ml) were seeded at 100 μl/well in 96 well plate and cultured overnight. After inhibitor treatment and infection described above, the cells were washed three times with PBS and cells were disrupted with 0.1% Triton-

X in PBS for 1min and 900 µl CDM was immediately added. Samples were diluted with CDM and cultured on BHIc plate overnight and numbers of colony were counted.

Phagocytosis

The activity of phagocytosis against *E. coli* was measured as previously described with slight modification [40, 41]. J774.1 cells (25×10^4 cells/ml) were seeded at 100 µl/well in 96 well plate or 500 µl/well in 24 well plate and cultured overnight. The cells were treated with the indicated concentration of Cucurbitacin I, Stattic, or DMSO control for 2h. After the treatment, the cells were infected with *E. coli* for 3h and treated with Gentamycin to kill extracellular bacteria. Then, the cells were washed three times with PBS and the cells were disrupted with 0.1% Triton-X in PBS for 1min followed by immediate addition of 900 µl PBS. CFU was counted as described above.

Laser Scanning Confocal Microscopy

J774.1 cells (25×10^4 cells/ml) were seeded at 500 µl/well in 24 well plate with 12 mm glass coverslips (Matsunami, Osaka, Japan) and cultured overnight. After treatment and infection with GFP-expressing *F. novicida* described above, the cells

were washed three times with PBS and fixed by 4% paraformaldehyde in PBS for 30min at room temperature. A FluoView FV100 confocal laser scanning microscope (Olympus) was used to obtain images of the cells.

Actin filaments

J774.1 cells (25×10^4 cells/ml) were seeded at 500 µl/well in 24 well plate with 12 mm glass coverslips (Matsunami) and cultured overnight. After the inhibitor treatment and infection with GFP-expressing *F. novicida*, the cells were washed three times with PBS and fixed by 4% paraformaldehyde in PBS. After washed three times with PBS, cells were permeabilized with 0.1% Triton-X in PBS for 5 min and washed three times with PBS. Then, cells were blocked with 2% bovine serum albumin in PBS for 1h and stained with 0.1 µM of Phalloidin-Rhodamine B isothiocyanate (P1951, Thermo Fisher Scientific K.K) for 2h. The cells were washed three times with PBS and observed using laser scanning confocal microscopy.

Western Blot analysis (WB)

WB was carried out according to the method described in previous study with modification [27, 36, 42]. J774.1 cells (25×10^4 cells/ml) were seeded at 500 µl/well in 24 well plate and cultured overnight. After the cells treated with 1 µM

Cucurbitacin I or 10 μM Stattic for 2h, cells were infected with *F. novicida*. The cells were washed twice by cold PBS and lysed with LDS sample buffer (Thermo Fisher Scientific K.K), and sonicated 10 times at 75% of output using ultrasonic homogenizer VP-050 (TAITEC, Saitama, Japan). Then, dithiothreitol was added to the samples at final concentration of 100 mM and boiled at 70°C for 10min. Each sample (30 μl) was separated on SDS-PAGE with a 4%–12% Bis-Tris Gel (Thermo Fisher) and were transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membrane was blocked with 5% skim milk for 1h and washed once with Tris-Buffered Saline (TBS) containing 0.1% tween20, then incubated with anti-phosphorylated STAT3 (1:20000, #94994S, Cell Signaling Technology, Massachusetts, USA) or anti-total STAT3 (1:10000, #9139, Cell Signaling Technology) primary antibodies overnight at 4°C. the primary antibodies overnight at 4°C. After the membranes were washed three times in TBS with 0.1% tween20, the membranes were incubated with 0.01 mg/ml of anti-mouse or rabbit IgG conjugated with horseradish peroxidase at room temperature for 2h. The membranes were developed using an enhanced chemiluminescence detection system (GE Healthcare Life Science, Little Chalfont, UK).

Lactic acid dehydration enzyme (LDH) assay

J774.1 cells (25×10^4 cells/ml) were seeded at 100 µl/well in 96 well plate and cultured overnight. The cells were treated with indicated concentration of inhibitors in 110 µl/well of culture medium for 24h. The plate was centrifuged at 300×g for 10min, and supernatants were collected. The LDH activity in the supernatants were measured using LDH cytotoxicity Detection kit (Takara bio, Shiga, Japan).

Statics analysis

Significant differences are determined by $P< 0.05$ or $P< 0.01$ using Student's T-test or Dunnett's test, Tukey-Kramer method, and indicate with * and ** respectively.

Results

Screening

To identify host factors important for the infection of *F. novicida*, 361 inhibitors were screened to identify the inhibitors that affect the growth of *F. novicida*. J774.1 cells treated with 1 or 10 µM of inhibitors for 2h were infected with GFP-expressing *F. novicida*, and fluorescence intensity was compared to

DMSO-treated control cells. The inhibitors were classified into "Promotion", "Suppression", "None"; the inhibitors with intensity > 5000 higher than the control at both of 1 and 10 μM were classified as "Promotion", the inhibitors with intensity > 4000 lower the DMSO control at both of 1 and 10 μM were classified as "Suppression", and the others were classified as "None" (Table 2). As the results, 8 inhibitors were classified as "Promotion", 56 inhibitors were classified as "Suppression", and 297 inhibitors were classified as "None" (Table 2). To confirm the effect of inhibitors, cells treated with inhibitors were observed with fluorescence microscopy. The results were observed with fluorescent microscopy and there were no inhibitors that damaged the cells and removed from the bottom of the wells.

Among those inhibitors, 3 inhibitors, No.54 (Cucurbitacin I), No.8 (Paclitaxel), and No.140 (SB218078) classified as "Suppression", "None", "Promotion" respectively were selected and the effect was confirmed. Cucurbitacin I and SB218078 showed significant difference of bacterial number of *F. novicida* in J774.1 cells at 10 μM (Fig 1). Cucurbitacin I suppressed the intracellular number of *F. novicida* compared to DMSO control. On the other hands, SB218078 promoted

the intracellular number of *F. novicida*. As the result of fluorescence microscopic observation, however, SB218078 was found to be auto-fluorescent (data not shown). Paclitaxel was no difference than DMSO.

Cucurbitacin I

The effect of inhibitors on *F. novicida* infection

Because the host factors important for *F. novicida* infection was searched for in this study, I focused on inhibitors classified as "Suppression". In particular, I selected Cucurbitacin I of JAK2/STAT3 pathway inhibitor and the effect on *F. novicida* infection was investigated.

To check whether Cucurbitacin I suppressed *F. novicida* infection, we treated J774.1 cells with Cucurbitacin I and infected with GFP-expressing *F. novicida* and the intracellular bacteria were observed by confocal microscopy. Cucurbitacin I-treated J774.1 cells were also infected with *F. novicida*, and the number of intracellular bacteria was measured by colony counting. As the results, Cucurbitacin I treatment decreased the number of intracellular *F. novicida* (Fig 2 A and C). These results suggest that JAK2/STAT3 pathway is important for *F. novicida* infection. To confirm the importance of JAK2/STAT3 pathway in *F.*

novicida infection, another JAK2/STAT3 pathway inhibitor Stattic was used. Stattic treatment decreased the number of intracellular *F. novicida* in both of microscopic observation and colony counting (Fig 2 B and D). Because 0.5 μM Cucurbitacin and 10 μM of Stattic at least were significantly effective, 1 μM Cucurbitacin and 10 μM of Stattic were used hereafter.

The effect of inhibitors on *F. novicida* growth in culture medium

To check the direct effect of inhibitors on the growth of *F. novicida*, Cucurbitacin I or Stattic was added into the growth medium and the growth was measured by optical density (λ=660 nm) and colony count. As the result, no significant differences were observed between Cucurbitacin I and DMSO control (Fig 3 A and C). In the presence of Stattic, on the other hands, the growth of *F. novicida* was slightly delayed at the early stage of growth. However, *F. novicida* reached to the same level of optical density at the late stage of growth regardless the presence of the inhibitor (Fig 3). Thus, Stattic delays *F. novicida* growth, but it grew to the same level as DMSO at the finally. Next, GFP-expressing *F. novicida* cultured with inhibitors were washed and infected to J774.1 cells, and intracellular *F. novicida* were observed with confocal microscopy. The same levels of

intracellular *F. novicida* was observed in the bacteria cultured with Cucurbitacin or Stattic compared to DMSO control (Fig 4). These results indicate that Cucurbitacin and Stattic have no direct effect to the growth and infectivity of *F. novicida*.

F. novicida infection to cells

Infection is established through following three steps, attachment, internalization, and proliferation [2, 3, 4, 5, 6, 37, 43]. To investigate which step of infection is affected by the inhibitors, at first the attachment of *F. novicida* was tested. J774.1 cells were treated with Cucurbitacin and Stattic, then infected with *F. novicida*. The bacteria number attached to cells at just after infection (10 and 30min) was measured by colony counting. As the result, no significant difference was observed between control DMSO and inhibitors (Fig 5). Next, to test the effect of inhibitors of internalization and proliferation, J774.1 cells were treated with inhibitors and infected with *F. novicida*. the cells were incubated for 1h to allow internalization of bacteria and attached bacterial cells were removed by gentamicin treatment. After 1.5 and 12h incubation, the number of internalized and proliferated *F. novicida* were measured. As the results, the intracellular *F. novicida*

of Cucurbitacin I and Stattic were significantly less than DMSO (Fig 6).

To determine which steps of internalization and proliferation were affected by inhibitors, proliferation of *F. novicida* was examined. J774.1 cells were infected with *F. novicida* and incubated for 1h to allow internalization of bacteria. Attached bacteria were removed by gentamicin treatment and infected cells were treated with inhibitors. The bacterial numbers in inhibitor-treated cells were not affected by inhibitors. There was no significant difference between DMSO and Cucurbitacin I or Stattic (Fig 7). These results indicate that Cucurbitacin I and Stattic affect the step of internalization of *F. novicida* infection.

Effect on Phagocytotic activity.

Because Cucurbitacin and Stattic affected internalization of *F. novicida*, the effect of inhibitors on phagocytosis was investigated using non-intracellular bacteria *E. coli* [40, 41]. J774.1 cells were treated with Cucurbitacin or Stattic and infected with *E. coli*. Cells were incurvated for 3h to allow phagocytosis and attached cells were removed by gentamicin treatment for 30min. At 3.5h post infection, the internal *E. coli* number was measured by colony counting. As the result, inhibitors decreased the number of internalized *E.coli* (Fig 8), indicating

Cucurbitacin and Stattic affect phagocytosis of host cells.

Effect of actin filaments

Phagocytosis results from polymerization, depolymerization, and rearrangement of actin [44]. To investigate the effect of inhibitors on actin polymerization, J774.1 cells were treated with Cucurbitacin or Stattic for 2h and infected with *F. novicida*, then actin was visualized by immunofluorescence microscopy. As the results, abnormal arrangements of actin were observed in inhibitor-treated cells compared to DMSO control (Fig 9). These results suggest that proper arrangement of actin is crucial for the infection of *F. novicida*.

Phosphorylation of STAT3

To determine whether *F. novicida* infection activates JAK2/STAT3 pathway or not, amount of intracellular phosphorylated-STAT3 (p-STAT3) were measured by western blotting. Treatment of Stattic for 2h were resulted in relative activation of phosphorylation of STAT3 compared to DMSO control and Cucurbitacin treatment. Without inhibitors (in DMSO control), infection of *F. novicida* tend to activate the phosphorylation of STAT3 at 10min post infection compared to inhibitors-treated cells, whereas phosphorylated STAT3 level was decreased to the

control level (at 60min post infection). In the presence of Cucurbitacin and Stattic, the enhancement of phosphorylated STAT3 at 10min post infection was not observed, indicating these inhibitors suppress the phosphorylation of STAT3 induced by *F. novicida* infection (Fig 10).

Cytotoxicity of inhibitors

To estimate the cytotoxicity of inhibitors, the activity of LDH released by inhibitor treatment was measured. The cytotoxicity of 1 and 10 μM Cucurbitacin I and 10 μM Stattic were increased compared to DMSO control. The cytotoxicity of 10 μM Stattic was higher than 1 and 10 μM Cucurbitacin I (Fig 11). This result indicates that these inhibitors possess cytotoxicity, but the cytotoxicity is weaker in Cucurbitacin I.

Discussion

To identify the host factors important for *Francisella* infection, 361 inhibitors were screened, and the inhibitors that affected *F. novicida* infection were identified. As the results, 8 inhibitors facilitated *F. novicida* infection and were classified as "Promotion", 56 inhibitors inhibited the infection and were classified as "Suppression", and 297 inhibitors failed to show any effect and were classified as

"None" (Table 2). It was previously reported that *F. tularensis* infection was inhibited by Wortmannin through the suppression of phagocytosis [4, 5, 6]. Although Wortmannin was classified in "None" in this screening, 10 μM Wortmannin (No. 75 and 261 of Table 3) inhibited the infection of *F. novicida*. In addition, to confirm the results of screening, Paclitaxel, SB218078, and Cucurbitacin I were purchased from another provide and, same results were obtained (Tables 2, Fig 1). These results indicate that screening method measuring fluorescence intensity of GFP derived from infected *F. novicida* is considered appropriate for the identification of inhibitors that affect the infectivity of bacteria.

Because the host factors important for *F. novicida* infection was searched for in this study, I focused on 56 inhibitors classified as "Suppression". Since most of inhibitors classified as "Suppression" possess antibiotic-property, I focused on the inhibitors without antibiotic-property such as inhibitors of JAK2/STAT3 pathway. In particular, I selected Cucurbitacin I for further study and the effect of the inhibitor on *F. novicida* infection was investigated. Cucurbitacin I is a triterpenoid compound derived from *Cucumis sativus L.* It inhibits JAK2 phosphorylation resulted in suppression levels of tyrosine phosphorylated STAT3 [27, 28]. In this

study, Cucurbitacin I inhibit the infection of *F. novicida*, even though it failed to affect the growth of *F. novicida* in culture medium (Figs 1-4). To confirm the involvement of JAK2/STAT3 pathway in *F. novicida* infection, another inhibitor of JAK2/STAT3 pathway, Stattic was tested. Stattic is a non-peptide small molecule and inhibit dimerization of STAT3 through SH2 domain [42, 45, 46, 47]. Similar to Cucurbitacin I, Stattic showed only slight effect on the growth of *F. novicida* in the culture medium, but strongly inhibited the *F. novicida* infection.

To examine which of the three steps of attachment, invasion and intracellular proliferation is inhibited by Cucurbitacin I or Stattic, Cells were treated with inhibitors and the effects of inhibitors were observed at different time points. Treatment of inhibitors failed to affect the attachment of *F. novicida* to the cells at 10 and 30min post -infection (Fig 5). These results indicate that Cucurbitacin I and Stattic do not suppress *F. novicida* attachment on the cell surface and the inhibitors affect the internalization or intracellular proliferation. Cucurbitacin or Stattic-treatment after infection failed to decrease the number of intracellular *F. novicida*, indicating that intracellular proliferation was not affected by the inhibitors. These results suggest that JAK2/STAT3 pathway is important for the

internalization step of *F. novicida*. The importance of JAK2/STAT3 pathway in bacterial infection was also reported in various other bacteria. In the infection of *Brucella abortus*, JAK2/STAT3 pathway is important for the intracellular survival of the bacteria [48]. In addition, JAK2/STAT3 pathway is important for the development of pulmonary fibrosis in *Mycobacterium tuberculosis* infection [49]. Thus, JAK2/STAT3 pathway is important for the infection and pathogenesis of various bacterial infection.

Francisella are ingested through the pseudopodial loop of macrophages and incorporated into spacious vacuoles with endosomal markers [21, 22]. The organism then escapes from the phagosomal membrane and replicates in the cytoplasm [23]. To examine which step of phagocytosis or escape from phagosome is the target of inhibitors, *E. coli*, a bacterium without ability of escape from phagosome was tested. As the result, the number of ingested intracellular *E.coli* was also decreased by Cucurbitacin I or Stattic treatment. This result indicates that Cucurbitacin I and Stattic inhibit the step of phagocytosis in *F. novicida* infection.

Since phagocytosis results from polymerization, depolymerization, and

rearrangement of actin [44], actin dynamics of *F. novicida* infected cells were observed. As the result, in Cucurbitacin or Stattic-treated cells, abnormal arrangements of actin were observed (Fig 9). These results suggest that JAK2/STAT3 pathway regulate actin dynamics followed by phagocytosis. This finding is consistent with the previous report that Cucurbitacin I inhibits cell motility or proliferation of cancer cell through interfering of actin dynamics [50, 51, 52]; Cucurbitacin I suppressed the cell's phagocytosis by regulating of actin dynamics through JAK2 inhibition. Although Stattic-treatment also showed abnormal actin dynamics, the structures of actin filaments were different from that of Cucurbitacin I-treated cells (Fig 9C).

Although there was no significant difference, *F. novicida* infection tended to enhance the STAT3 phosphorylation just after infection (Fig 10). thinking about the importance of JAK2/STAT3 to induce phagocytosis, this result indicates that activation of JAK2/STAT3 pathway by *F. novicida* infection may enhance the phagocytosis of host cells and facilitate the internalization of the bacteria.

Conclusion

In this study, I identified Cucurbitacin I as an inhibitor of *F. novicida*

infection, and demonstrated that JAK2/STAT3 pathway is important for actin dynamics resulting in phagocytosis. In infection of other intracellular bacteria, JAK2/STAT3 pathway is important for the intracellular growth or pathogenesis [48, 49]. Moreover, Cucurbitacin I showed antimicrobial effect through autophagy [33]. Therefore, inhibitors such as Cucurbitacin I and Stattic can be utilized as antimicrobial agent, and JAK2/STAT3 pathway can be a therapeutic target of infection with intracellular bacteria as well. Because the cytotoxicity of Cucurbitacin I was weaker than Stattic, Cucurbitacin I was further studied for the protist infection models using *Paramecim* in chapter 2.

Tables

Table 1 List of inhibitors in screening

No.	Plate	Well	Category	Compound	Concentrations
1	1	1- B	antitumor (thymidylate synthetase)	5-FU	2mM
2	1	1- C	antitumor (aminopeptidase B)	Bestatin	2mM
3	1	1- D	antitumor (DNA)	Bleomycin sulfate	2mM
4	1	1- E	antitumor (DNA)	Cisplatin	2mM
5	1	1- F	antitumor (DHFR)	Methotrexate	2mM
6	1	1- G	antitumor (DNA)	Mitomycin C	2mM
7	1	1- H	antitumor (tubulin)	Vinblastine sulfate	2mM
8	1	2- A	antitumor (tubulin)	Paclitaxel	2mM
9	1	2- B	antitumor (AR)	Flutamide	2mM
10	1	2- C	antitumor (DNA)	Daunorubicin, HCl	2mM
11	1	2- D	antitumor (DNA)	Doxorubicin, HCl	2mM
12	1	2- E	antitumor (ER)	Tamoxifen, citrate	2mM
13	1	2- F	antitumor (RNA)	Actinomycin D	2mM
14	1	2- G	antitumor (topo I)	Camptothecin	2mM
15	1	2- H	antitumor (topo I/II)	Aclarubicin	2mM
16	1	3- A	antitumor (topo II)	Etoposide (VP-16)	2mM
17	1	3- B	actin filament	Cytochalasin D	2mM
18	1	3- C	adenylcyclase	2',5'-dideoxyadenosine	2mM
19	1	3- D	AKT	AKT inhibitor	2mM
20	1	3- F	Bcr-Abl	AG957	2mM
21	1	3- G	CAMKII	KN93	2mM
22	1	3- H	caspase	Z-VAD-FMK	2mM
23	1	4- A	CDC2	Kenpaullone	2mM
24	1	4- B	CDK2	Purvalanol A	2mM
25	1	4- C	CDK4	3-ATA	2mM
26	1	4- D	CDKs	Olomoucine	2mM
27	1	4- E	CKII	TBB	2mM
28	1	4- F	COX-1	Sulindac sulfide	2mM
29	1	4- G	COX-1	Valeryl salicylate	2mM
30	1	4- H	COX-2	NS-398	2mM
31	1	5- A	COX	Sodium salicylate	2mM
32	1	5- B	cyclicphosphodiesterase	Theophylline	2mM
33	1	5- C	DNA methyltransferase	Azacytidine	2mM
34	1	5- D	DNA polymerase	Aphidicolin	2mM
35	1	5- E	EGFR	AG1478	2mM
36	1	5- F	EGFR, topoII	Genistein	2mM
37	1	5- G	farnesyltransferase	Manumycin A	2mM
38	1	5- H	farnesyltransferase	FTI-276	2mM
39	1	6- A	Flk-1	SU1498	2mM
40	1	6- B	geranylgeranyltransferase I	GGTI-286	2mM
41	1	6- C	GR	Dexamethasone	2mM
42	1	6- D	GSK-3	GSK-3 inhibitor II	2mM
43	1	6- E	HDAC	Scriptaid	2mM
44	1	6- F	HDAC	Trichostatin A	2mM
45	1	6- G	HER2 (erbB2/neu), EGFR	AG825	2mM
46	1	6- H	protein synthesis	Cycloheximide	2mM
47	1	7- A	HMG-CoA reductase	Lovastatin	2mM
48	1	7- B	HSP90	Radicicol	2mM
49	1	7- C	HSP90	17-AAG	2mM
50	1	7- D	IGF-1R	AG1024	2mM

No.	Plate	Well	Category	Compound	Concentrations
51	1	7- E	iNOS	1400W, HCl	2mM
52	1	7- F	iNOS	AMT, HCl	2mM
53	1	7- G	Jak-2	AG490	2mM
54	1	7- H	Jak-2	Cucurbitacin I	2mM
55	1	8- A	JNK	SP600125	2mM
56	1	8- B	lck (p56), TYK	Damnacanthal	2mM
57	1	8- C	MEK	PD 98059	2mM
58	1	8- D	MEK	U0126	2mM
59	1	8- E	methionine aminopeptidase	Fumagillin	2mM
60	1	8- F	MMP	GM 6001	2mM
61	1	8- G	NF-kB	N-Acetyl-L-cysteine	2mM
62	1	8- H	NOS	Aminoguanidine, HCl	2mM
63	1	9- A	NOS	L-NMMA	2mM
64	1	9- B	p38 (MAPK)	PD169316	2mM
65	1	9- C	p38 (MAPK)	SB 203580	2mM
66	1	9- D	p70 S6K	Rapamycin	2mM
67	1	9- E	PARP	NU1025	2mM
68	1	9- F	PARP-1	Benzamide	2mM
69	1	9- G	PC-PLC	D609	2mM
70	1	9- H	PDE	IBMX	2mM
71	1	10- A	PDE (cAMP)	Ro-20-1724	2mM
72	1	10- B	PDE (cGMP)	Zaprinast	2mM
73	1	10- C	PDGFR	AG1296	2mM
74	1	10- D	PI3K	LY294002	2mM
75	1	10- E	PI3K	Wortmannin	2mM
76	1	10- F	PKA	H-89, HCl	2mM
77	1	10- G	PKC	Bisindolymaleimide I, HCl	2mM
78	1	10- H	PKC, PKA	H-7	2mM
79	1	11- A	PKC, PKA, PKG, MLCK	Staurosporine	2mM
80	1	11- B	PLA2	cPLA2inhibitor	2mM
81	1	11- C	PLA2	OBAA	2mM
82	1	11- D	PP2A	Cantharidin	2mM
83	1	11- E	PP2A	Cytostatin	2mM
84	1	11- F	PP2B/cyclophilin	Cyclosporin A	2mM
85	1	11- G	PP2B/FKBP	FK-506	2mM
86	1	11- H	proteasome	MG-132	2mM
87	1	12- A	proteasome	Lactacystin	2mM
88	1	12- B	ribonucleotide reductase	Hydroxyurea	2mM
89	1	12- C	ROCK	HA1077	2mM
90	1	12- D	ROCK	Y27632	2mM
91	1	12- E	Src, Fyn, Lck	PP1 (analog)	2mM
92	1	12- G	tubulin depolymerization	Nocodazole	2mM
93	1	12- H	tyr phosphatase (PTP)	Dephostatin	2mM
94	2	1- B	p53	Pifithrin-a (cyclic)	2mM
95	2	1- C	p53 activator	PRIMA-1	2mM
96	2	1- D	5α-reductase	Finasteride	2mM
97	2	1- E	aromatase	Aminoglutethimide	2mM
98	2	1- F	aromatase	Formestane	2mM
99	2	1- G	progesterone receptor	Mifepristone	2mM
100	2	1- H	acetyl-CoA carboxylase (ACC)	TOFA	2mM

No.	Plate	Well	Category	Compound	Concentrations
101	2	2- A	aminopeptidase A	Amastatin	2mM
102	2	2- B	aminopeptidase M	Actinonin	2mM
103	2	2- C	F1-ATPase	Oligomycin	2mM
104	2	2- D	V-ATPase	Bafilomycin A1	2mM
105	2	2- E	Bcl-2	HA 14-1	2mM
106	2	2- F	Bcl-XL	BH3I-1	2mM
107	2	2- G	Burton's tyrosine kinase(BTK)	LFM-A13	2mM
108	2	2- H	Burton's tyrosine kinase(BTK)	Terreic acid	2mM
109	2	3- A	calpain	E-64d	2mM
110	2	3- B	calpain, cathepsin B, L	ALLN	2mM
111	2	3- C	cathepsin B	CA-074	2mM
112	2	3- D	cathepsin D	Pepstatin A	2mM
113	2	3- E	cathepsin G	Z-GLF-CMK	2mM
114	2	3- F	CCR2	RS 102895	2mM
115	2	3- G	CCR3	SB 328437	2mM
116	2	3- H	CXCR2	SB 225002	2mM
117	2	4- A	CXCR4	AMD3100 octahydrochloride	2mM
118	2	4- B	Cdc25	NSC95397	2mM
119	2	4- D	Na channel	Amiloride	2mM
120	2	4- E	Na channel	Lidocaine	2mM
121	2	4- F	Na ionophore	Monensin	2mM
122	2	4- G	Na/K ATPase	Ouabain	2mM
123	2	4- H	Na/K/Mg ATPase	Sanguinarine	2mM
124	2	5- A	K channel	Glibenclamide	2mM
125	2	5- B	K channel	Dequalinium	2mM
126	2	5- C	K channel opener	Diazoxide	2mM
127	2	5- D	K ionophore	Valinomycin	2mM
128	2	5- E	K ionophore	Nigericin	2mM
129	2	5- F	Ca channel	Diltiazem	2mM
130	2	5- G	Ca channel	Nifedipine	2mM
131	2	5- H	Ca channel, MDR	Verapamil	2mM
132	2	6- A	MDR	PGP-4008	2mM
133	2	6- B	BCRP	Fumitremorgin C	2mM
134	2	6- C	Ca ionophore	A23187	2mM
135	2	6- D	Ca ionophore	Ionomycin	2mM
136	2	6- E	Ca-ATPase	Thapsigargin	2mM
137	2	6- F	Ca-ATPase	t-Butylhydroquinone (BHQ)	2mM
138	2	6- G	Cl channel	N-phenylanthranilic acid	2mM
139	2	6- H	Cl channel	DIDS	2mM
140	2	7- A	Chk 1	SB 218078	2mM
141	2	7- B	Chk 1, 2	Debromohymenialdisine (DBH)	2mM
142	2	7- C	mitochondrial complex I	Rotenone	2mM
143	2	7- D	mitochondrial complex III	Antimycin A1	2mM
144	2	7- E	CRM1	Leptomycin B*	0.2mM
145	2	7- F	DAG kinase	R59022	2mM
146	2	7- G	DAG kinase	Dioctanoylglycol	2mM
147	2	7- H	DAG lipase	RHC80267	2mM
148	2	8- A	DAG acyltransferase (DGAT)	Xanthohumol	2mM
149	2	8- B	fatty acid synthase (FAS)	C75	2mM
150	2	8- C	FAS	Cerulenin	2mM

No.	Plate	Well	Category	Compound	Concentrations
151	2	8- D	glycosylation	Tunicamycin	2mM
152	2	8- E	glucosidase I, II	Deoxynojirimycin	2mM
153	2	8- F	a-mannosidase	Swainsonine	2mM
154	2	8- G	guanylate cyclase	LY 83583	2mM
155	2	8- H	guanylate cyclase	ODQ	2mM
156	2	9- A	HAT	Anacardic acid	2mM
157	2	9- B	HIF	Chetomin	2mM
158	2	9- C	HIF-1a hydroxylase	Dimethyloxalylglycine	2mM
159	2	9- D	kinesin Eg5	HR22C16	2mM
160	2	9- E	kinesin Eg5	Monastrol	2mM
161	2	9- F	lipoxygenase	Nordihydroguaiaretic acid (NDGA)	2mM
162	2	9- G	12, 15-lipoxygenase	ETYA	2mM
163	2	9- H	12-lipoxygenase	Baicalein	2mM
164	2	10- A	Mdm2	Nutlin-3	2mM
165	2	10- B	Mdm2	MDM2 inhibitor	2mM
166	2	10- C	monoamine oxidase	Phenelzine	2mM
167	2	10- D	monoamine oxdase B	Deprenyl	2mM
168	2	10- E	mitochondrial permeability transition pore (MPTP)	Decylubiquinone	2mM
169	2	10- F	MPTP	Ro 5-4864	2mM
170	2	10- G	MPTP opener	Lonidamine	2mM
171	2	10- H	myosin light chain kinase	ML-7	2mM
172	2	11- A	O6-methylguanine-DNA methyltransferase (MGMT)	Benzylguanine	2mM
173	2	11- B	ornithine decarboxylase (ODC)	DFMO	2mM
174	2	11- C	PKG	KT 5823	2mM
175	2	11- D	PKG	Rp-8-CPT-cGMPS	2mM
176	2	11- E	PPAR-a	MK 886	2mM
177	2	11- F	PPAR-a activator	Clofibrate	2mM
178	2	11- G	PPAR-g	BADGE	2mM
179	2	11- H	PPAR-g activator	Troglitazone	2mM
180	2	12- A	reverse transcriptase	AZT	2mM
181	2	12- B	reverse transcriptase	Nalidixic acid	2mM
182	2	12- C	RNA polymerase	a-Amanitin	2mM
183	2	12- D	telomerase	MST-312	2mM
184	2	12- E	telomerase	b-Rubromycin	2mM
185	2	12- F	TGF-b receptor	SB 431542	2mM
186	2	12- G	spermidine/spermine N1-acetyltransferase (SSAT) activator	N1,N12-Diethylspermine (BESpm)	2mM
187	2	12- H	sphingosine N-acyltransferase	Fumonisin B1	2mM
188	3	1- B	AK	ABT-702	2mM
189	3	1- C	AKT	Akt Inhibitor IV	2mM
190	3	1- D	AKT	Akt Inhibitor VIII, Isozyme-Selective, Akti-1/2	2mM
191	3	1- E	AKT	Akt Inhibitor XI	2mM
192	3	1- F	AMPK	compound C	2mM
193	3	1- G	ATM	ATM/ATR kinase inhibitor	2mM
194	3	1- H	ATM	ATM kinase inhibitor	2mM
195	3	2- A	Aurora	Aurora kinase/cdk inhibitor	2mM
196	3	2- B	Aurora	Aurora kinase inhibitor II	2mM
197	3	2- C	Aurora	Aurora kinase inhibitor III	2mM
198	3	2- D	Bcr-abl	AG957	2mM
199	3	2- E	BTK	LFM-A13	2mM
200	3	2- F	BTK	Terreic acid	2mM

No.	Plate	Well	Category	Compound	Concentrations
201	3	2- G	CAMKII	KN-93	2mM
202	3	2- H	CAMKII	KN-62	2mM
203	3	3- A	CAMKII	Lavendustin C	2mM
204	3	3- B	CDK	Kenpaullone	2mM
205	3	3- C	CDK	purvalanol A	2mM
206	3	3- D	CDK	Olomoucine	2mM
207	3	3- E	CDK	Alsterpaullone, 2-cyanoethyl	2mM
208	3	3- F	CDK	Cdk1/2 inhibitor III	2mM
209	3	3- G	CDK	Cdk2/9 inhibitor	2mM
210	3	3- H	CDK	NU6102	2mM
211	3	4- A	CDK	Cdk4 inhibitor	2mM
212	3	4- B	CDK	NSC625987	2mM
213	3	4- C	Chk	SB218078	2mM
214	3	4- D	Chk	isogranulatimide	2mM
215	3	4- E	Chk	Chk2 inhibitor	2mM
216	3	4- F	Chk	Chk2 inhibitor II	2mM
217	3	4- G	CK	Ellagic acid	2mM
218	3	4- H	CK	TBB	2mM
219	3	5- A	CK	DMAT	2mM
220	3	5- B	CK	D4476	2mM
221	3	5- C	Clk	TG003	2mM
222	3	5- D	DGK	Diacylglycerol kinase inhibitor II	2mM
223	3	5- E	DNA-PK	IC60211	2mM
224	3	5- F	eEF2	TX-1918	2mM
225	3	5- G	EGFR	BPIQ- II	2mM
226	3	5- H	EGFR	AG1478	2mM
227	3	6- A	EGFR	AG490	2mM
228	3	6- B	FGFR	SU4984	2mM
229	3	6- C	FGFR	SU5402	2mM
230	3	6- D	Flt-3	Flt-3 Inhibitor	2mM
231	3	6- E	Fms	cFMS Receptor Tyrosine Kinase Inhibitor	2mM
232	3	6- F	Fyn	SU6656	2mM
233	3	6- G	GSK	GSK-3 inhibitor IX	2mM
234	3	6- H	GSK	1-Azakenpaullone	2mM
235	3	7- A	GSK	indirubin-3'-monoxime	2mM
236	3	7- B	HER2	AG825	2mM
237	3	7- C	IGF-IR	AG1024	2mM
238	3	7- D	IGF-IR	AGL 2263	2mM
239	3	7- E	IKK	BMS-345541	2mM
240	3	7- F	IKK	IKK-2 inhibitor VI	2mM
241	3	7- G	IRAK	IRAK-1/4 inhibitor	2mM
242	3	7- H	Jak	JAK Inhibitor I	2mM
243	3	8- A	Jak	JAK3 Inhibitor VI	2mM
244	3	8- B	JNK	SP600125	2mM
245	3	8- C	JNK	JNK inhibitor VIII	2mM
246	3	8- D	Lck	Damnacanthal	2mM
247	3	8- E	Lck	PP2	2mM
248	3	8- F	MAPK	ERK inhibitor II	2mM
249	3	8- G	MEK	PD98059	2mM
250	3	8- H	MEK	U-0126	2mM

No.	Plate	Well	Category	Compound	Concentrations
251	3	9- A	MEK	MEK inhibitor I	2mM
252	3	9- B	Met	SU11274	2mM
253	3	9- C	MLCK	ML-7	2mM
254	3	9- D	p38	SB202190	2mM
255	3	9- E	p38	SB239063	2mM
256	3	9- F	PDGFR	AG1296	2mM
257	3	9- G	PDGFR	SU11652	2mM
258	3	9- H	PDGFR	PDGF receptor tyrosine kinase inhibitor V	2mM
259	3	10- A	PDGFR	PDGF receptor tyrosine kinase inhibitor IV	2mM
260	3	10- B	PI3K	LY-294002	2mM
261	3	10- C	PI3K	Wortmannin	2mM
262	3	10- D	PKA	H-89	2mM
263	3	10- E	PKA	4-cyano-3-methylisoquinoline	2mM
264	3	10- F	PKC	Bisindolymaleimide I, HCl	2mM
265	3	10- G	PKC	Go7874	2mM
266	3	10- H	PKG	Rp-8-CPT-cGMPS	2mM
267	3	11- A	PKG	KT5823	2mM
268	3	11- B	PKR	PKR inhibitor	2mM
269	3	11- C	Raf	RAF1 kinase inhibitor I	2mM
270	3	11- D	Raf	ZM 336372	2mM
271	3	11- E	ROCK	H-1152	2mM
272	3	11- F	ROCK	Y-27632	2mM
273	3	11- G	Hsp90	radicicol	2mM
274	3	11- H	Src	PP1 analog	2mM
275	3	12- A	Syk	Syk inhibitor	2mM
276	3	12- B	TGF-ßRI	SB431542	2mM
277	3	12- C	TGF-ßRI	TGF-b RI kinase inhibitor II	2mM
278	3	12- D	Tpl2	Tpl2 kinase inhibitor	2mM
279	3	12- E	TrKA	TrkA inhibitor	2mM
280	3	12- F	VEGFR	VEGFR receptor tyrosine kinase inhibitor II	2mM
281	3	12- G	VEGFR	VEGF recptor 2 kinase inhibitor I	2mM
282	3	12- H	VEGFR	SU1498	2mM
283	4	2- B	Bcr-Abl	nilotinib	2mM
284	4	2- C	Multi-kinases	sorafenib	2mM
285	4	2- D	mTOR	temsirolimus	2mM
286	4	2- E	EGFR/Her2	lapatinib	2mM
287	4	2- F	Bcr-Abl/Kit	imatinib mesylate	2mM
288	4	2- G	Multi-kinases	sunitinib malate	2mM
289	4	2- H	EGFR	gefitinib	2mM
290	4	3- A	HDAC	vorinostat	2mM
291	4	3- B	EGFR	erlotinib	2mM
292	4	3- C	Proteasome	bortezomib	2mM
293	4	3- D	Bcr-Abl/Src	dasatinib	2mM
294	4	3- E	mTOR	everolimus	2mM
295	4	3- F	Multi-kinases	pazopanib	2mM
296	4	3- G	Rho/SRF	CCG-1423	2mM
297	4	3- H	PIM	PIM1/2 Kinase Inhibitor V	2mM
298	4	4- A	PIM	PIM1 Inhibitor II	2mM
299	4	4- B	Hedgehog	AY 9944	2mM
300	4	4- C	Hedgehog	cyclopamine	2mM

No.	Plate	Well	Category	Compound	Concentrations
301	4	4- D	Hedgehog	Jervine	2mM
302	4	4- E	STAT3	WP1066	2mM
303	4	4- F	STAT3	5,15-DPP	2mM
304	4	4- G	Wnt	IWP-2	2mM
305	4	4- H	Wnt	IWR-1-endo	2mM
306	4	5- A	Wnt	FH535	2mM
307	4	5- B	Notch	DAPT	2mM
308	4	5- C	tankyrase-selective PARP	XAV939	2mM
309	4	5- D	pan-PARP	PJ-34	2mM
310	4	5- E	PARP-1/2-selective	Olaparib	2mM
311	4	5- F	antipsychotic drug	chlorpromazine hydrochloride	2mM
312	4	5- G	depression treatment	desipramine hydrochloride	2mM
313	4	5- H	golgi inhibitor	brefeldin A	2mM
314	4	6- A	stress inducer	anisomycin	2mM
315	4	6- B	thalidomide family	thalidomide	2mM
316	4	6- C	thalidomide family	lenalidomide	2mM
317	4	6- D	retinoids	tretinoin	2mM
318	4	6- E	retinoids	tamibarotene	2mM
319	4	6- F	DNA alkylation	temozolomide	2mM
320	4	6- G	EML4-ALK	crizotinib	2mM
321	4	6- H	mTOR	Torkinib	2mM
322	4	7- A	lipase	orlistat	2mM
323	4	7- B	AR	MDV3100	2mM
324	4	7- C	caspase activator	PAC-1	2mM
325	4	7- D	blc-2	ABT-737	2mM
326	4	7- E	G9a	UNC0638	2mM
327	4	7- F	G9a	BIX01294	2mM
328	4	7- G	LSD1	S2101 (LSD1 inhibitor II)	2mM
329	4	7- H	PRMT1	AMI-1	2mM
330	4	8- A	p300	C646	2mM
331	4	8- B	SIRT1	SIRT1 inhibitor III	2mM
332	4	8- C	SIRT1/2	Tenovin-6	2mM
333	4	8- D	HDAC8	PCI-34051	2mM
334	4	8- E	BRD4 bromodomain	(+)-JQ1	2mM
335	4	8- F	Telomerase	TMPyP4	2mM
336	4	8- G	PARP	BSI-201 (Iniparib)	2mM
337	4	8- H	PARP	ABT-888 (Veliparib)	2mM
338	4	9- A	PARP	AG014699 (Rucaparib)	2mM
339	4	9- B	PARP	MK-4827 (Niraparib)	2mM
340	4	9- C	Aurora	ENMD-2076	2mM
341	4	9- D	Aurora	MLN8237	2mM
342	4	9- E	Survivin	YM155	2mM
343	4	9- F	PDK1	OSU-03012	2mM
344	4	9- G	IGF-IR	OSI-906	2mM
345	4	9- H	c-Met	PF-04217903	2mM
346	4	10- A	DNMT	Decitabine	2mM
347	4	10- B	Multi-kinases	Vandetanib	2mM
348	4	10- C	Multi-kinases	Axitinib	2mM
349	4	10- D	BRAF	Vemurafenib	2mM

No.	Plate	Well	Category	Compound	Concentrations
350	4	10- E	JAK	Ruxolitinib	2mM
351	4	10- F	Hedgehog	Vismodegib	2mM
352	4	10- G	GLI1	Gant61	2mM
353	4	10- H	FGFR	PD173074	2mM
354	4	11- A	ALK	A83-01	2mM
355	4	11- B	GSK-3	BIO	2mM
356	4	11- C	GSK-3	TWS119	2mM
357	4	11- D	GSK-3	CT99021	2mM
358	4	11- E	TGFb-R	LY2157299	2mM
359	4	11- F	TGFb-R	SD208	2mM
360	4	11- G	ALK	LDN193189	2mM
361	4	11- H	ROCK	Thiazovivin	2mM

Table 2 List of inhibitors classified

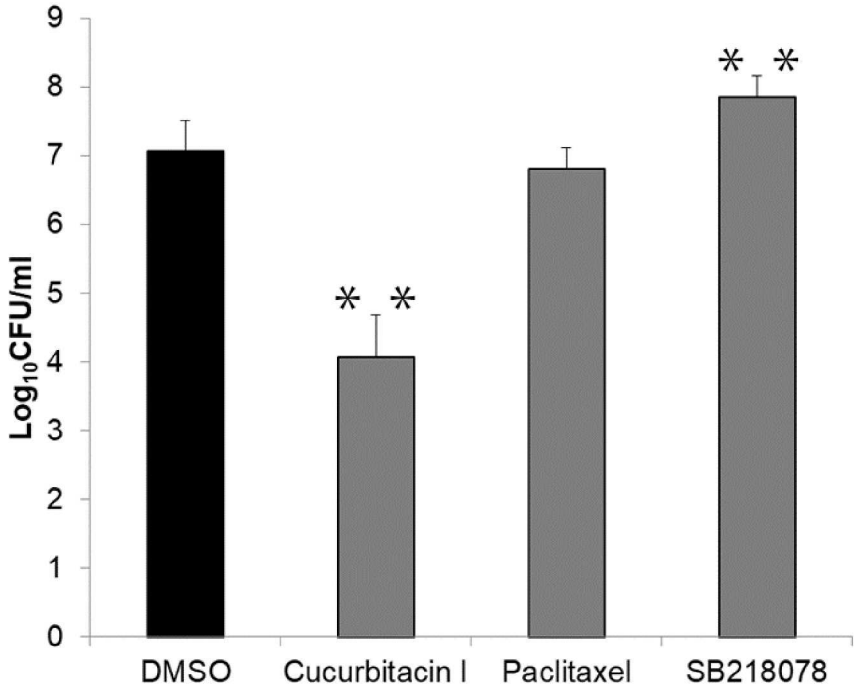
A. Promotion

No.	Category	Compound
2	antitumor (aminopeptidase B)	Bestatin
21	CAMKII	KN93
25	CDK4	3-ATA
27	CKII	TBB
32	cyclicphosphodiesterase	Theophylline
41	GR	Dexamethasone
43	HDAC	Scriptaid
52	iNOS	AMT, HCl

B. Suppression

No.	Category	Compound
17	actin filament	Cytochalasin D
54	Jak-2	Cucurbitacin I
86	proteasome	MG-132
94	p53	Pifithrin-a (cyclic)
95	p53 activator	PRIMA-1
97	aromatase	Aminoglutethimide
98	aromatase	Formestane
99	progesterone receptor	Mifepristone
100	acetyl-CoA carboxylase (ACC)	TOFA
101	aminopeptidase A	Amastatin
102	aminopeptidase M	Actinonin
104	V-ATPase	Bafilomycin A1
105	Bcl-2	HA 14-1
106	Bcl-XL	BH3I-1
107	Burton's tyrosine kinase(BTK)	LFM-A13
109	calpain	E-64d
111	cathepsin B	CA-074
112	cathepsin D	Pepstatin A
113	cathepsin G	Z-GLF-CMK
114	CCR2	RS 102895
115	CCR3	SB 328437
117	CXCR4	AMD3100 octahydrochloride
118	Cdc25	NSC95397
121	Na ionophore	Monensin
122	Na/K ATPase	Ouabain
123	Na/K/Mg ATPase	Sanguinarine
124	K channel	Glibenclamide
128	K ionophore	Nigericin
130	Ca channel	Nifedipine
136	Ca-ATPase	Thapsigargin
142	mitochondrial complex I	Rotenone
144	CRM1	Leptomycin B*
157	HIF	Chetomin
188	AK	ABT-702
198	Bcr-abl	AG957
211	CDK	Cdk4 inhibitor
212	CDK	NSC625987
267	PKG	KT5823
268	PKR	PKR inhibitor
273	Hsp90	radicicol
283	Bcr-Abl	nilotinib
284	Multi-kinases	sorafenib
285	mTOR	temsirolimus
287	Bcr-Abl/Kit	imatinib mesylate
288	Multi-kinases	sunitinib malate
289	EGFR	gefitinib
290	HDAC	vorinostat
292	Proteasome	bortezomib
298	PIM	PIM1 Inhibitor II
300	Hedgehog	cyclopamine
301	Hedgehog	Jervine
302	STAT3	WP1066
303	STAT3	5,15-DPP
313	golgi inhibitor	brefeldin A
314	stress inducer	anisomycin
342	Survivin	YM155

Figures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Cucurbitacin I
Paclitaxel
SB218078

Fig. 1 Comparison of Paclitaxel, Cucurbitacin I and SB218078.

J774.1 cells treated with 10 μM of Paclitaxel, Cucurbitacin I, or SB218078 were infected with *F. novicida*, then treated with Gentamycin. After incubation for 24h, CFU of intracellular *F. novicida* was counted. Mean ± SD, Dunnett's test, *: P=0.05, **: P=0.01

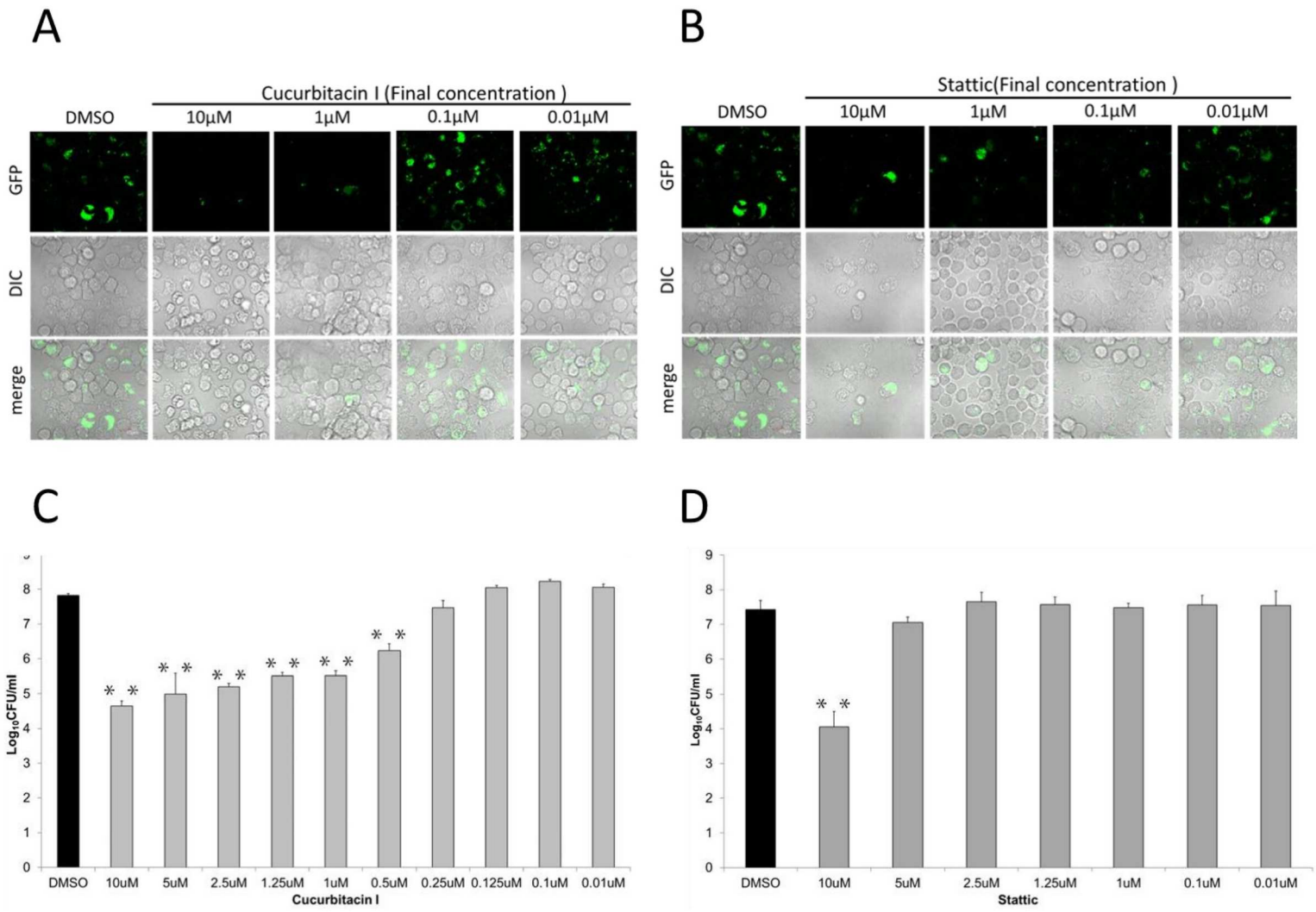

A
Cucurbitacin I (Final concentration)
DMSO
10μM
1μM
0.1μM
0.01μM
GFP
DIC
merge
B
Stattic(Final concentration)
DMSO
10μM
1μM
0.1μM
0.01μM
GFP
DIC
merge
C
Log10CFU/ml
DMSO
10uM
5uM
2.5uM
1.25uM
1uM
0.5uM
0.25uM
0.125uM
0.1uM
0.01uM
Cucurbitacin I
D
Log10CFU/ml
DMSO
10uM
5uM
2.5uM
1.25uM
1uM
0.1uM
0.01uM
Stattic

Fig. 2 Comparison of the suppression of *F. novicida* infection by the inhibitor's concentration

J774.1 cells treated with 0.01 to 10 μM of Cucurbitacin I (A, C) or Stattic (B, D) were infected with *F. novicida* (C, D) or GFP-expressing *F. novicida* (A, B). Cells were treated with gentamicin and incubated for 12h. Then Cells were observed with confocal microscopy (A, B), or intracellular bacterial number was counted (C, D). Mean±SD, Dunnett's test, P=0.05, **: P=0.01

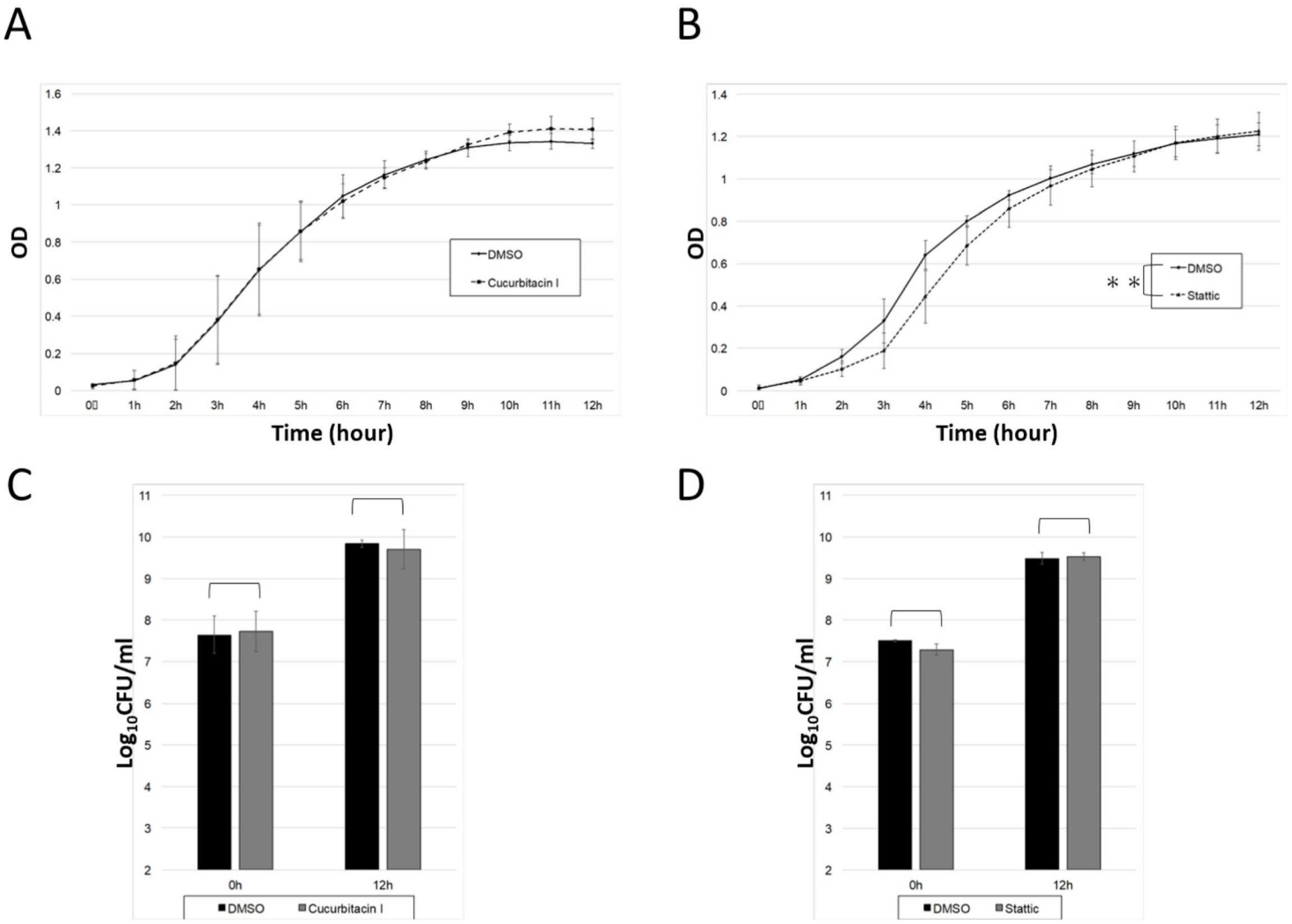

A
OD
Time (hour)
DMSO
Cucurbitacin I
B
OD
Time (hour)
DMSO
Stattic
C
Log10CFU/ml
0h
12h
DMSO
Cucurbitacin I
D
Log10CFU/ml
0h
12h
DMSO
Stattic

Fig. 3 *F. novicida* growth for 12h in BHIc

F. novicida was cultured in BHIc with Cucurbitacin I (A, C) or Stattic (B, D) and optical density (λ=660 nm) was measured at indicated time point (A, B). CFU at start and end points (0 h and 12h) was counted (C, D). Mean±SD, Tukey–Kramer method (A, C), *: P=0.05, Student t-test (B, D), *: p=0.05

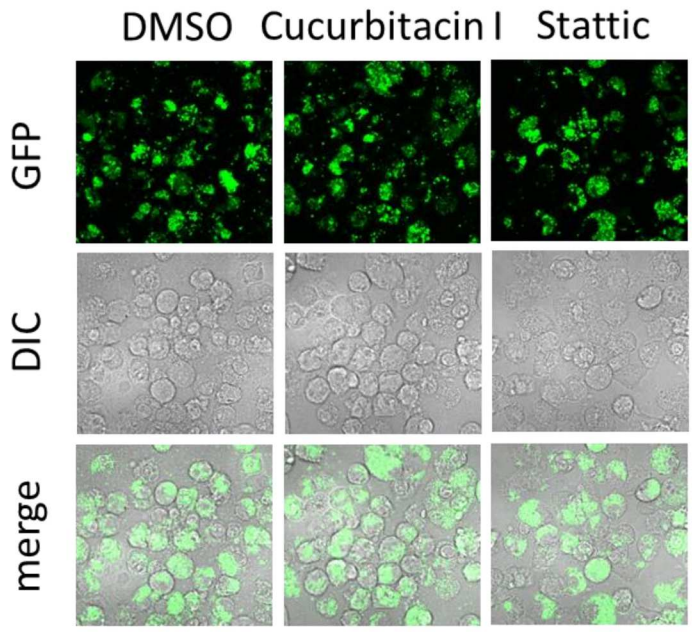


Fig. 4 Comparison of *F. novicida* infection which cultured with inhibitors

F. novicida was cultured in BHIc with Cucurbitacin I or Stattic. J774.1 cells were infected with inhibitor-treated *F. novicida* and observed at 12h post infection.

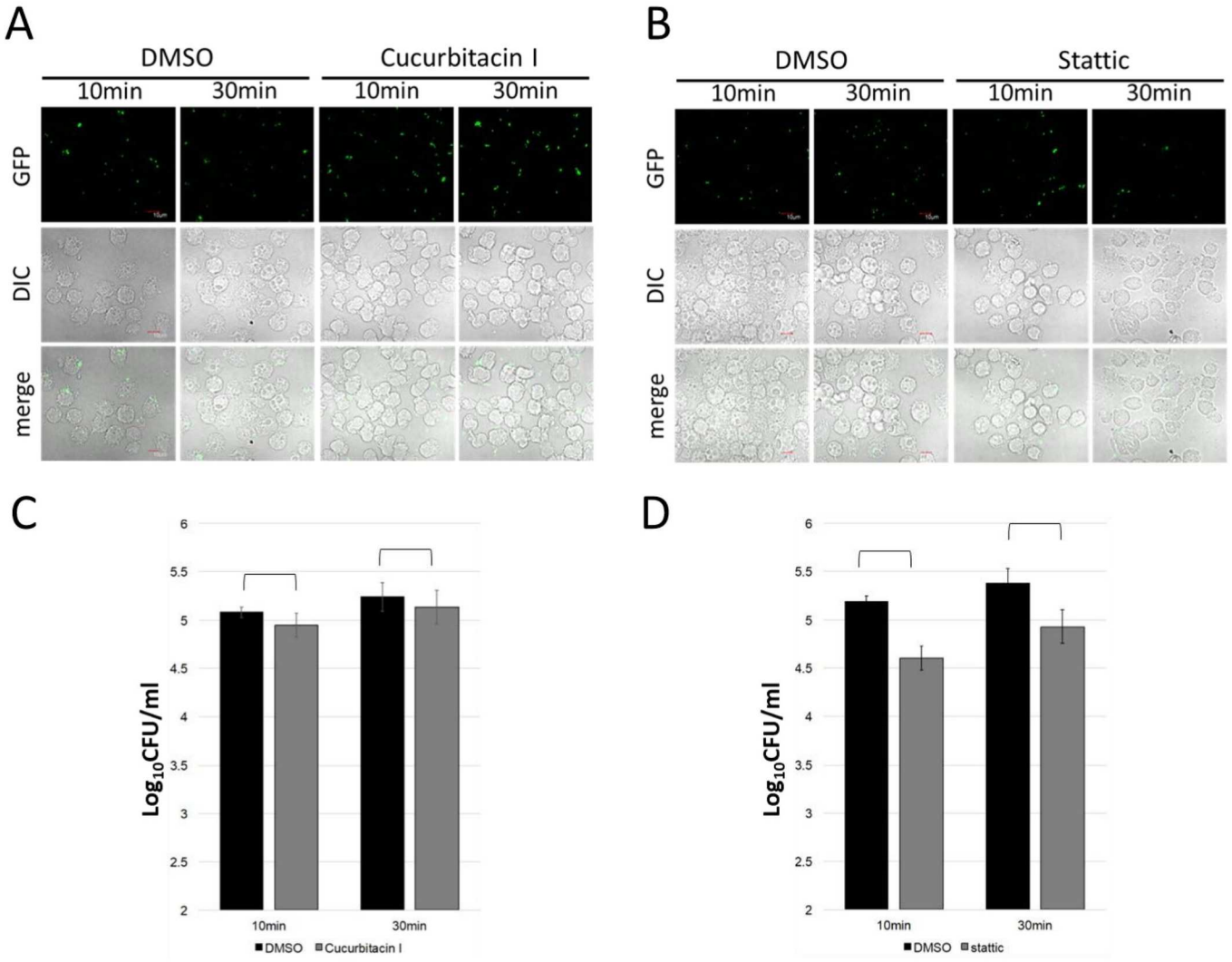

A
DMSO
Cucurbitacin I
10min
30min
10min
30min
GFP
DIC
merge
B
DMSO
Stattic
10min
30min
10min
30min
GFP
DIC
merge
C
Log10CFU/ml
6
5.5
5
4.5
4
3.5
3
2.5
2
10min
30min
DMSO
Cucurbitacin I
D
Log10CFU/ml
6
5.5
5
4.5
4
3.5
3
2.5
2
10min
30min
DMSO
stattic

Fig. 5 attachment on cell surface

J774.1 cells treated with 1 μM of Cucurbitacin I (A, C) or 10 μM Stattic (B, D) were infected with *F. novicida* (C, D) or GFP-expressing *F. novicida* (A, B). Cells were observed with confocal microscopy (A, B), or intracellular bacterial number was counted (C, D) at 10 or 30min post infection. Mean±SD, Student T-test, *: p=0.05.

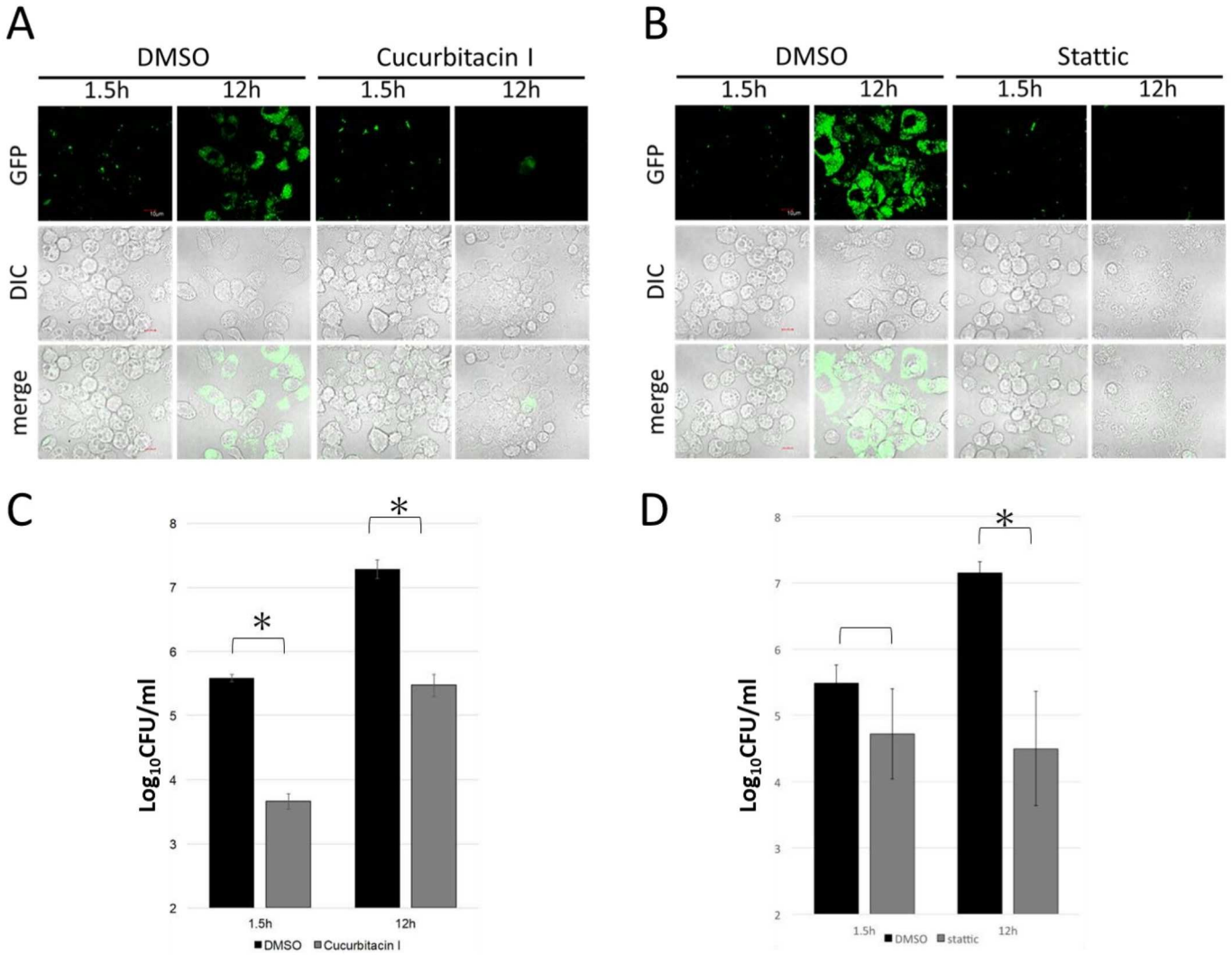

A
DMSO
Cucurbitacin I
1.5h
12h
1.5h
12h
GFP
DIC
merge
B
DMSO
Stattic
1.5h
12h
1.5h
12h
GFP
DIC
merge
C
Log10CFU/ml
8
7
6
5
4
3
2
*
*
1.5h
12h
DMSO
Cucurbitacin I
D
Log10CFU/ml
8
7
6
5
4
3
2
*
1.5h
12h
DMSO
stattic

Fig. 6 Internalization and proliferation in cells

J774.1 cells treated with 1 μM of Cucurbitacin I (A, C) or 10 μM of Stattic (B, D) were infected with *F. novicida* (C, D) or GFP-expressing *F. novicida* (A, B). Cells were treated with gentamicin and incubated for 1.5 or 12h. Then Cells were observed with confocal microscopy (A, B), or intracellular bacterial number was counted (C, D). Mean±SD, Student T-test, *: p=0.05.

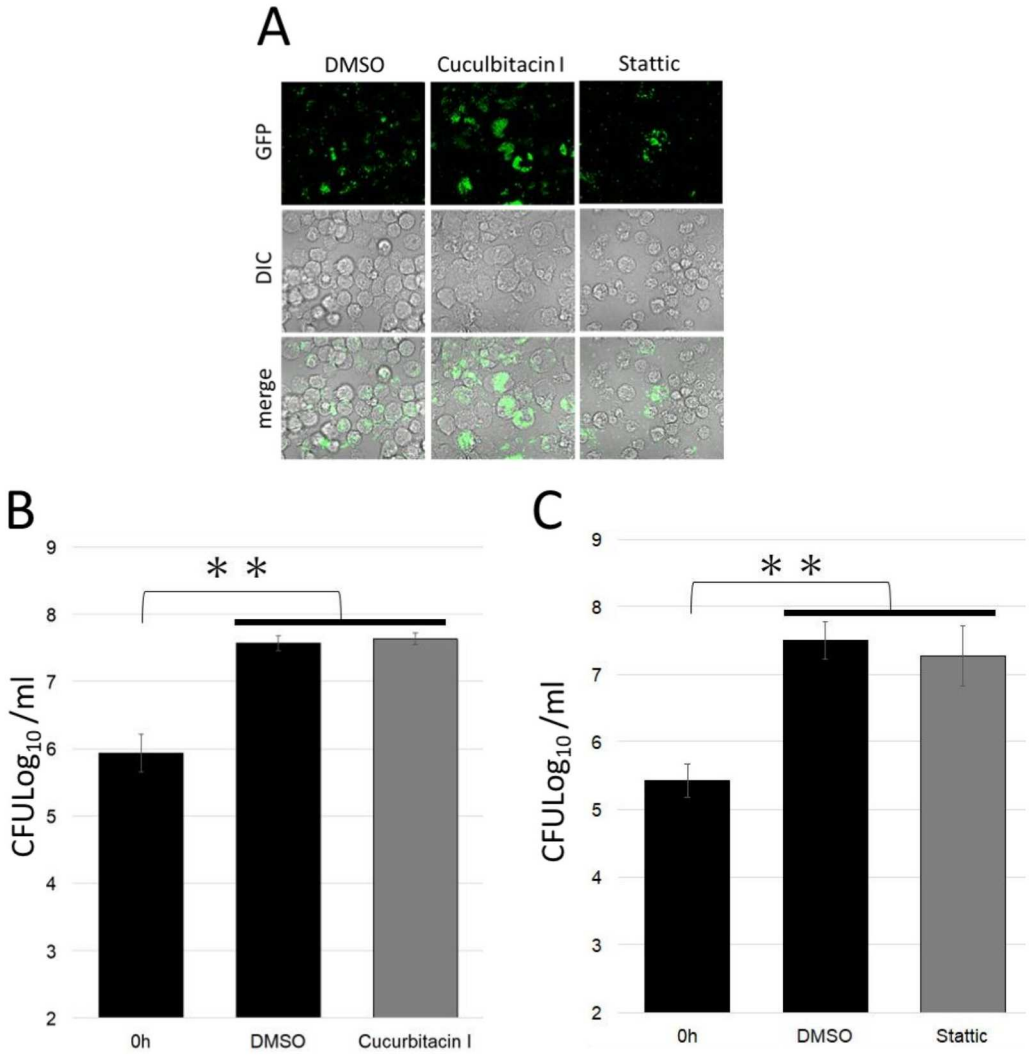

A
DMSO
Cuculbitacin I
Stattic
GFP
DIC
merge
B
C
CFULog10/ml
* *
0h
DMSO
Cucurbitacin I
Stattic

Fig. 7 Proliferation in cells

J774.1 cells were infected with *F. novicida* (B, C) or GFP-expressing *F. novicida* (A). Cells were treated with gentamicin and cultured with 1 μM of Cucurbitacin I (A, B) or 10 μM of Stattic (A, C) for or 12h. Then Cells were observed with confocal microscopy(A), or intracellular bacterial number was counted (B, C). Mean±SD, Dunnett's test, **: P=0.01.

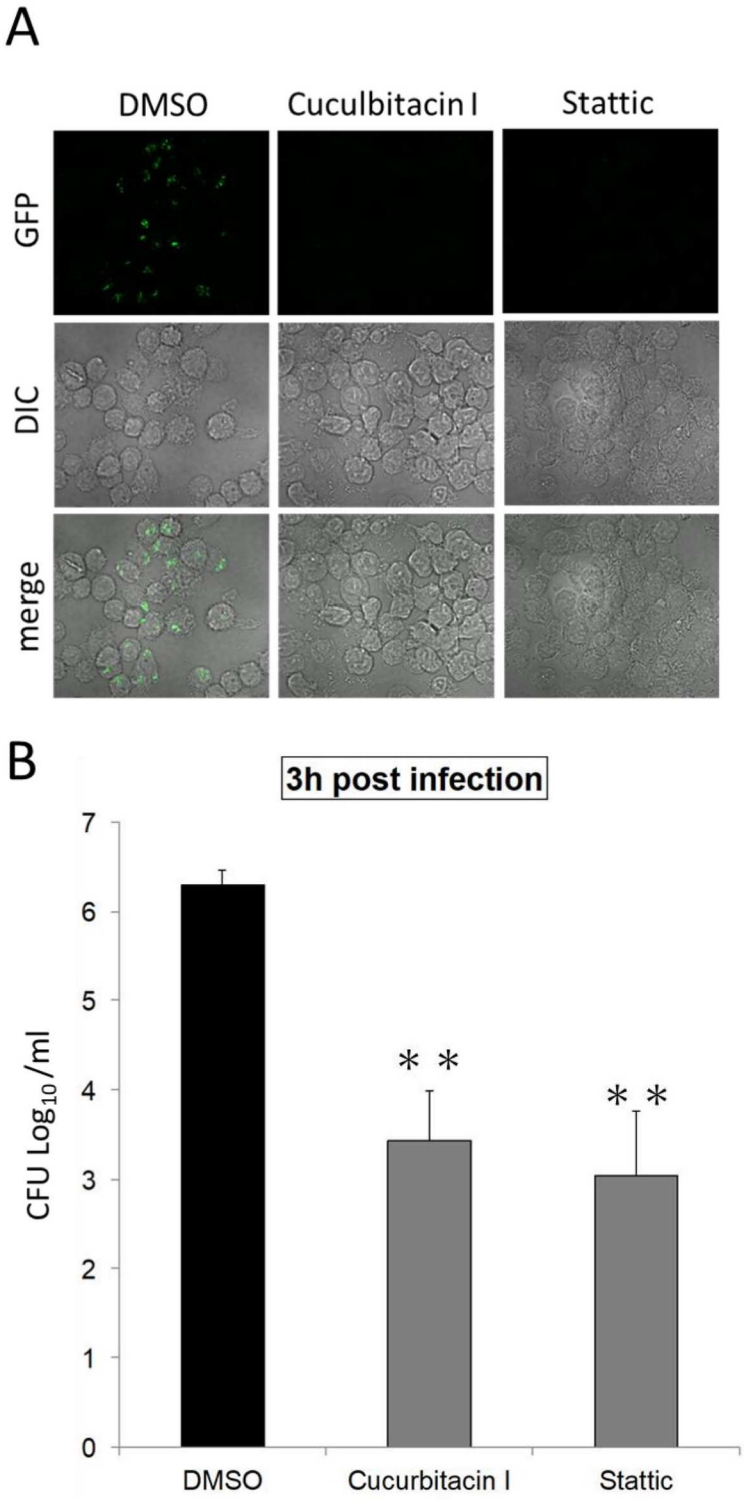

A
DMSO
Cuculbitacin I
Stattic
GFP
DIC
merge
B
3h post infection
CFU Log10/ml
7
6
5
4
3
2
1
0
* *
* *
DMSO
Cucurbitacin I
Stattic

Fig. 8 Phagocytosis

J774.1 cells treated with 1 μM of Cucurbitacin I (A, B) or 10 μM of Stattic (A, B) were infected with *E. coli* (B) or GFP-expressing *E.coli* (A). Cells were treated with gentamicin and incubated for 3h. Then Cells were observed with confocal microscopy (A), or intracellular bacterial number was counted (B). Mean±SD, Dunnett's test, **: P=0.01.

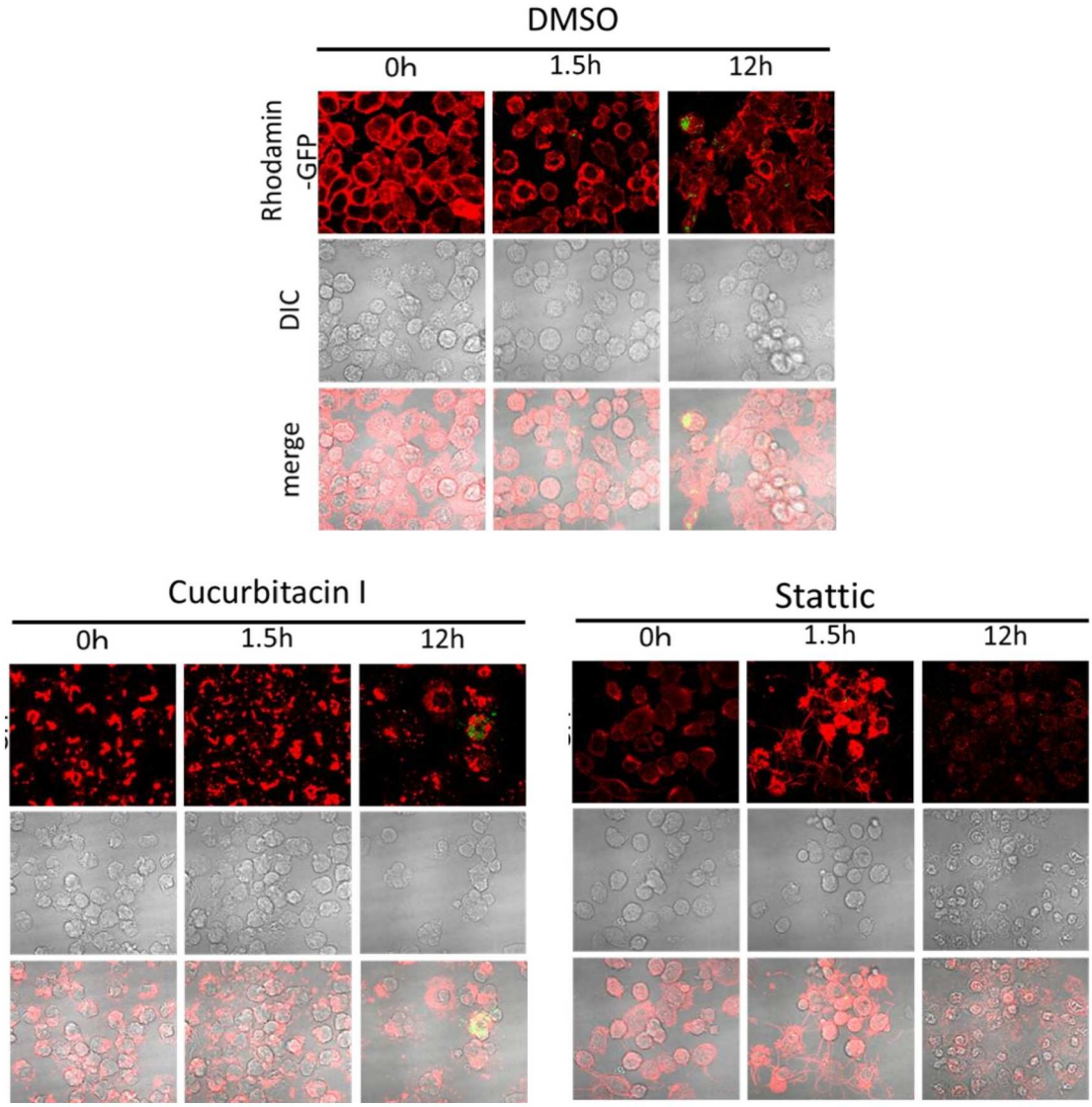


Fig. 9 Actin filaments

J774.1 cells treated with 1 μM of Cucurbitacin I or 10 μM of Stattic were infection with GFP-expressing *F. novicida* for indicated time. Cells were stained with Phalloidin-Rhodamine and observed by confocal microscopy.

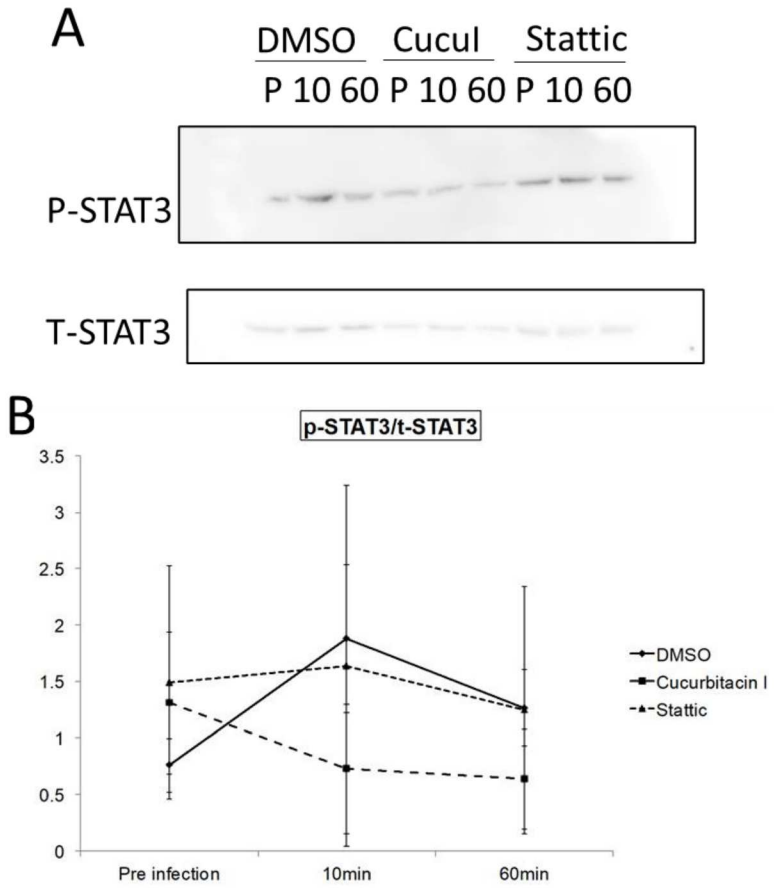


Fig. 10 Comparison of p-STAT3 expression

J774.1 cells treated with 1 μM of Cucurbitacin I or 10 μM of Stattic were infection with *F. novicida* for 10 or 60min. Cells were collected and the amount of total and phosphorylated STAT3 was examined by western blotting (A). The intensity of each band from 3 independent experiments was calculated (B). Mean±SD, Dunnett's test, *: P=0.05, **: P=0.01

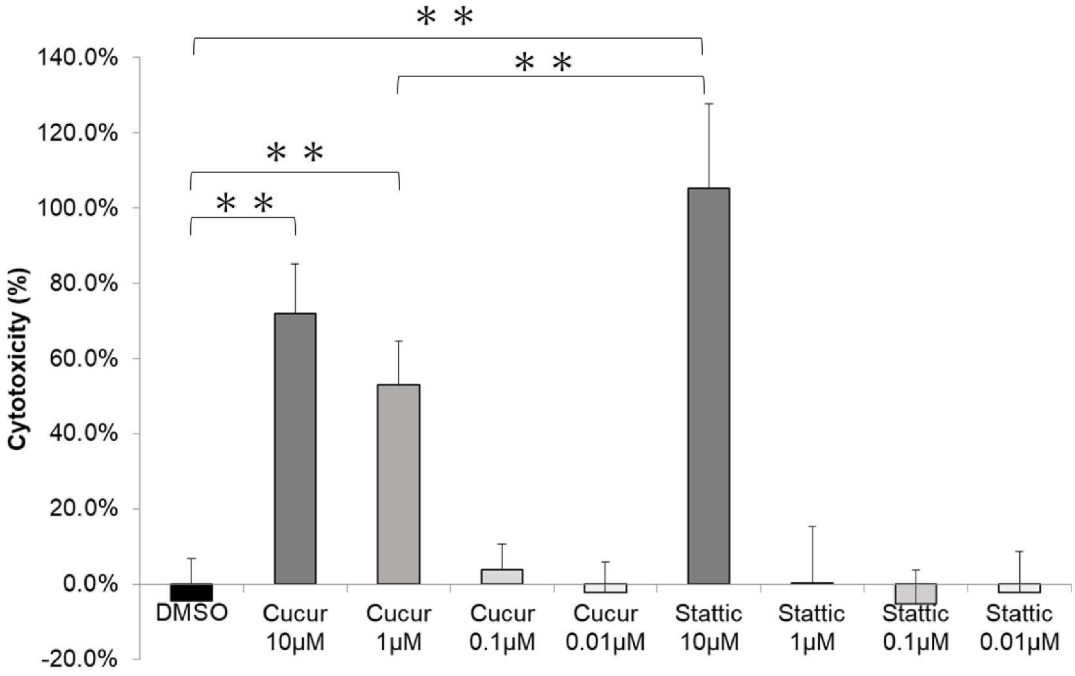


Fig. 11 Cytotoxicity

J774.1 cells were treated with indicated concentration of inhibitors and LDH rerelease in the supernatant was measured. Mean±SD, Tukey–Kramer method, **: P=0.01

Chapter 2 Development of protist infection model using *Paramecium*.

Introduction

Paramecium spp. single-celled and free-living protists, are general ciliates found in freshwaters, such as ponds, lakes, and rivers. They are well studied in various fields as ideal model organisms since they are easy to deal with [7, 8, 9]. The unique and complex reproduction process of *Paramecium*, in which asexual binary fusion and several types of sexual processes like conjugation and autogamy are involved, has already been revealed [15, 16, 17, 18, 53]. Conjugation can occur only among different mating types of the same syngen. Probably each *Paramecium* has some syngens, and in some cases, the type of syngen has been recognized as a cryptic species. For example, *P. aurelia* and *P. jenningsi* have been considered to comprise a group of 16 and 3 cryptic species [54, 55]. Each syngen is genetically isolated from other syngens, but it has been impossible to distinguish them morphologically. These different syngen groups are now defined as separate species within the *P. aurelia* complex of sibling species [56, 57, 58, 59].

In our previous studies, I have referred to the possibility that *Paramecium*

can be a natural host for pathogenic bacteria in the environment [10, 11, 12]. I have also analyzed the relationship between the host competency of *Paramecium* and its species or strains and reported that *Legionella pneumophila* can establish intracellular symbiotic relationships with a large majority of strains of *P. caudatum*; however, such stable relationships have not been established in specific strains of *P. caudatum*, *P. bursaria*, or *P. multimicronucleatum* [10, 60]. In addition, *P. bursaria* can be a protist model of *Francisella* infection [13]. As it is currently unknown what factors in *Paramecium* determine their ability to act as hosts for pathogenic bacteria, the possibility that the syngen is an important factor related to this cannot be ruled out. From the perspective of controlling infectious diseases, understanding the natural host is of great public health significance. In this regard, it is essential to establish a method to quickly understand the characteristics of the *Paramecium* strains, including syngens, and to evaluate whether they can be risk factors for infection. Moreover, easy and rapid identification methods are essential to select suitable strains from large numbers of *Paramecium* strains, including field strains for developing bacterial infection models. However, easy, rapid, and reliable method to distinguish *Paramecium*

species, strains, syngens, and mating types are currently not available.

Random amplified polymorphic DNA (RAPD) analysis is one of the molecular biological methods for determining genetic diversity by analyzing DNA sequence homology or polymorphisms [61, 62, 63]. In this assay, the genomic DNA of the target organism is used as a template, and DNA fragments are amplified by PCR using primers with random sequences. By comparing the appearance pattern of the DNA fragments by electrophoresis, genetic diversity can be determined. The major advantages of this method are that it is quick and easy to obtain results and it does not require information on the genomic DNA of the target organism.

Because of these advantages, this analysis has been applied to the identification and discrimination of microbial and plant species [63, 64, 65], phylogenetic analysis, and epidemiological fields [66, 67, 68]. The identification and comparative analysis of *Paramecium* strains using RAPD analysis have also been reported [69, 70, 71, 72, 73]. RAPD analysis is a useful tool for species identification and classification in *Paramecium*, which is difficult to distinguish morphologically.

In this study, I prepared several strains of various *Paramecium* spp. with

conformed syngen and mating type, by obtaining them from the National Bio Resource Project (NBRP), and attempted to apply RAPD analysis to construct a method for identifying these strains and their syngens in particular. In addition, various *Paramecium* species were tested as a protist host model for *Francisella* infection. It was found that the response to infection varied from species to species, indicating the importance of the rapid identification of *Paramecium* species to develop an appropriate infection model.

Materials and Methods

All experiments were conducted in compliance with the institutional biosecurity guidelines and were approved by Yamaguchi University.

Paramecium Strains

P. caudatum, *P. tetraurelia*, and *P. bursaria* were obtained from the NBRP (http://nbrpcms.nig.ac.jp/paramecium/?lang=en). Table 1 lists all strains used in first analysis. Table 2 lists all strains used in Second analysis. *P. caudatum* dYDRM-3E and dYDRM-3O strains, *P. bursaria* YKK10w and YDS1w strains were used in infection model.

Inhibitors

Cucurbitacin I (Merck, Darmstadt, German) was dissolved in DMSO (Fujifilm, Osaka, Japan) at 2 mM concentration. The same amount of the inhibitors added to culture medium (Inhibitor 0.5 µl per Medium 100 µl) at 10 µM of final concentration.

Bacteria strain and culture condition

Francisella tularensis subspecies *novicida* ATCC15482 strain (*F. novicida*) was cultured aerobically at 37°C with Brain heart infusion broth (Becton and Dickinson company, New Jersey, USA) suppled with cysteine (BHIc), BHIc plates containing 1.5% Agar (Fujifilm) [34]. I used Green Fluorescent Protein (GFP)-expressing *F. novicida* for microscopic observation, and the production of GFP-expressing *F. novicida* was cultured with BHIc containing 2.5 µg/ml chloramphenicol [36].

Bacterial concentrations were adjusted to the optical density (OD, λ=595 nm).

Isolation of Genomic DNA from *Paramecium*

Genomic DNA was isolated from 3 mL of *Paramecium* cell culture (including approximately 3000 cells) using a DNA extraction kit (QIAGEN, Venlo, Netherlands), following the manufacturer's instructions. The cells were washed

with PBS twice to remove extracellular bacteria before applying them to the kit. The DNA samples were frozen at −30°C until use.

RAPD Analysis

RAPD-PCR

RAPD analysis was performed as described previously [70], with some modifications. Briefly, RAPD–PCR was conducted in a 10 µL reaction mixture comprising 2.8 µL of PCR master mix (KOD-Plus-Neo, TOYOBO, Osaka, Japan), 1 µL of primer (10 µM), 1 µL of template DNA (adjusted to 10 ng/µL), and 5.2 µL of nuclease-free water. The PCR program consisted of first, four cycles at a denaturation temperature of 94°C for 5min, followed by primer annealing at 35°C for 5min, and finally, an elongation at 72°C for 2min. The subsequent 36 cycles consisted of denaturation at 94°C for 1min, primer annealing at 40°C for 1min, and elongation at 72°C for 2min. A final elongation step was extended to 5min at 72°C. The fragments were separated by electrophoresing at 100 V for 30min on 1.8% agarose gel with DNA ladder markers (Kapa Biosystems, Bath, UK). The gels were stained with ethidium bromide and visualized using a gel imaging system (ATTO, Tokyo, Japan). All RAPD–PCR were repeated at least three times to confirm the

reproducibility of the band patterns. Table 3 lists the primers used in this study.

DNA extraction and cloning

The PCR program was same as that of first analysis. The fragments were separated by electrophoresing at 100 V for 30min on 2% agarose gel. The gels were stained with ethidium bromide and visualized using the gel imaging system (ATTO). All RAPD-PCR analyses were repeated at least thrice to confirm the reproducibility of the band patterns. DNA sequencing After electrophoresis on a 2% agarose gel, RAPD-PCR products (several single bands that differed in size and presence or absence depending on the strain) were extracted using the QIAEx II Gel Extraction Kit (Qiagen) according to the manufacturer's instructions and used as template DNA. PCR was performed using KOD-Plus-Neo polymerase (Toyobo) with primers designed for cloning the PCR products into EcoRI cleaved p-Cold TF DNA vector (Takara Bio). Cloning was performed using an In-Fusion HD Cloning Kit (Takara Bio). The inserted DNA was amplified by pCold-F1 and p-Cold-R primers. These PCR products were purified and submitted to sequencing analysis using an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) with the Big Dye Terminator version 3.1 Cycle Sequencing Kit (Thermo Fisher

Scientific, Waltham, MA).

Conventional and multiplex PCR for distinguishing *P. caudatum* strains

Conventional PCR was carried out using KOD-Plus-Neo polymerase. The reaction conditions were 2min at 94˚C, followed by 30 cycles of 10s denaturation at 94˚C, 30s annealing at 60˚C, 30s extension at 68˚C, and 7min final extension at 68˚C. The fragments were separated by electrophoresing at 100 V for 30min on 1% agarose gel. Multiplex PCR was carried out using the Multiplex PCR Assay Kit version 2 (Takara Bio). The reaction conditions were 1min at 94˚C, followed by 30 cycles of 30s denaturation at 94˚C, 45s annealing at 57˚C, 45s extension at 72˚C, and 10min final extension at 72˚C. The fragments were separated by electrophoresing at 100 V for 30min on 3% agarose gel. Gel staining and visualization were carried out as described above. Based on the result of sequence (Table 4), specific primers were designed to identify *P. caudatum* strains. 18S rRNA was used as a control gene to check the quality of the template DNA. The primers used in this assay are also listed in Table 5.

Infection model

To examine the cytotoxicity of inhibitors against *Paramecium* strains, 500 µl

of *Paramecium* strains were treated with indicated concentration of Cucurbitacin I, Stattic, or DMSO control for 48h. After the treatment, 100 µl of *Paramecium* strains were dropped on Petri dish (NISSUI PHARMACEUTICAL CO. LTD. Tokyo, Japan) and observed with microscope (CKX53, Olympus Corporation, Tokyo, Japan), then the number of moving *Paramecium* strains was counted.

To examine the infectivity of *Francisella* to *Paramecium* strains, 500 µl of *Paramecium* strains were treated with Cucurbitacin I, Stattic, or DMSO control for 2h. After the treatment, the *Paramecium* strains were infected with GFP-expressing *F. novicida* ATCC15482 strain for 24h. *Paramecium* cells were fixed with 10% paraformaldehyde in PBS for 30min. *Paramecium* cells were washed with PBS and observed using Laser Scanning Confocal Microscopy (FV1000-D, Olympus Corporation).

Statics analysis

Significant differences are determined by $P < 0.05$ using Student's T-test and indicate with *.

Results

Development of RAPD method for *Paramecium*

Several studies about RAPD analysis applied for *Paramecium* species distinction have been reported [70, 74, 75]. In this study, I first applied the reported primers and methods of RAPD analysis for some *Paramecium* strains obtained from NBRP. Although a previous study reported that only one primer (Ro-460-04; primer-4 in this study) gave robust band patterns [70], I confirmed that other random primers work for various *Paramecium* strains and that specific band patterns dependent on each strain are observed in four *Paramecium* strains (two strains of *P. caudatum*, one strain of *P. tetraurelia*, and one strain of *P. bursaria*) (Fig 1). Comparing the results between *P. caudatum* RB-1 and TAZ0462, explicit bands or band patterns that define the species of *Paramecium* were not identified. However, some primers such as primer-2, -3, or -9 showed a very clearly and distinctly different pattern of bands in all strains used in this study and might be used for rough identification of *Paramecium* species. Next, I performed the same RAPD analysis using DNA samples isolated only in the culture medium of *Paramecium*, with bacteria that are used as feeds for *Paramecium*, since it is impossible to deny the genomic contamination of the bacteria. As expected, some PCR products were observed in all experiments using the 10 random primers,

although the band patterns were different from those of the *Paramecium* DNA samples. Similarly, *P. bursaria* is well known to maintain *Chlorella* cells as an endosymbiont [76, 77, 78]. Thus, the effect of the existence of *Chlorella* DNA should be considered in this assay, using a sample of *P. bursaria*. I have prepared a pair of two *P. bursaria* strains, YDS1 and HA1, which either maintain *Chlorella* cells (YDS1g and HA1g) or do not (YDS1w and HA1w), and evaluated the changing of band patterns. Consequently, it was found that the band patterns changed depending on the presence or absence of *Chlorella*, although there was a difference in degree depending on the primer used. Furthermore, the sharpness of the bands was attenuated, and the appearance of these bands tended to become smear-like in the presence of *Chlorella* (YDS1g and HA1g), compared with strains without *Chlorella* (YDS1w and HA1w) (Fig 2).

Next, I applied this RAPD system to investigate a simplified method for identifying syngens of *Paramecium* strains. Since multiple strains were available for each syngen in NBRP, *P. caudatum* was adopted for this examination. A total of 10 *P. caudatum* strains (five strains of syngen 6 and five strains of syngen 12) were compared. Ten different random primers (1–10) were used, as in the above

experiment. Consequently, it was reconfirmed that the band patterns were not completely consistent among the strains of the same species, similar to the results observed between RB-1 and TAZ0462 strains (Fig 1). Additionally, syngen 6 strains No. 3 (YDRM6) and No. 4 (YDRM11), or syngen 12 strains No. 1 (My43C3d) and No. 2 (KGS1), tended to show very similar band patterns in all primers' results. By contrast, syngen 12 strain No. 3 (SBK2019-3b1) tended to show a very different band pattern from the other four strains of syngen 12 (Fig 3). When these results were grouped by the primers used, there were cases where (1) all 10 strains showed almost similar patterns (primer-3, primer-4, primer-7, and primer-8), (2) all 10 strains showed almost disparate patterns (primer-1, primer-9, and primer-10), and (3) each syngen showed a somewhat coherent pattern (primer-2, primer-5, and primer-6). The bands surrounded by a white box in the results of primer-5 (Fig 3) were clearly visible as single bands in all five strains of syngen 12 but did not exist as distinct common bands in any of the five strains of syngen 6. Thus, I conducted a comparative analysis for other strains of *P. caudatum* (syngens 1, 3, and 4) by the RAPD method using primer-5. This target band that was specifically observed in strains of syngen 12 was also observed in the Ai51 strain (syngen 1) but not in

other syngen strains. The other band patterns of My43C3d and Ai51 were also almost identical. Although I additionally compared one strain each of the mating types O and E, there was no clear trend or specific band depending on the mating type.

Development of new PCR method for *Paramecium* using RAPD and multiplex PCR

I analyzed more strains using only the random primer-02, -03, and -05 and there were differences in band patterns were found between strains (Figs 5–7). In particular, results using primer-05 for *P. caudatum* (Fig 5C), primer-02 for *P. tetraurelia* (Fig 6A), and primer-03 for *P. bursaria* (Fig 7B) showed different patterns for each strain and were most effective in roughly identifying strains. Especially, No. 9 (51), one of the standard strains of *P. tetraurelia*, and No. 11 (Yad1g1N), a standard strain of *P. bursaria*, showed different numbers and patterns of bands compared to other strains (Figs 6 and 7). In contrast, some strains of *P. caudatum* showed quite similar band patterns among the recommended and standard strains. It was slightly more difficult to distinguish recommended and standard strains of *P. caudatum* by the RAPD method alone

than *P. tetraurelia* and *P. bursaria*. The RAPD method did not reveal any specific band patterns that could distinguish between syngens and mating types in *P. caudatum*. In addition, *P. bursaria* showed similar band patterns in strains with *Chlorella* (Nos. 1, 3, 4, 5, 7, 9, and 11) and without *Chlorella* (Nos. 2, 6, 8, and 10). No distinct bands specific to strains with *Chlorella* were observed in any results. As expected, the same parental strain of *P. bursaria* with and without *Chlorella*, symbiotic green alga, showed very similar band patterns, for example, No.7 (YKK3g) and No.8 (YKK3w), or No.9 (HA1g) and No.10 (HA1w). However, there were cases where the band pattern was similar, although the strains were different, as in No. 3 (Dd1g) and No. 4 (KM2g).

This analysis attempted to develop a method for more strictly distinguishing strains in *P. caudatum*. First, specific primers were designed based on the sequence information (Table 4) obtained from RAPD-PCR products (Fig 8). Next, comparative analysis was performed using these primers on 26 strains and another strain of *P. caudatum*, which is not designated as the standard or recommended strain in the NBRP. As a result, in the PCRs targeting the five regions named as Pc-1 to Pc-5, the patterns of the bands were differentiated among each strain, and

it was possible to distinguish some of the strains, including the standard strain (No. 14; dKNZ-12O), by comprehensively determining the results of these five individual PCRs (Fig 8B). This study also successfully distinguished strains (e.g., Nos. 4 and 5 and Nos. 10 and 11) that were difficult to distinguish clearly since the band patterns were similar in all cases using random primers in the RAPD method. Finally, this study investigated a simple method to distinguish *P. caudatum* strains by multiplex PCR. Although the PCR reagents used in Fig 8 had low reproducibility of the results, as some bands did not appear, the results were very reproducible, reflecting very well the PCR results performed individually targeting Pc_1 to Pc_5 using multiplex PCR-specific reagents (Fig 9). A maximum of five single bands (No. 4) was obtained with the predicted size, and no smearing or nonspecific bands due to primer-dimer were observed. As a result, by conducting this multiplex PCR once, it was possible to easily distinguish between several standard and recommended strains of *P. caudatum.*

Infection model

I tested several *Paramecium* strains as the infection model for *Francisella* infection. Four strains of *Paramecium* including *P. caudatum* dYDRM-3E and

dYDRM-3O strains, *P. bursaria* YDS1w and YKK10w were infected with *F. novicida*. As the result, *P. bursaria* YDS1w and YKK10w were successfully infected with *F. novicida*, while *P. caudatum* dYDRM-3E and dYDRM-3O failed to maintain *F. novicida* inside the cells (Fig 11, DMSO control). Because Cucurbitacin I was found to be a possible therapeutic agent for *Francisella* infection in chapter 1, effect of Cucurbitacin I in *Paramecium* infection model was tested. Cucurbitacin I did not show any cytotoxicity in all four *Paramecium* strains (Fig 10). Cucurbitacin-I treatment decreased the infection of *F. novicida* only in *P. bursaria* YKK10w, but failed to decrease the infection in other three strains (Fig 11). These results indicate that the response of *Paramecium* against *Francisella* infection differ from strain to strain.

Discussion

Development of RAPD method for *Paramecium*

Many species of *Paramecium* have been reported to be widely isolated from aquatic environments worldwide. Several syngens and different mating types are also known to exist in each species [79, 80, 81]. Although the identification of species or syngens is one of the most fundamental tasks in understanding the

distribution and ecology of *Paramecium* in the environment, it is extremely difficult to distinguish them easily and rigorously via morphological methods. Although genomic information, such as 18S rRNA and COI mtDNA genes, has also been used for their classification [82, 83], such genetic methods are limited, since the small number of species and strains have undergone whole-genome analysis because of the lack of basic information about them, such as their number of chromosomes. Given this situation, RAPD analysis is best suited for the identification of species or strains in *Paramecium* because it does not require detailed genomic information of the target organism. In fact, attempts to use RAPD analysis to identify *Paramecium* species and compare them by their strains have already been reported [70, 74, 75]. In this study, I also performed RAPD analysis using the reported random primers and confirmed different band patterns (number of visualized bands and their sizes) between strains (Fig 1). These results suggest that it is feasible to determine the identity of *Paramecium* strains by using multiple results of the RAPD analysis with different random primers in combination. One of the disadvantages of RAPD analysis is that it requires highly standardized experimental procedures because of its sensitivity to the reaction conditions of

PCR. Additionally, RAPD analysis generally requires well-purified, high-molecular-weight DNA isolated from the target organism. This means that precautions must be heeded to avoid the contamination of the DNA sample because short random primers can amplify DNA fragments from various organisms. When cultured and maintained strains of *Paramecium* are used in RAPD analysis, it is not possible to avoid DNA contamination from the bacteria (such as *Klebsiella* or *Enterobacter*) that are fed as food. In the present experiment, several clear bands were still observed, using any of *P. caudatum* Ai51 (Fig 4). Previous reports suggested the genetic differences among collection localities are greater than those among syngens [56, 84]. My43C3d and Ai51 were collected from the same place (Table 1), although their syngens and mating types were different. Since the band patterns of the two strains are very similar (Fig 4), it is highly possible that such regional factors have a stronger influence on the results of our RAPD method than syngen, which should be taken into consideration in future studies. However, it shows the potential of this method for use in narrowing down syngen groups and in screening tests.

Development of new PCR method for Paramecium using RAPD and

multiplex PCR

The RAPD method is a useful approach in First analysis, including the identification of *Paramecium* strains. However, one of the disadvantages that must be considered is the reproducibility of the tests and the quality of the template DNA that may affect the results. By investigating the design of random primers and reaction conditions, it is expected to improve the outcomes. However, the suitability of the RAPD analysis for each target species is likely to be variable, since it was more difficult to detect differences in band patterns of *P. caudatum* (Fig 5) than in those of *P. tetraurelia* (Fig 6) or *P. bursaria* (Fig 7) in RAPD analysis performed under the same conditions. RAPD-PCR products were extracted from agarose gels to design primers specific for *P. caudatum*, and sequence analysis was performed (Table 4A). Results showed that most PCR products were amplified from *Paramecium* DNA, including Pc_1 to Pc_5, also used in the following analysis. The results included that the PCR product was amplified from the genomic DNA of a bacteria (Enterobacter aerogenes) fed to *Paramecium*, but the rate was extremely low with only one sample out of 16 samples extracted from gel. Therefore, it would be reasonable to conclude that the band patterns observed in the RAPD method

under the method and conditions described in this study are mainly genomic products of *Paramecium*. The sequences (Pc_1–Pc_5) used in the primer design for *P. caudatum*-specific PCR (Figs 8B and 9) were analyzed for sequence homology in the database. However, all of them corresponded to genes of unknown function and their surrounding regions in *P. caudatum* and did not match the sequences of genes whose specific functions were identified (Table 6). This study would reveal genes involved in phenotypic determinations, such as syngen or mating types, also important as classification factors for *Paramecium* strains, but no such findings were obtained. Although several reports have referred to genes involved in determining mating types of *Paramecium* [85, 86, 87, 88, 89, 90], genetic information on syngens of *Paramecium* is relatively lacking. Thus, the general method of identifying syngens remains deeply dependent on mating tests. As whole-genome sequences of many *Paramecium* strains are developed in the future, and more information on the function of each gene is revealed, the relationship between target DNA sequences employed in this study and the diversity of the strains will be clarified, leading to the identification of novel genes that determine syngens or mating types. The multiplex PCR method used in this study was only

applied to the analysis of *P. caudatum*. Still, it is highly possible that the method can be improved to simplify the distinction and identification of other *Paramecium* strains, including *P. tetraurelia* and *P. bursaria*, in a similar manner. This study did not attempt to perform the method because there were not enough strains of these two species to determine the utility compared to *P. caudatum*. It is important to continue to examine the utility and generality of this method by preparing more strains of *Paramecium* species other than *P. caudatum*. There may be some strains to which the present analysis method cannot be applied in such investigations. In particular, because the purified genome DNA from *P. bursaria* strains that maintain symbiont *Chlorella* will certainly be a mix of the genome of *Chlorella* and that of the host *P. bursaria*, it is necessary to consider this effect in the RAPD-PCR process. It is also important to consider the existence of endosymbiotic bacteria, such as *Holospora* and *Legionella*, when studying *P. caudatum* strains that maintain them [10, 91]. It is necessary to ensure species specificity by combining multiplex PCR as in this method; at the same time, it is important to modify this method to the most appropriate one by changing the target sequence for each *Paramecium* species or strain for use.

Infection model

As it was previously reported from my laboratory that *Paramecium* can be a natural host for pathogenic bacteria in the environment [10, 11, 12], I tested several *Paramecium* strains for developing a new infection model for *Francisella* infection. Among four strains of *Paramecium* (*P. caudatum* dYDRM-3E and dYDRM-3O, *P. bursaria* YDS1w and YKK10w), *P. bursaria* YDS1w and YKK10w showed ability to be infected with *F. novicida*. This result is consistent with the previous result from my laboratory that *P. bursaria* can be a protist model of *Francisella* infection [13]. Although both of two strains maintained *F. novicida* inside the cells, the location of *F. novicida* was different. In YDS1w the bacterial cells were located at the center of the cytoplasm of *Paramecium* cells, while in YKK10w the bacterial cells are located at the edge of cytoplasm where usually *Chlorella* cells are located.

Because Cucurbitacin I was found to be a possible therapeutic agent against *Francisella* infection, we tested the effect of Cucurbitacin I in *Paramecium* infection model. Among the four infected *Paramecium* strains. Only *P. bursaria* YKK1w strain showed reduction of intracellular *F. novicida* in the presence of

Cucurbitacin I (Fig 11D). Cucurbitacin I is an inhibitor of JAK2/STAT3 pathway, and this inhibitor block the phosphorylation of JAK2 in particular. As the result of BLAST search, *Paramecium* have a JAK2-like protein, and the C-terminal domain of JAK2-like protein is similar to JAK2 protein with approximately 55% of similarity. In some *Paramecium* strains Cucurbitacin I may affect the activity of JAK2-like protein.

In this experiment for infection model, it was found that the response against *Francisella* differed strain to strain. This indicates that a large number of *Paramecium* strains should be tested to select appropriate strains for developing infection models for *Francisella* infection.

Conclusion

Although RAPD is a simple and rapid method for strain identification, the problem of reproducibility and the effect of the presence of DNA other than that of the target organism must be considered when identifying *Paramecium* strains. The multiplex PCR method applying the results of the RAPD method reported in this study is considerably more reliable than the RAPD method in terms of reproducibility. Multiplex PCR is also a method that can disregard unexpected

contamination of DNA from nontarget organisms. In this study, I could provide a specific method for distinguishing the standard strains of several *Paramecium* species. Multiplex PCR method is considered to be a useful classification tool for *Paramecium* model. In addition, I tested various *Paramecium* strains for infection model of *Francisella* infection. The response of *Paramecium* strain against bacterial infection differed strain to strain, suggesting the necessity of testing a large number of *Paramecium* including stock library such as NBRP and field strains to develop an apropriate models. From these aspects, rapid, easy, and reliable method to identify *Paramecium* species, strains, syngens, and mating types is definitely important. Considering the application of this method to the identification of larger number of *Paramecium* strains in the future, it is necessary to verify the accuracy and validity of this method.

Tables

Table 1 List of *Paramecium* strains used in First analysis

No.	Species	Strain Name	NBRP ID	Syngen	Mating Type	Collected location	Reference
1	*P. caudatum*	Ai51	PC012013A	1	E	Miyagi Pref, Japan	
2		Mmn64	PC011011A	1	O	Miyagi Pref, Japan	
3		TAZ0462	PC032004A	3	E	Ishikawa Pref, Japan	
4		HK-F3	PC031030A	3	O	Ishikawa Pref, Japan	
5		RB-1	PC042001A	4	E	Stuttgart, Germany	
6		OW8	PC041004A	4	O	Okinawa Pref, Japan	
7		YR1504-2	PC062012A	6	E	Yamaguchi Pref, Japan	
8		YR1504-6	PC061016A	6	O	Yamaguchi Pref, Japan	
9		YDRM6	PC062131A	6	E	Yamaguchi Pref, Japan	
10		YDRM11	PC062138A	6	E	Yamaguchi Pref, Japan	
11		YDRM26	PC061132A	6	O	Yamaguchi Pref, Japan	
12		My43C3d	PC121015B	12	O	Miyagi Pref, Japan	[Sawka N P. A., 2021]
13		KGS1	PC121086A	12	O	Kagoshima Pref, Japan	
14		SBK2019-3b1	PC122105A	12	E	Miyagi Pref, Japan	
15		dKNZ1207x1209-1	PC122029A	12	E	Ishikawa Pref, Japan	
16		dKNZ1207x1209-3	PC121031A	12	O	Ishikawa Pref, Japan	
17	*P. tetraurelia*	51	PA040011A	-	E	Indiana, USA	
18	*P. bursaria*	Yad1g1N	PB031010B	3	I	Yamaguchi Pref, Japan	[Tsukii Y, 1988]
19		YDS1g	PB032031A	3	II	Yamaguchi Pref, Japan,	
20		YDS1w	PB000061A	3	II		
21		HA1g	PB034004A	3	IV	Aomori Pref, Japan,	
22		HA1w	PB034007A	3	IV		

Table 2 List of *Paramecium* strains used in second analysis

Species	No.	Strain Name	NBRP ID	Syngen	Mating Type	note
P. caudatum	1	dYDRM-3E	PC032039A	3	E	recommended strain
	2	dYDRM-3O	PC031040A	3	O	recommended strain
	3	G3-402	PC031044A	3	O	recommended strain
	4	dOW-4E	PC042005A	4	E	recommended strain
	5	dOW-4O	PC041006A	4	O	recommended strain
	6	dCRT-5E	PC052001A	5	E	recommended strain
	7	dCRT-5O	PC051002A	5	O	recommended strain
	8	YDRM-6E	PC062131A	6	E	recommended strain
	9	YDRM-6O	PC061132A	6	O	recommended strain
	10	SBK2019-12E	PC122022A	12	E	recommended strain
	11	SBK2019-12O	PC121226A	12	O	recommended strain
	12	My43C3d	PC121015B	12	O	recommended strain [Catania F, 2009]
	13	dKNZ-12E	PC122029A	12	E	standard strain
	14	dKNZ-12O	PC121031A	12	O	standard strain
	15	Myn92	PC012002A	1	E	
	16	BAT-CIA3	PC012001A	1	E	
	17	Ai102	PC011016A	1	O	
	18	Mmn64	PC011011A	1	O	
	19	YR1504-2	PC062012A	2	E	
	20	YDRM20	PC032036A	3	E	
	21	YDRM46	PC032037A	3	E	
	22	TAZ0462	PC032004A	3	E	
	23	RB-1	PC042001A	4	E	
	24	YR1504-6	PC061016A	6	O	
	25	YDRM28	PC062134A	6	O	
	26	SBK2019-3b1	PC122105A	12	E	
P. tetraurelia	1	st110-1a	PA041001A	-	O	recommended strain
	2	st110-1b	PA042002A	-	E	recommended strain
	3	rie-1	PA042018A	-	E	recommended strain
	4	rie-2	PA041019A	-	O	recommended strain
	5	SSZ1	PA042017A	-	E	recommended strain
	6	KMA21	PA041022A	-	O	recommended strain
	7	ds4-2 (VIII)	PA042004A	-	E	standard strain
	8	ds4-2 (VII)	PA041003A	-	O	standard strain
	9	51	PA040011A	-	E	standard strain
P. bursaria	1	YKK10g	PB031015A	B1 or R3	I	recommended strain
	2	YKK10w	PB031016A	B1 or R3	I	recommended strain
	3	Dd1g	PB032001A	B1 or R3	II	recommended strain
	4	KM2g	PB031002A	B1 or R3	II	recommended strain
	5	YDS1g	PB032031A	B1 or R3	II	recommended strain
	6	YDS1w	PB032061A	B1 or R3	II	recommended strain
	7	YKK3g	PB033046A	B1 or R3	III	recommended strain
	8	YKK3w	PB033049A	B1 or R3	III	recommended strain
	9	HA1g	PB034004A	B1 or R3	IV	recommended strain
	10	HA1w	PB034007A	B1 or R3	IV	recommended strain
	11	Yad1g1N	PB031010B	B1 or R3	I	standard strain [Kodama Y, 2011]

Table 3 List of random primers used in RAPD PCR

No.	Primer Name	Sequence (5'-3')	References
1	Ro-460-01	TGCGCGATCG	[Tsukii Y, 1994; Stoeck T, 1998]
2	Ro-460-02	GCAGGATACG	
3	Ro-460-03	CTGCGATACC	
4	Ro-460-04	GCAGAGAAGG	
5	Ro-460-05	CTAGCTCTGG	
6	Ro-460-06	GTAGCCATGG	
7	Ro-460-07	AACGTACGCG	
8	Ro-460-08	CGATGAGCCC	
9	Ro-460-09	CGCTGTTACC	
10	Ro-460-10	CTAGGTCTGC	

Table 4 Result of DNA sequencing

A. DNA sequencing result 1

Pc_1 primers were designed from this sequence (underlined).

Template DNA: No.13 Primer: 02
GAGGGATCCGAATTGCAGGATACGGGATATATGTACATGTGAATGGTGCCAAAT ACGAAGGGTATTGGAGGAACGACCTTCAAGATGGTTATGGGATTGAGACTTGG GCAGATGGTAGTAAGTATGAAGGGTTTTATGCAAATGGGAAGAAAGATGGTTAG GGAAAATATGAATGGCCTGATGGTAGTAAGTACAATGGTCTATGGAAAGAGAAT TAAATAGATGGAATGGGTACATATGAATGGTAGGATGGAAGGAAGGTATTTGAG CTTATTAATTAGTATTGTGGAGAATGGCAATAAAATTTTATGCATGGAAAAGGTA AATATATTTGGAGGGATGGTCGTCAGTATGATGGAGAGTTTTAGTTAGATAAAA AATCAGGCTTTGGGGTATATGTGTGGGAGGATGGTAGAAGATATGAAGGAATGT GGGAAAATAACAAACAGCATGGAGAGGGTAAATACTACGAACCAGACGGAGTG ATGAGAAGAGGCTTATGGGAAGATGGAAAAAGAGTAAAATGGGTTGACGAGTG ACATATATAACACTATCTCAAATCAAAGAATCGAGTGAGGCTGAGGCTTCTTGC AGCAAATTTAAAATAAGGGCTTTGCATTCNTCTACTCCATCTTCGCTGAATTGAG CCAAGTGTTCTTTTATGTAGAGAACAAATTCATTTTCCTGTGATTCAGTAAGTTTT TCAGCGACATAGACAAGCCATTATTCGTCAGATGTGTAGTTTTTATCTCTAAAAT TGATGAGATGTTTAATCTTTTAGGTGTACTCAACTTTGGCTTTCTTTTGTTGATCC ATGAGGATGGCATCGTACTTTGATTGTAGTTATTCATCTCGAATAGAGAAGTATG TATAACTTTGCAAAAATTCTGCCCTCAATTCTTGGAGTTCTTATTTTATGTAGTTTT GAGTAAAGAATTAATCAAAGTTATCTAAAGTAAGTACTCTTAGGGCATCATCAAC TTTTCTTTGTAATGTTTCCAATTCATAAACTCCTATGCTTCTTAAACATTTTGAGC AAGCTGATACAATTTTTGAAGAATCTTTCTCCTTAACTGGCATTCTGTAAATGGC TGGGCAGTTGTGAAGGGAGCATTTAGGTTGATAAACAGATTTCCTATCTGGTAT GATATCTGACATATCATTTGTCGAAAACATAAGAGGTGACATTGCTTATTATTAA TAGAGTGTTTGTGGTTAAAAGGAAAGTGAATTGGATGAGACATTCTATTTGTATT GTCTTATCATATCAATGACCCATATCAGGGCCTTTCGATTGGTAGGTTCTTTCTG TATCATACCAGTCAGAATGCTTGCTATGATTGATTTACGATTTGGTAATCTACTTG GCTTATATTCAGCCTTGCGAGGATAATATGGTTCATTTCTAATTTTAATGAAGACT TCATCTTCTGACATACCATCATAAAATGGTGAGCCATGTAATAATTCATCAAACA TTAACCCTAAGGCCCAAACATCAACTGTTTTATCGTATCCTGCAATTCAAGCTGT CGA

B. DNA sequencing result 2

Pc_2 primers were designed from this sequence (underlined).

Template DNA: No.12 Primer: 05
GAATTCTAGCTCTGGACAGAATAGTGACTCTTCTTGCATGAACTGCACATAAATT GGTGTCTTCAAATAATCCAACTAGATATGCTTCAGTAGCTTCTTAAAGAGCTATA ACAGCTGAGCTTTGAAATCTGAGTTCCTTTTAGACTTAGCTTGCTATTTCTCTAAC CAATCTTTAGAATGGAAGCTTTCTGATTAAGAGTTGAGTGGACTTTTAGTATTTTC TAATCTCCCTCAAAGCAACAGTTCCTGGTCGAAATTTGTGTGGTTTCTTTAAACC TCCAGATACTGGGGGTGTGGAGTAATATTTTATAGGTTTCTTAGGAGTGTTGTCT CTCGGTGGTTATTTGATTCTTGCCATTATTATTAAGTTGATTTTTTGATCAATTGTT ATTGATTAATTGATGCATTAGTACCTCATTACCTACGTTAAAATCATGAATCTTAT CTCATATAAATTTAATCTAATTTGTCTAAAGATTTTATCAATCCTATGAATGCAAA CATGAATATTTTAAGACGTAAAATTAAATAATTAATGGAAGCCAAGAAGATCATC GGATTAGGAAGTCCCCTGTTAGATATTCAAGCTGAGGTGTCTGCTGAGTTTTTAG AGAAATATGGCTTAACCCTCAATAACACATATTTCGCTGAAGAAAAGCACATGC CATTGTATGAAGACTTAATTAACATACCAACTCACTCTCACGTCCCAGGAGGTA ATATACCATTTAATGAGGTTCCGCACTTAACACCATTCGACTTGCAAGATGGATG GCCCAAGCAGGACCAGAATAGGTGAAATTCATCGGATGCGTCGGAAAAGACAA ATTCGCTAAAATGCTTATCGAAGTCACCAATTCAGATAGTGTCACAACATTATTC GATGAATAAGACTAACCCACAGGAAAGTGTGGTGTGTTGTTATGTAATAAGGAC AGGTATAATTTCATATAACAAAGGTGTTTGGTTCCATTGATTGGTTCAGCTGCAC ATTTATCGTAAGAGTATGTCGAATAACACATCAATGACATCAAAACAGCCACCG TCTTATTCAGTGAAGTGTATTTCCTTTATCCCAGAGCTAGAATTC

C. DNA sequencing result 3

Pc_3 primers were designed from this sequence (underlined).

Template DNA: No.12 Primer: 02
GGGCCCTCCGCGGGTCTGGTGCCACGCGGTAGTGGTGGTATCGAAGGTAGGCA TATGGAGCTCGGTACCCTCGAGGGATCCGAATTGCAGGATACGGCAATGTAAAT GCTGAATGTAAAAGATGCACTACAAATTGTGATAAATGTTTTGGTATAGGTCCAG ATGAGTGTACAGATTGCTCAAGTGGATATTATTTCCTCCACAACTAATGTTATGG TAGATGTCCTAAAAATTATGTTGGAATAAGACCATAATATATGTGTAAGTGCATA TTTGAAAATTGTGTATCTTGTACAGAGACATAATTCAATCTTGATAATGTATGTTA TGATAAATGTCCTAATGGAACATTTGGATTTAATGGATTATGTATAAAGTGTGAT AAAAGTTGTGGAACTTGTTCAGGAGAGAGTTTTGATTAATGTGACTCATGTAGTT TTCCTTTAATCTTTTATTAACACACCTGCCTTAGTTAATGTGAAAACAATACATAT CATGATATTACTACAAATGAATGTCTTTTATGCGATGATTCTTGTTTAGCTTGTAC AGGACCACATTTAATTGATTGTACTGCTTGCAAAAATGAATAATTGTTAAATGTT GATGGATCATGTTAGGACTAATGTACATCTGATTCATATGTTATTATTTCTGAAA GAAGATGTTTAGCATGTCATCCAACTTGTTTAACATGTTTTGGTGGGATGATTAA TAATTGTTTGACATGTAAGAAATTAATTTATTTGAATGAATGTGTTGATACGTATC CTGCAATTCAAGCTTGTCGACCTGCAGTCTAGATAGGTAATCTCTGCTTAAAAGC ACAGA

D. DNA sequencing result 4

Pc_4 primers were designed from this sequence (underlined).

Template DNA: No.13 Primer: 04
GAATTGCAGAGAAGGGTAATACTTCATTAGTTTTAGATAGATCACCACCTAAATA AGTATGTATAATACCTTTTGTATCTAAGTAGGCTTTAAATTGATTTACTGCTTATT TATCAATTAATCCTTTTCTCATTGACAATGGATGAATAGGGTAGATGAGAAAATA GATTTAAATATAAAACTCCAACTTCCAAGAATCATGATCTTATCGTTATGAGATT GGGATATAGCGACTCCAAGATTCAAGAAATTTATCTCTTTCACCCCTATCAAAAT GTCCAAGAATGACATATTCTGTTTCACTTCCCTATTCAGTTGCAAATTATTATTCT ATCCGAAATTGTTCCAATGTTTTGAAACAATTACATCTAACCAAATAATCCTATA CAATATTGTTCAATTGTTCCACTAAATTTCCTCCTTAATCAACTGATTTATCAGCC TAAATATAACAAATTGTTACAGATTATACAGCAGATTGAACTCTCTTTTATGGAG GGTCTACTTTGGAATTGCGACTTAATCCAGAATTTACTTTAGCTCTTACATCCTTC TCTGCAATTC

E. DNA sequencing result 5

Pc_5 primers were designed from this sequence (underlined).

Template DNA: No.13 Primer: 09
GAATTCGCTGTTACCAATTAGACATCATGGAAGAAATACTGGGGAGGTGCAGGA ATATCGGTTGATATTTTTACGAATTCCTCCTCAGTTATGTTTTATAATGCCCAATT CTTAGCCTTCTACTCACCGATAGTGCTAAATGTTCCAGCGCCTATAGCTTTCCCA CATGCTGATCCAGCCCCATGTCCAGGGTATAGGACTACATCATCATTGAGGGTT ATGACCTTGTCCCTTAATGAATGGTAGAGCAGGGAGGCAAGCTTTTCTGTTGAC AAGCCTGTAGCTCTTGATGCTAGGTCTGGTCGACCAACTTCTTCTAGGAACAAA GTGTCTCCACTGAATACACATCGATCCTTGCCTTCATCGACCAATACAAAGCAG CTTGATTCGAGTGTATGCCCTGGAGTGTGTAGTACTCTCAGTTGCACATGTCCAA GGGGTAATAATTCTTTATTATTCCTAATAGTACCTTCGTCAGAAGCAATTTTGGCT TCATACTTGGTAACAGCGAATTC

Table 5 Primers used in Multiplex PCR

Primer name	Sequence (5'-3')	Expected size of the PCR product (bp)	References
Pc_1 F	GGATATATGTACATGTGAAT	1487	This study
Pc_1 R	ATAAAACAGTTGATGTTTGG		This study
Pc_2 F	TAGCTCTGGACAGAATAGTG	1078	This study
Pc_2 R	TAGCTCTGGGATAAAGGAAA		This study
Pc_3 F	GCAGGATACGGCAATGTAAA	689	This study
Pc_3 R	GCAGGATACGTATCAACACA		This study
Pc_4 F	GCAGAGAAGGGTAATACTTC	554	This study
Pc_4 R	GCAGAGAAGGATGTAAGAGC		This study
Pc_5 F	GCTGTTACCAATTAGACATC	503	This study
Pc_5 R	CGCTGTTACCAAGTATGAAG		This study
18S F	GATGGTAGTGTATTGGAC	618	[Sawka-Gądek N, 2021]
18S R	TTGGCAAATGCTTTCGC		

Table 6 Quick identification chart for each standard strain

Name of standard strains	Method	Result	Approximate size	Related figure
P. caudatum dKNZ-12O	Multiplex PCR	Pc_1 (-) Pc_2 (-) Pc_3 (-) Pc_4 (+) Pc_5 (+)	- - - 554 bp 553 bp	Figure 8B and 9
P. tetraurelia 51	RAPD PCR analysis (Random primer 02)	Single band	3000 bp	Figure 6A
P. bursaria Yad1g1N	RAPD PCR analysis (Random primer 03)	Two distinct bands	4000 bp 2000 bp	Figure 7B

Figures

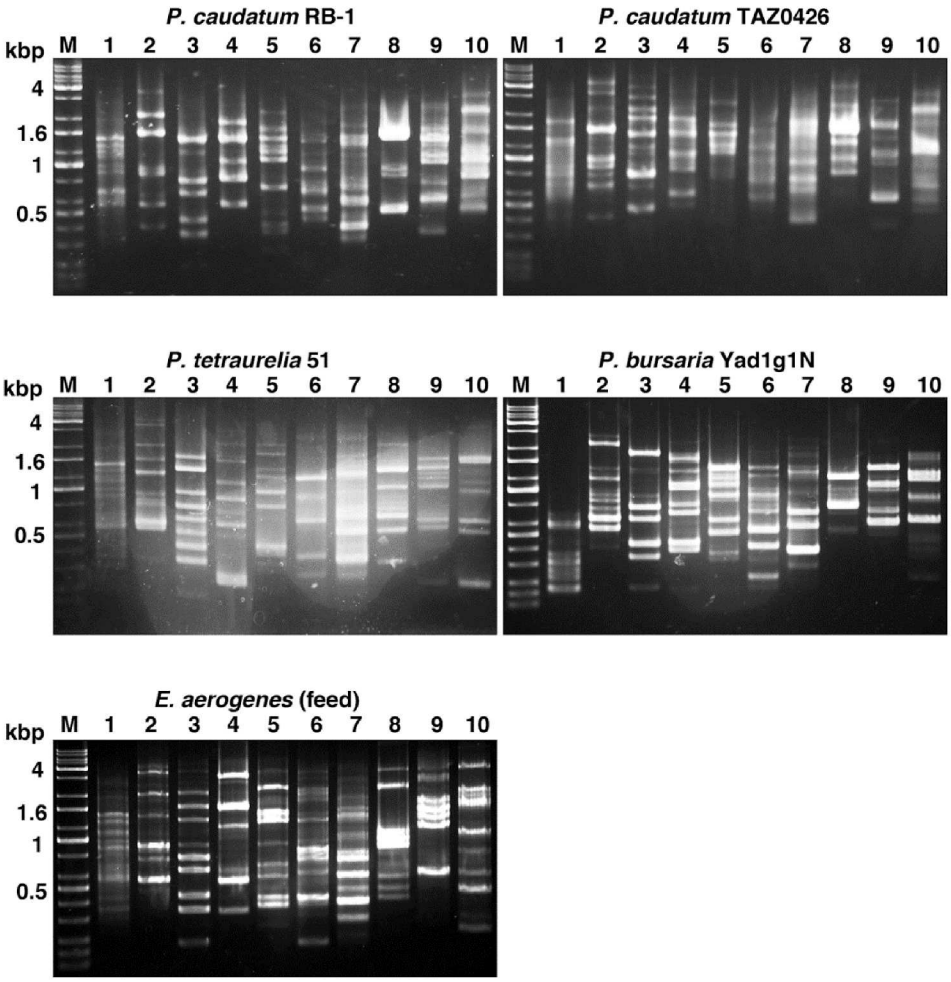

P. caudatum RB-1
P. caudatum TAZ0426
P. tetraurelia 51
P. bursaria Yad1g1N
E. aerogenes (feed)
kbp
M 1 2 3 4 5 6 7 8 9 10
4
1.6
1
0.5

Fig. 1 RAPD reaction products using primers 1–10 separated in an agarose gel

Results of RAPD analysis using template DNA from *P. caudatum* RB-1, *P. caudatum* TAZ0462, *P. tetraurelia* 51, *P. bursaria* Yad1g1N, and only culture medium with *E. aerogenes* (feed) are shown. The number of each lane indicates the random primer used in the reaction.

M: weight marker

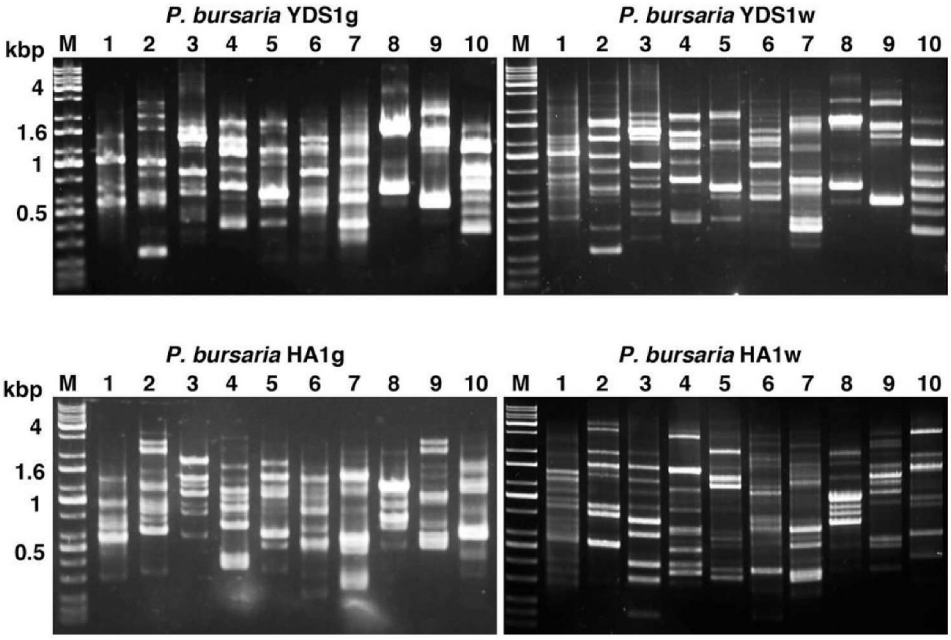


Fig. 2 Difference in RAPD reaction products between *P. bursaria* with and without Chlorella

Results of RAPD analysis using template DNA from *P. bursaria* YDS1g, *P. bursaria* YDS1w, *P. bursaria* HA1g, and *P. bursaria* HA1w are shown. The number of each lane indicates the random primer used in the reaction.

M: weight marker

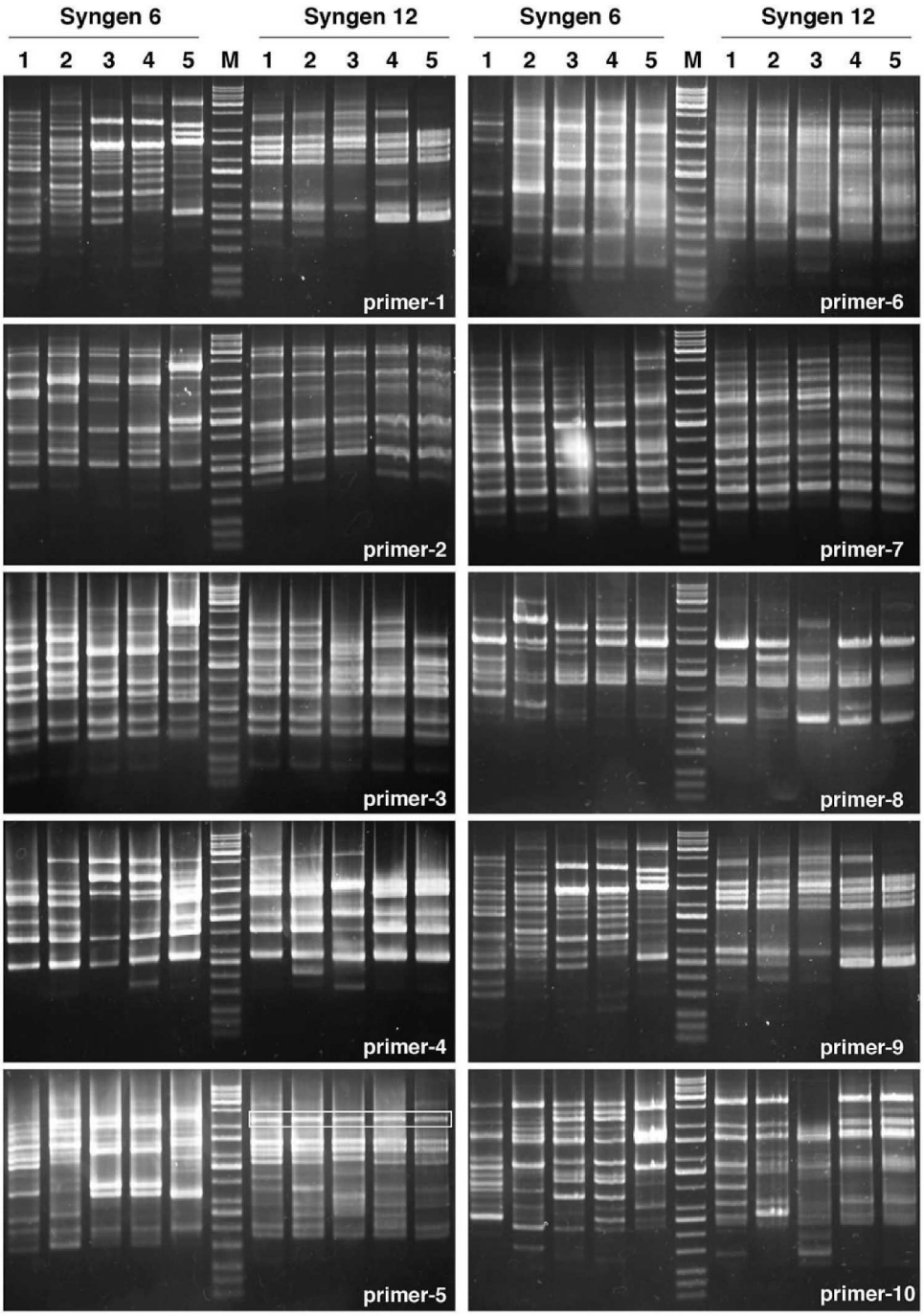

Syngen 6
Syngen 12
Syngen 6
Syngen 12
1 2 3 4 5 M 1 2 3 4 5
1 2 3 4 5 M 1 2 3 4 5
primer-1
primer-6
primer-2
primer-7
primer-3
primer-8
primer-4
primer-9
primer-5
primer-10

Fig. 3 Comparative analysis between *P. caudatum* syngen 6 strains and syngen 12 strains

Results of RAPD analysis using template DNA from *P. caudatum* syngen 6 strains (1, YR1504-2; 2, YR1504- 6; 3, YDRM6; 4, YDRM11; and 5, YDRM26) and syngen 12 strains (1, My43C3d; 2, KGS1; 3, SBK2019-3b1; 4, dKNZ1207×1209–1; and 5, dKNZ1207×1209–3) are shown.

M: weight marker

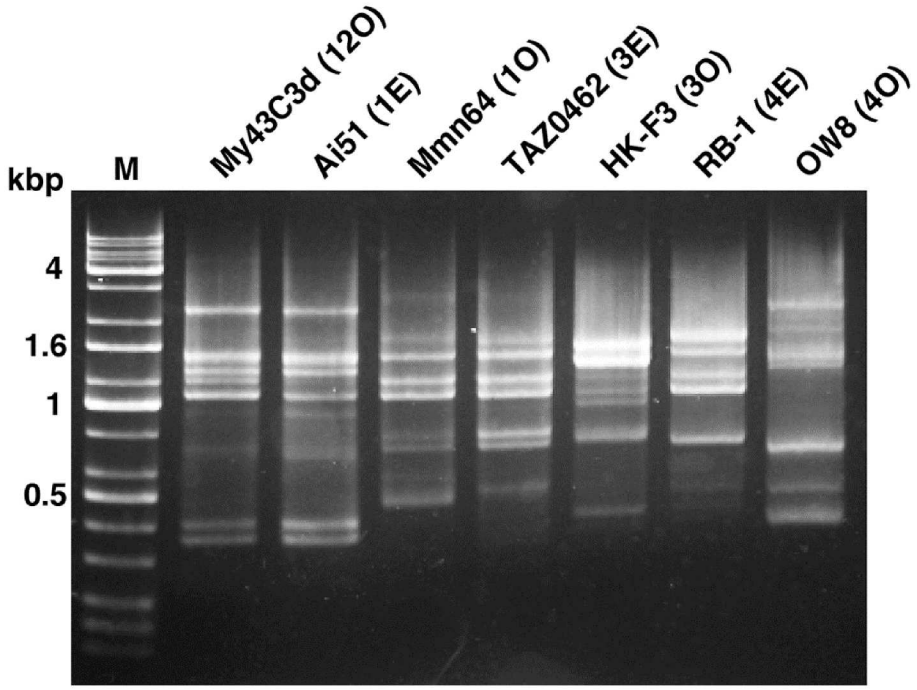


Fig. 4 Evaluating the specificity of PCR product observed in *P. caudatum* syngen 12 strains

Results of RAPD analysis using template DNA from *P. caudatum* syngens 1, 3, 4, and 12 strains are shown. Each strain name, together with its syngen and mating type, is indicated.

M: weight marker

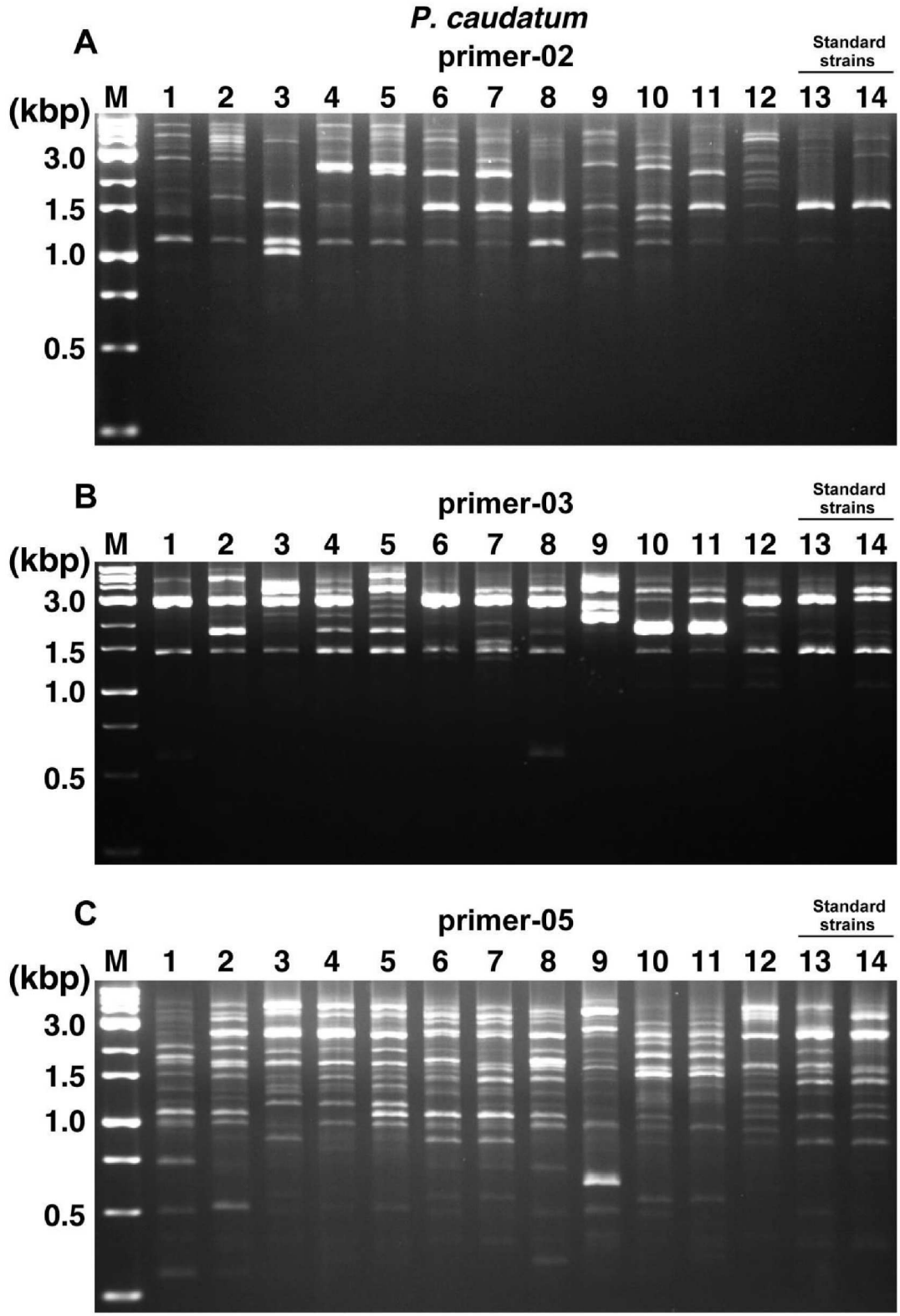

A
P. caudatum
primer-02
Standard strains
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14
(kbp)
3.0
1.5
1.0
0.5
B
primer-03
Standard strains
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14
(kbp)
3.0
1.5
1.0
0.5
C
primer-05
Standard strains
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14
(kbp)
3.0
1.5
1.0
0.5

Fig. 5 RAPD analysis using template DNA from *P. caudatum* strains

RAPD analysis using template DNA from 12 recommended strains (Nos. 1–12) and 2 standard strains (Nos. 13 and 14) of *P. caudatum*. The random primers used in each reaction are presented at the top of the figures (A, primer-02. B, primer-03. C, primer-05).

M: weight marker

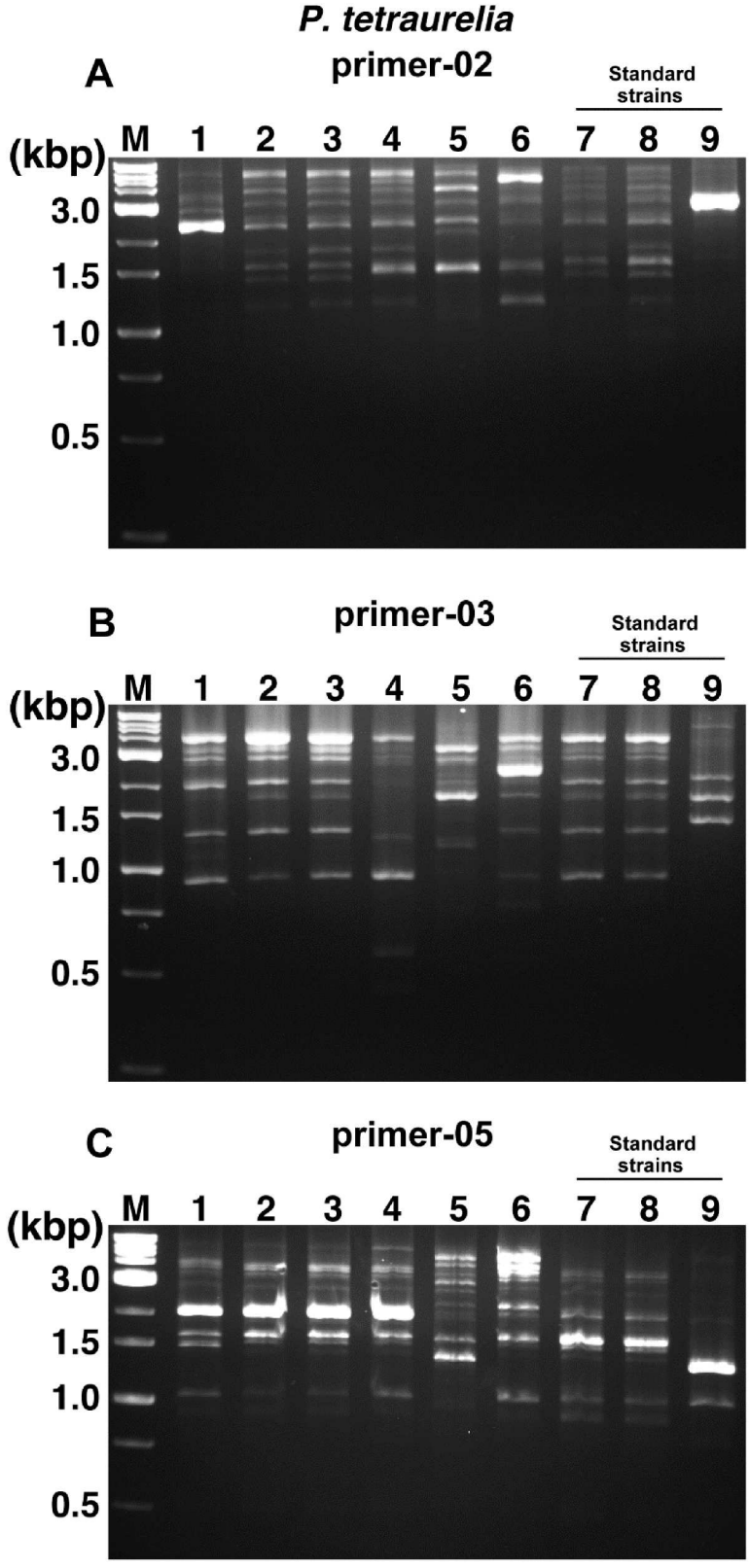

P. tetraurelia
A
primer-02
Standard strains
M 1 2 3 4 5 6 7 8 9
(kbp)
3.0
1.5
1.0
0.5
B
primer-03
Standard strains
M 1 2 3 4 5 6 7 8 9
(kbp)
3.0
1.5
1.0
0.5
C
primer-05
Standard strains
M 1 2 3 4 5 6 7 8 9
(kbp)
3.0
1.5
1.0
0.5

Fig 6 RAPD analysis using template DNA from *P. tetraurelia* strains

RAPD analysis using template DNA from 6 recommended strains (Nos. 1–6) and 3 standard strains (Nos. 7–9) of *P. tetraurelia*. The random primers used in each reaction are presented at the top of the figures (A, primer-02. B, primer-03. C, primer-05).

M: weight marker

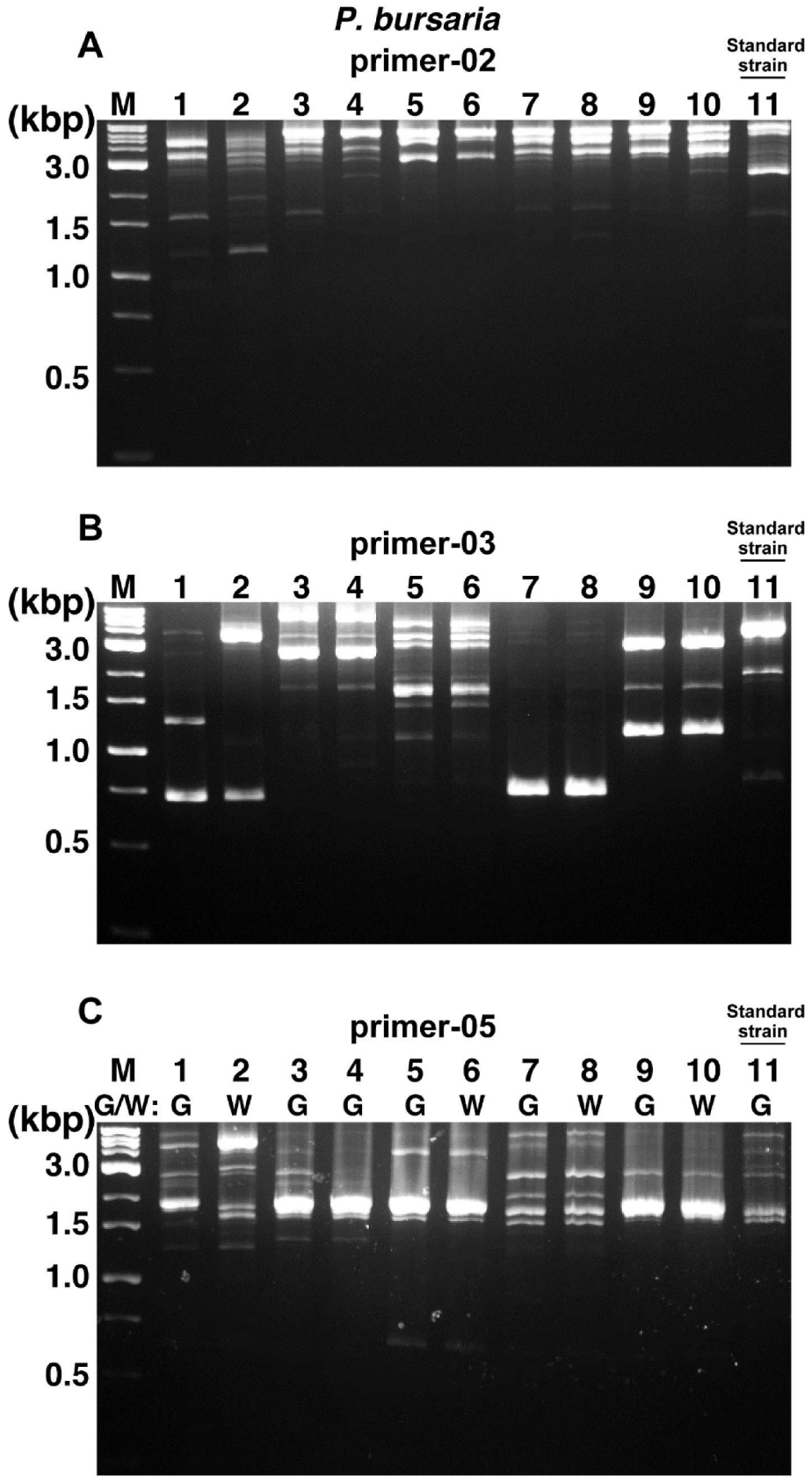

P. bursaria
A
primer-02
Standard strain
M 1 2 3 4 5 6 7 8 9 10 11
(kbp)
3.0
1.5
1.0
0.5
B
primer-03
Standard strain
M 1 2 3 4 5 6 7 8 9 10 11
(kbp)
3.0
1.5
1.0
0.5
C
primer-05
Standard strain
M 1 2 3 4 5 6 7 8 9 10 11
G/W: G W G G G W G W G W G
(kbp)
3.0
1.5
1.0
0.5

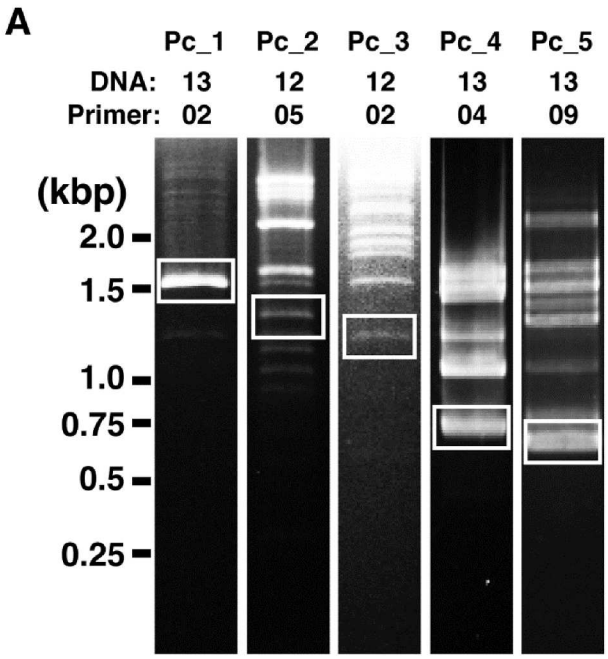
Fig. 7 RAPD analysis using template DNA from *P. bursaria* strains

RAPD analysis using template DNA from 10 recommended strains (Nos. 1–10) and a standard strain (No. 11) of *P. bursaria*.

G: strain with Chlorella.

W: strain without Chlorella. The random primers used in each reaction are presented at the top of the figures (A, primer-02. B, primer-03. C, primer-05).

M: weight marker.


A
Pc_1
Pc_2
Pc_3
Pc_4
Pc_5
DNA: 13 12 12 13 13
Primer: 02 05 02 04 09
(kbp)
2.0
1.5
1.0
0.75
0.5
0.25

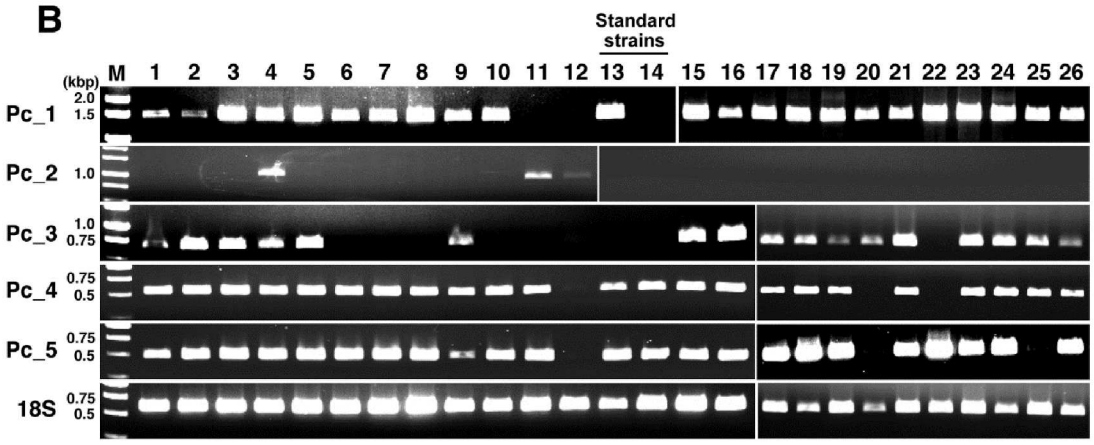

B
Standard strains
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26
(kbp)
Pc_1 2.0 1.5
Pc_2 1.0
Pc_3 1.0 0.75
Pc_4 0.75 0.5
Pc_5 0.75 0.5
18S 0.75 0.5

Fig. 8 Selecting the target gene region to identify strains based on the RAPD-PCR and PCR results for these target sequences.

A. RAPD-PCR was conducted individually using template DNA from No. 12 or 13 of *P. caudatum* and random primer-02, 04, 05, and 09, individually. Single bands which were selected and submitted to sequencing analysis were surrounded by white boxes.

B. PCR results using template DNA from 26 strains of *P. caudatum* are shown. Pc_1 to Pc_5 and 18S indicate target region names.

18S: 18S rRNA gene.

M: weight marker.

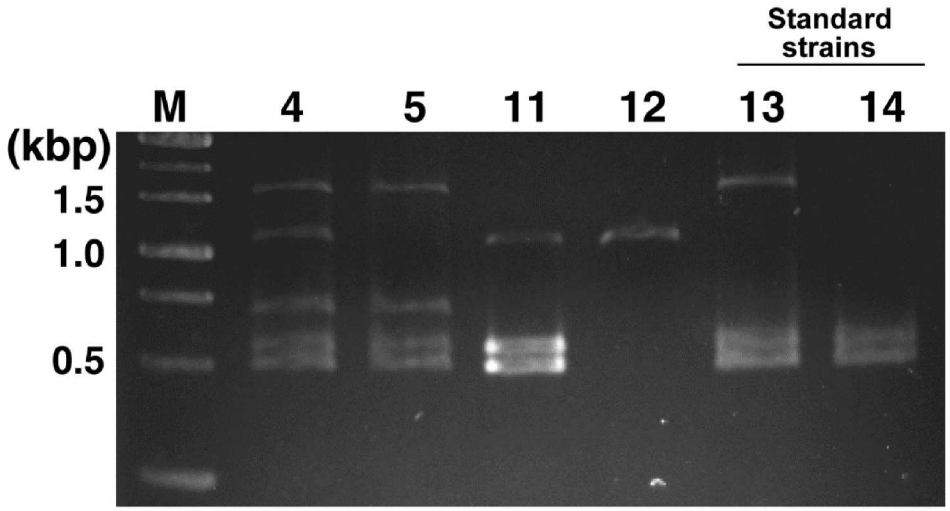


Fig. 9 Multiplex PCR

Multiplex PCR results using template DNA from 6 strains (Nos. 4, 5, and 11–14) of *P. caudatum*.

M: weight marker

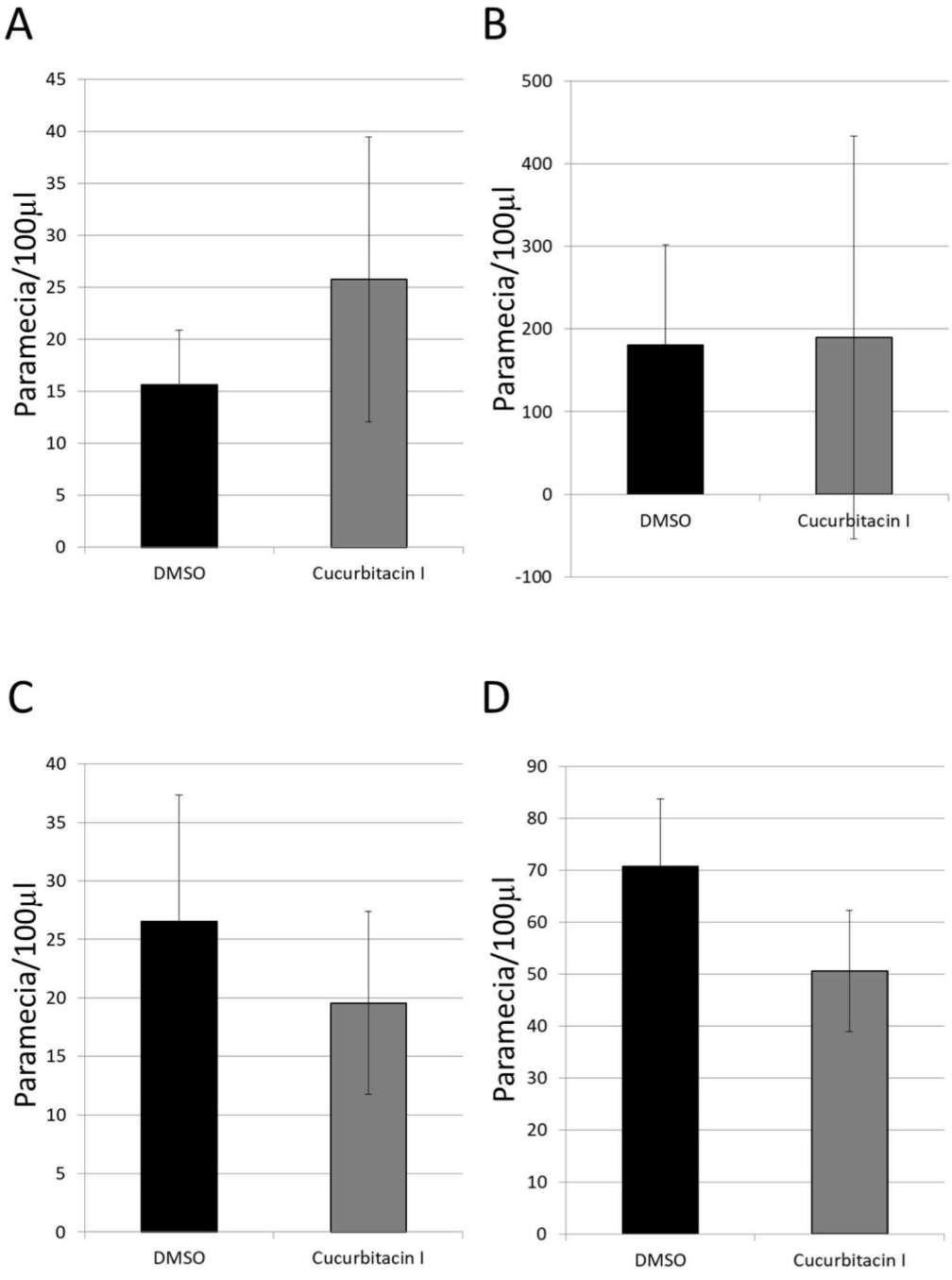

A
Paramecia/100µl
45
40
35
30
25
20
15
10
5
0
DMSO
Cucurbitacin I
B
Paramecia/100µl
500
400
300
200
100
0
-100
DMSO
Cucurbitacin I
C
Paramecia/100µl
40
35
30
25
20
15
10
5
0
DMSO
Cucurbitacin I
D
Paramecia/100µl
90
80
70
60
50
40
30
20
10
0
DMSO
Cucurbitacin I

Fig. 10 Survival *Paramecium*

P. caudatum dYDRM-3E (A) and dYDRM-3O (B), *P. bursaria* YKK10w (C) and YDS1w(D) were cultured with 1 μM of Cucurbitacin I or 10 μM of Stattic for 48 h and moving Paramecium cells were counted by microscopic observation. Mean ± SD, Student t-test, *: p=0.05

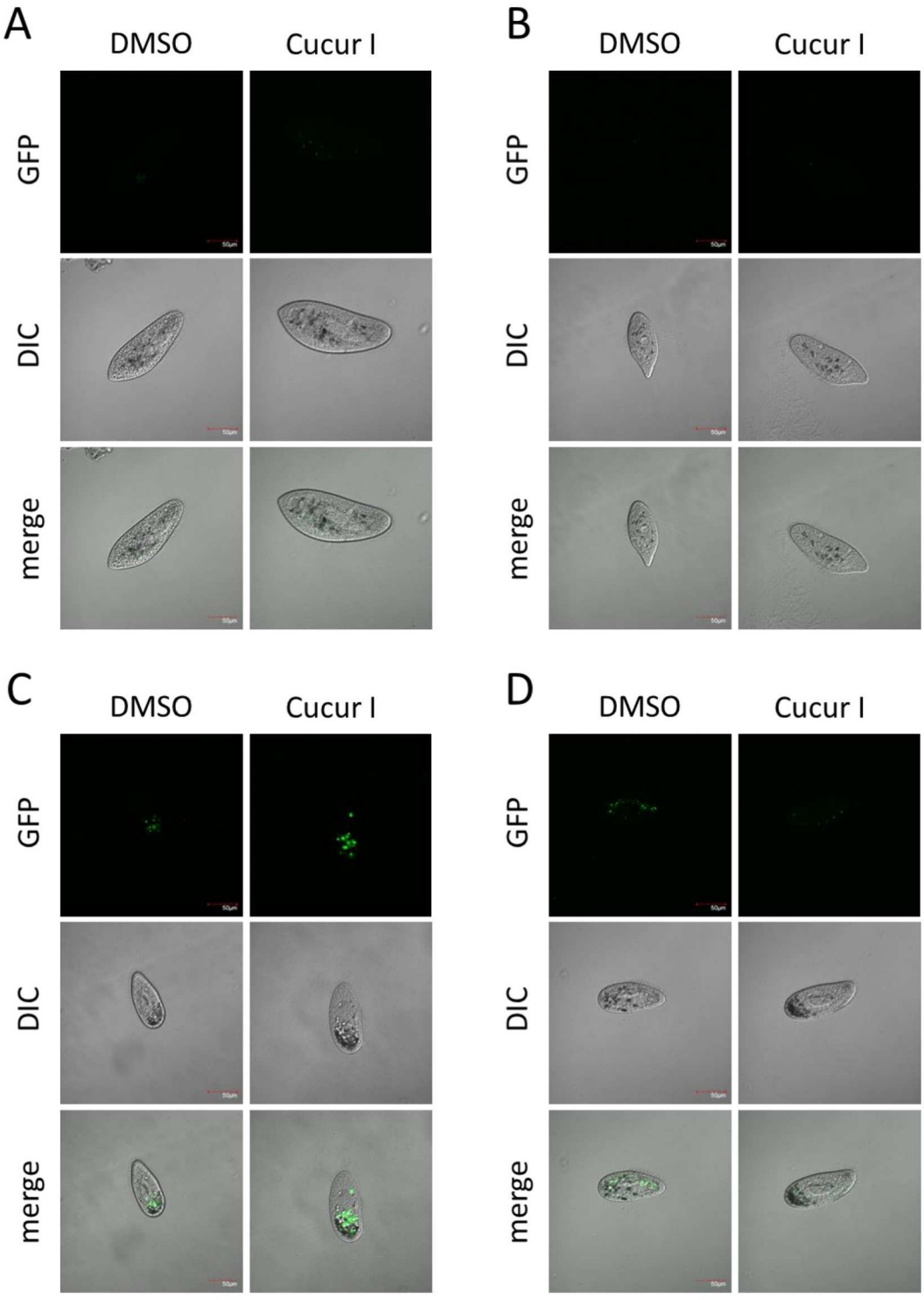

A
DMSO
Cucur l
GFP
DIC
merge
B
DMSO
Cucur l
GFP
DIC
merge
C
DMSO
Cucur l
GFP
DIC
merge
D
DMSO
Cucur l
GFP
DIC
merge

Fig. 11 Comparison of *F. novicida* in cells

P. caudatum dYDRM-3E (A) and dYDRM-3O (B), *P. bursaria* YKK10w (C) and YDS1w(D) were cultured with 1 μM of Cucurbitacin I or 10 μM of Stattic for 2 h. *Paramecium* cells were infected with GFP-expressing *F. novicida* for 24h. cells were fixed and observed with confocal microscopy.

Reference

[1] Jill Ellis, Petra C F Oyston, Michael Green, Richard W Titball, "Tularemia," Clin Microbiol Rev 15, 631-646., 2002.

[2] Edward A. Medina, Ian R. Morris, Michael T. Berton, "Phosphatidylinositol 3-Kinase Activation Attenuates the TLR2-Mediated Macrophage Proinflammatory Cytokine Response to Francisella tularensis Live Vaccine Strain.," J Immunol. 15;185(12):7562-72. doi: 10.4049/jimmunol.0903790. Epub 2010 Nov 22., 2010.

[3] Riccardo D' Elia, Dominic C. Jenner, Thomas R. Laws, Margaret G.M. Stokes, Matthew C. Jackson, Angela E. Essex-Lopresti & Helen S. Atkins, "Inhibition of Francisella tularensis LVS infection of macrophages results in a reduced inflammatory response: evaluation of a therapeutic strategy for intracellular bacteria.," FEMS Immunol Med Microbiol.;62(3):348-61. doi: 10.1111/j.1574-695X.2011.00817.x., 2011.

[4] Linda A. Lieberman, Darren E. Higgins, "A Small-Molecule Screen Identifies the Antipsychotic Drug Pimozide as an Inhibitor of Listeria monocytogenes Infection," ANTIMICROBIAL AGENTS AND CHEMOTHERAPY p.756-764 Vol. 53, No. 2, 2009.

[5] Linda A. Lieberman, Darren E. Higgins, "Inhibition of Listeria monocytogenes infection by neurological drugs," Int J Antimicrob Agents. 35(3): 292 doi:10.1016/j.ijantimicag.2009.10.011., 2010.

[6] ILAN ROSENSHINE, VINCENT DURONIO, B. BRETT FINLAY, "Tyrosine Protein Kinase Inhibitors Block Invasin-Promoted Bacterial Uptake by Epithelial Cells," INFECTION AND IMMUNITY, p.2211-2217 Vol. 60, No. 6, 1992.

[7] Steinman RM, Mellman IS, Muller WA, Cohn ZA, "Endocytosis and the recycling of plasma membrane.," J Cell Biol 96:1-27, 1983.

[8] Plattner H, "Membrane trafcking in protozoa SNARE proteins, H+-ATPase, actin, and other key players in ciliates.," Int Rev Cell Mol Biol 280:79-184. https://doi.org/10.1016/S1937-6448(10)80003-6, 2010.

[9] Soares H, Sunter JD, Wloga D, Joachimiak E, Miceli C, "Trypanosoma, Paramecium and Tetrahymena: from genomics to fagellar and ciliary structures and cytoskeleton dynamics.," Eur J Protistol 76:125722. https://doi.org/10.1016/j.ejop.2020.125722, 2020.

[10] Watanabe K, Nakao R, Fujishima M, Tachibana M, Shimizu T, Watarai M, "Ciliate Paramecium is a natural reservoir of Legionella pneumophila.," Sci Rep 6:24322. https://doi.org/10.1038/srep24322, 2016.

[11] Nishida T, Hara N, Watanabe K, Shimizu T, Fujishima M, Watarai M, "Crucial role of Legionella pneumophila TolC in the inhibition of cellular trafcking in the protistan Host Paramecium tetraurelia.," Front Microbiol 9:800. https://doi.org/10., 2018.

[12] Watanabe K, Suzuki H, Nishida T, Mishima M, Tachibana M, Fujishima M, Shimizu T, Watarai M, "Identifcation of novel Legionella genes required for endosymbiosis in Paramecium based on comparative genome analysis with Holospora spp.," FEMS Microbiol Ecol 94.

https://doi.org/10.1093/femsec/fy162, 2018.

[13] Kenta Watanabe, Akane Motonaga, Masato Tachibana, Takashi Shimizu and Masahisa Watarai, "Francisella novicida can utilize Paramecium bursaria as its potential host.," Environmental Microbiology Reports 14(1), 50-59, 2022.

[14] Steinman RM, Mellman IS, Muller WA, Cohn ZA, "Endocytosis and the recycling of plasma membrane.," J Cell Biol 96:1-27, 1983.

[15] Hiwatashi K, "Determination and inheritance of mating type in Paramecium caudatum.," Genetics 58:373-386, 1968.

[16] Hiwatashi K, "Conjugation in protozoa. Tanpakushitsu kakusan koso.," Protein Nucleic Acid Enzym 43:337-345, 1998.

[17] Betermier M, Duharcourt S, "Programmed rearrangement in ciliates: Paramecium.," Microbiol Spect 2. https://doi.org/10.1128/microbiolspec.MDNA3-0035-2014, 2014.

[18] Sawka N, "Mating types in Paramecium and a molecular approach to their determination.," Folia Biol 60:3-9. https://doi.org/10.3409/fb60_1-

2.03-09, 2012.

[19] L D Anthony, R D Burke, F E Nano, "Growth of Francisella spp. in rodent macrophages.," Infect Immun. 59(9):3291-6. doi: 10.1128/iai.59.9.3291-3296.1991., 1991.

[20] Luke C Kingry, Jeannine M Petersen, "Comparative review of Francisella tularensis and Francisella novicida.," Front Cell Infect Microbiol. 13;4:35. doi: 10.3389/fcimb.2014.00035., 2014.

[21] Daniel L Clemens, Bai-Yu Lee, Marcus A Horwitz, "Virulent and avirulent strains of Francisella tularensis prevent acidification and maturation of their phagosomes and escape into the cytoplasm in human macrophages.," Infect Immun. 72(6):3204-17. doi: 10.1128/IAI.72.6.3204-3217.2004., 2004.

[22] Daniel L Clemens, Bai-Yu Lee, Marcus A Horwitz, "Francisella tularensis enters macrophages via a novel process involving pseudopod loops.," Infect Immun. 73(9):5892-902. doi: 10.1128/IAI.73.9.5892-5902.2005., 2005.

[23] Igor Golovliov, Vladimir Baranov, Zuzana Krocova, Hana Kovarova, Anders Sjöstedt, "An attenuated strain of the facultative intracellular bacterium Francisella tularensis can escape the phagosome of monocytic cells.," Infect Immun. 71(10):5940-50. doi: 10.1128/IAI.71.10.5940-5950.2003., 2003.

[24] Claire Checroun, Tara D Wehrly, Elizabeth R Fischer, Stanley F Hayes, Jean Celli, "Autophagy-mediated reentry of Francisella tularensis into the endocytic compartment after cytoplasmic replication.," Proc Natl Acad Sci U S A. 26;103(39):14578-83. doi: 10.1073/pnas.0601838103. Epub 2006 Sep 18., 2006.

[25] Audrey Chong, Jean Celli, "The Francisella intracellular life cycle: toward molecular mechanisms of intracellular survival and proliferation.," Front Microbiol. 28;1:138. doi: 10.3389/fmicb.2010.00138. eCollection 2010., 2010.

[26] Audrey Chong, Tara D Wehrly, Robert Child, Bryan Hansen, Seungmin Hwang, Herbert W Virgin, Jean Celli, "Cytosolic clearance of

replication-deficient mutants reveals Francisella tularensis interactions with the autophagic pathway.," Autophagy. 8(9):1342-56. doi: 10.4161/auto.20808. Epub 2012 Aug 6., 2012.

[27] Michelle A. Blaskovich, Jiazhi Sun, Alan Cantor, James Turkson, Richard Jove, and Saı ¨ d M. Sebti, "Discovery of JSI-124 (Cucurbitacin I), a Selective Janus Kinase/Signal Transducer and Activator of Transcription 3 Signaling Pathway Inhibitor with Potent Antitumor Activity against Human and Murine Cancer Cells in Mice.," CANCER RESEARCH 63, 1270-1279, 2003.

[28] Pia M. Sörensen, Roxana E. Iacob, Marco Fritzsche, John R. Engen, William M. Brieher, Guillaume Charras, and Ulrike S. Eggert, "The natural product cucurbitacin E inhibits depolymerization of actin filaments," ACS Chem Biol; 7(9): 1502-1508. doi:10.1021/cb300254s., 2012.

[29] Hans Dieter Hofmann, Matthias Kirsch, "JAK2-STAT3 signaling: A novel function and a novel mechanism.," JAKSTAT. 1;1(3):191-3. doi:

10.4161/jkst.20446., 2012.

[30] Kiu H, Nicholson SE, "Biology and significance of the JAK/STAT signalling pathways.," Growth Factors. 30(2):88-106. doi: 10.3109/08977194.2012.660936, 2012.

[31] Hao Wu, Min Huang, Peng Cao, Tongshan Wang, Yongqian Shu, Ping Liu, "MiR-135a targets JAK2 and inhibits gastric cancer cell proliferation.," Cancer Biol Ther. 2012;13(5):281-288. doi: 10.4161/cbt.18943, 2012.

[32] Murray PJ, "The JAK-STAT signaling pathway: input and output integration.," J Immunol. 178(5):2623-2629. doi: 10.4049/jimmunol.178.5.2623, 2007.

[33] Yao Wu, Hongying Chen, Ruli Li, Xiaoxiao Wang, He Li, Juanjuan Xin, Zhiqiang Liu, Sisi Wu, Wei Jiang, Ling Zhu, "Cucurbitacin-I induces hypertrophy in H9c2 cardiomyoblasts through activation of autophagy via MEK/ERK1/2 signaling pathway.," Toxicol Lett. 15;264:87-98. doi: 10.1016/j.toxlet.2016.11.003. Epub 2016 Nov 9., 2016.

[34] Patrick Mc Gann, David A. Rozak, Mikeljon P. Nikolich, Robert A. Bowden, Luther E. Lindler, Mark J. Wolcott, Raju Lathigra, "A novel brain heart infusion broth supports the study of common Francisella tularensis serotypes.," J Microbiol Methods.80(2):164-71 doi: 10.1016/j.mimet. 2009.12.005. Epub 2009 Dec 11., 2010.

[35] S. C. NAGLE, JR., R. E. ANDERSON, AND N. D. GARY, U. S. Army Chemical Corps, Fort Detrick, Frederick, Maryland, "CHEMICALLY DEFINED MEDIUM FOR THE GROWTH OF PASTEURELLA TULARENSIS," J Bacteriol. 1960 Apr;79(4):566-71. doi: 10.1128/jb.79.4.566-571.1960., 1960.

[36] Takemasa Nakamura, Takashi Shimizu, Fumiya Inagaki, Shoma Okazaki, Shib Shankar Saha, Akihiko Uda, Kenta Watanabe and Masahisa Watarai, "Identification of Membrane-Bound Lytic Murein Transglycosylase A (MltA) as a Growth Factor for Francisella novicida in a Silkworm Infection Model," Front Cell Infect Microbiol. 22;10: 581864. doi:10.3389/fcimb.2020.581864. eCollection 2020., 2021.

[37] BJØRN-ERIK KRISTIANSEN, LISBETH RUSTAD, ODDVAR SPANNE, BJARNE BJORVATN, "Effect of Subminimal Inhibitory Concentrations of Antimicrobial Agents on the Piliation and Adherence of Neisseria meningitidis.," ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, p. 731-734 Vol. 24, No. 5, 1983.

[38] Dieter W. Gump, Owen W. Nadeau, Gregory M. Hendricks, Diane H. Meyer, "Evidence that bismuth salts reduce invasion of epithelial cells by enteroinvasive bacteria.," Med Microbiol Immunol 181:131-143, 1992.

[39] Paul H. M. Savelkoul, Bas Kremer, Johannes G. Kusters, Bernard A. M. van der Zeijist, Wim Gaastra, "Invasion of Hela cells by Bordetella bronchiseptica.," Microbial pathogenesis 14: 161-168, 1993.

[40] Keqiang Chen, Teizo Yoshimura, Wanghua Gong, Cuimeng Tian, Jiaqiang Huang, Giorgio Trinchieri and Ji Ming Wang, "Requirement of CRAMP for mouse macrophages to eliminate phagocytosed E. coli through an autophagy pathway.," Journal of Cell Science 134,

jcs252148. doi:10.1242/jcs.252148, 2021.

[41] Douglas A. Drevets, Beth P. Canono, Priscilla A. Campbell, "Measurement of Bacterial Ingestion and UNIT 14.6 Killing by Macrophages," Current Protocols in Immunology 14.6.1-14.6.17, 2015.

[42] George A. Timmons, Richard G. Carroll, James R. O' Siorain, Mariana P. Cervantes-Silva, Lauren E. Fagan, Shannon L. Cox, Eva Palsson-McDermott, David K. Finlay, Emma E. Vincent, Nicholas Jones, Annie M. Curtis, "The Circadian Clock Protein BMAL1 Acts as a Metabolic Sensor In Macrophages to Control the Production of Pro IL-1 β .," ORIGINAL RESEARCH published: doi: 10.3389/fimmu.2021.700431, 2021.

[43] BARRY I. EISENSTEIN, ITZHAK OFEK, EDWIN H. BEACHEY, "Interference with the Mannose Binding and Epithelial Cell Adherence of Escherichia coli by Sublethal Concentrations of Streptomycin.," J Clin Invest. 63(6):1219-28. doi: 10.1172/JCI109417., 1979.

[44] Robin C May, Laura M Machesky, "Phagocytosis and the actin

cytoskeleton.,” J Cell Sci. 114(Pt 6):1061-77. doi: 10.1242/jcs.114.6.1061., 2001.

[45] Chris S Mantsounga, Cadence Lee, Jade Neverson, Sheila Sharma, Abigail Healy, Joshua M Berus, Crystal Parry, Nicolle M Ceneri, Francesc López-Giráldez, Hyung J Chun, Qing Lu, Frank Sellke, Gaurav Choudhary, Alan R Morrison, “Macrophage IL-1 β promotes arteriogenesis by autocrine STAT3- and NF- κ B-mediated transcription of pro-angiogenic VEGF-A,” Cell Reports 38, 110309, https://doi.org/10.1016/j.celrep.2022.110309, 2022.

[46] Taito Miyamoto, Ryusuke Murakami, Junzo Hamanishi, Kenji Tanigaki, Yuko Hosoe, Nathan Mise, Shiro Takamatsu, Yuka Mise, Masayo Ukita, Mana Taki, Koji Yamanoi, Naoki Horikawa, Kaoru Abiko, Ken Yamaguchi, Tsukasa Baba, Noriomi Matsumura, Masaki Mandai, “B7-H3 Suppresses Antitumor Immunity via the CCL2-CCR2-M2 Macrophage Axis and Contributes to Ovarian Cancer Progression.,” Cancer Immunol Res; 10(1), 2022.

[47] Jochen Schust, Bianca Sperl, Angela Hollis, Thomas U. Mayer, Thorsten Berg, "Stattic: A Small-Molecule Inhibitor of STAT3 Activation and Dimerization.," Chem Biol. 13(11):1235-42. doi: 10.1016/j.chembiol.2006.09.018., 2006.

[48] Yi J, Wang Y, Zhang J, Xu J, Li T, Chen C, "Effects of JAK2 / STAT3 Signaling Pathway Activation on Intracellular Survival of Brucella.," Pak Vet J, 38(2): 153-158. http://dx.doi.org/10.29261/pakvetj/2018.048, 2018.

[49] Kenji Izuhara, Simon J. Conway, Bethany B. Moore, Hisako Matsumoto, Cecile T. J. Holweg, John G. Matthews, Joseph R. Arron, "Roles of Periostin in Respiratory Disorders.," Am J Respir Crit Care Med. 1;193(9):949-56., 2016.

[50] Hui GUO, Shan KUANG, Qiao-ling SONG, Man LIU, Xiao-xiao SUN, Qiang YU, "Cucurbitacin I inhibits STAT3, but enhances STAT1 signaling in human cancer cells in vitro through disrupting actin filaments," Acta Pharmacologica Sinica 39: 425-437., 2018.

[51] David A. Knecht, Rebecca A. LaFleur, Alem W. Kahsai, Christian E. Argueta, Anwar B. Beshir, Gabriel Fenteany, "Cucurbitacin I Inhibits Cell Motility by Indirectly Interfering with Actin Dynamics," PLoS One. 24;5(11):e14039. doi: 10.1371/journal.pone.0014039., 2010.

[52] Knecht DA, LaFleur RA, Kahsai AW, Argueta CE, Beshir AB, "Cucurbitacin I Inhibits Cell Motility by Indirectly Interfering with Actin Dynamics.," PLoS ONE 5(11): e14039. doi:10.1371/journal.pone.0014039, 2010.

[53] Sonneborn TM, "Paramecium tetraurelia.," 著: *Handbook of genetics, vol 2.*, New York, Plenum Press, 1974, p. pp 469-594.

[54] Potekhin A, Mayén-Estrada R, "Paramecium diversity and a new member of the Paramecium aurelia species complex described from Mexico.," Diversity 12:197. https://doi.org/10.3390/d12050197, 2020.

[55] PrzyboŚ E, Tarcz S, "Global molecular variation of Paramecium jenningsi complex (Ciliophora, Protista): a starting point for further, detailed biogeography surveys.," Syst Biodivers 17:527-539.

https://doi.org/10.1080/14772000.2019.1643424, 2019.

[56] Hori M, Tomikawa I, Przyboś E, Fujishima M, "Comparison of the evolutionary distances among syngens and sibling species of Paramecium.," Mol Phylogenet Evol 38:697-704. https://doi.org/10.1016/j.ympev.2005.09.006, 2006.

[57] Catania F, Wurmser F, Potekhin A, Przybos E, Lynch M, "Genetic diversity in the Paramecium aurelia species complex.," Mol Biol Evol 26:421-431. https://doi.org/10.1093/molbevmsn266, 2009.

[58] Sonneborn TM, "The Paramecium aurelia complex of fourteen sibling species.," Trans Am Microsc Soc 94:155-178, 1975.

[59] Aufderheide KJ, Daggett PM, Nerad TA, "Paramecium sonneborni new species a new member of the Paramecium aurelia species complex.," J Protozool 30:128-131, 1983.

[60] Watanabe K, Higuchi Y, Shimmura M, Tachibana M, Fujishima M, Shimizu T, Watarai M, "Peculiar Paramecium hosts fail to establish a stable intracellular relationship with Legionella pneumophila.," Front

Microbiol 11:596731. https://doi.org/10.3389/fmicb.2020.596731, 2020.

[61] Williams JG, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV, "DNA polymorphisms amplifed by arbitrary primers are useful as genetic markers.," Nucleic Acids Res 18:6531-6535. https://doi.org/10.1093/nar/18.22.6531, 1990.

[62] Hadrys H, Balick M, Schierwater B, "Applications of random amplifed polymorphic DNA (RAPD) in molecular ecology.," Mol Ecol 1:55-63. https://doi.org/10.1111/j.1365-294x.1992. tb00155.x, 1992.

[63] Powell W, Orozco-Castillo C, Chalmers KJ, Provan J, Waugh R, "Polymerase chain reaction-based assays for the characterisation of plant genetic resources.," Electrophoresis 16:1726-1730. https://doi.org/10.1002/elps.11501601285, 1995.

[64] Hofstra H, van der Vossen JM, van der Plas J, "Microbes in food processing technology.," FEMS Microbiol Rev 15:175-183. https://doi.org/10.1111/j.1574-6976.1994.tb00133.x, 1994.

[65] Saunders NA, J Infect 38:80-86. https://doi.org/10.1016/s0163-4453(99),

1999.

[66] Kuznetsova MV, Gizatullina JS, "Epidemiological characteristics of uropatogenic isolates of Escherichia coli in hospitals.," Klin Lab Diagn 66:248-256. https://doi.org/10.51620/0869-2084-2021-66-4-248-256, 2021.

[67] Jaafar JN, Bhore SJ, Phua KK, "Non-specifcity of sequence characterised amplifed region as an alternative molecular epidemiology marker for the identifcation of Salmonella enterica subspecies enterica serovar Typhi.," BMC Res Notes 11:766. https://doi.org/10.1186/s13104-018-3870-z, 2018.

[68] Reinoso EB, Bettera SG, "Random amplifed polymorphic DNA PCR in the teaching of molecular epidemiology.," Biochem Mol Biol Educ 44:391-396. https://doi.org/10.1002/bmb.20957, 2016.

[69] Tsukii Y, "Genetic diversity among natural stocks of Paramecium caudatum revealed by RAPD markers.," Eur J Protistol 32:165-169, 1996.

[70] Stoeck T, Schmidt HJ, "Fast and accurate identifcation of European species of the Paramecium aurelia complex by RAPDfngerprints.," Microb Ecol 35:311-317. https://doi.org/10.1007/s002489900086, 1998.

[71] Przyboś E, Prajer M, Greczek-Stachura M, "Molecular analysis (RAPD-PCR) of inter-strain hybrids of the Paramecium aurelia species complex (Ciliophora, Protozoa).," Folia Biol 53:115-122. https://doi.org/10.3409/173491605775142846, 2005.

[72] Maciejewska A, "Molecular phylogenetics of representative Paramecium species.," Folia Biol 55:1-8. https://doi.org/10.3409/ 173491607780006335, 2007.

[73] Greczek-Stachura M, Tarcz S, Przyboś E, "Intra-specifc diferentiation of Paramecium bursaria strains by molecular methods-preliminary studies.," Folia Biol 58:35-45. https://doi.org/10. 3409/fb58_1-2.35-45, 2010.

[74] Przyboś E, Tarcz S, Skoblo I, "First American stand of Paramecium novaurelia and intra-specifc diferentiation of the species.," Folia Biol

55:53-63. https://doi.org/10.3409/1734916077 80006371, 2007.

[75] Przyboś E, Tarcz S, Greczek-Stachura M, Surmacz M, Potekhin A, Rautian M, "Molecular studies on intraspecifc diferentiation of Paramecium dodecaurelia, with description of new stands of the species (Protozoa, Ciliophora).," Folia Biol 56:249-262., 2008.

[76] Karakashian MW, " Symbiosis in Paramecium bursaria.," Symp Soc Exp Biol 29:145-173 39, 1975.

[77] Iwai S, Fujiwara K, Tamura T, "Maintenance of algal endosymbionts in Paramecium bursaria: a simple model based on population dynamics.," Environ Microbiol 18:2435-2445. https://doi.org/10.1111/1462-2920.13140, 2016.

[78] Song C, Murata K, Suzaki T, "Intracellular symbiosis of algae with possible involvement of mitochondrial dynamics.," Sci Rep 7:1221. https://doi.org/10.1038/s41598-017-01331-0, 2017.

[79] Sonneborn TM, "Breeding systems, reproductive methods, and species problem in Protozoa.," 著: *The Species Problem.*, Washington DC,

American Association for the Advancement of Science, 1957, p. pp 155-324.

[80] Greczek-Stachura M, Potekhin A, Przyboś E, Rautian M, Skoblo I, Tarcz S, "Identifcation of Paramecium bursaria syngens through molecular markers-comparative analysis of three loci in the nuclear and mitochondrial DNA.," Protist 163:671-685. https://doi.org/10.1016/j.protis.2011.10.009, 2012.

[81] Wang Y, Wang Y, Sheng Y, Huang J, Chen X, Al-Rasheid KAS, Gao S, "A comparative study of genome organization and epigenetic mechanisms in model ciliates, with an emphasis on Tetrahymena, Paramecium and Oxytricha.," Eur J Protistol 61:376- 387., 2017.

[82] Shakoori FR, Tasneem F, Al-Ghanim K, Mahboob S, Al-Misned F, Jahan N, Shakoori AR, "Variability in secondary structure of 18S ribosomal RNA as topological marker for identifcation of Paramecium species.," J Cell Biochem 115:2077-2088. https://doi.org/10.1002/jcb.24885, 2014.

[83] Przyboś E, Rautian M, Beliavskaia A, Sebastian T, "Evaluation of the molecular variability and characteristics of Paramecium polycaryum and Paramecium nephridiatum, within subgenus Cypriostomum (Ciliophora, Protista).," Mol Phylogenet Evol 132:296-306. https://doi.org/10.1016/j.ympev.2018.12.003, 2019.

[84] Tsukii Y, "Evolution of mitochondrial DNA in Paramecium caudatum," Jpn J Genet 69:307-319. https://doi.org/10.1266/jjg.69.307, 1994.

[85] Butzel HM, "Mating type mutations in variety 1 of Paramecium aurelia, and their bearing upon the problem of mating type determination.," Genetics 40:321-330, 1955.

[86] Taub SR, "The genetic control of mating type diferentiation in Paramecium.," Genetics 48:815-834 , 1963.

[87] Byrne BC, "Mutational analysis of mating type inheritance in syngen 4 of PARAMECIUM Aurelia.," Genetics 74:63-80, 1973.

[88] Tsukii Y, Hiwatashi K, "Genes controlling mating-type specifcity in

PARAMECIUM CAUDATUM: three loci revealed by Intersyngenic crosses.," Genetics 104:41-62, 1983.

[89] Singh DP, Saudemont B, Guglielmi G, Arnaiz O, Goût JF, Prajer M, Potekhin A, Przybòs E, Aubusson-Fleury A, Bhullar S, Bouhouche K, Lhuillier-Akakpo M, Tanty V, Blugeon C, Alberti A, Labadie K, Aury JM, Sperling L, Duharcourt S, Meyer E, "Genome-defence small RNAs exapted for epigenetic mating-type inheritance.," Nature 509:447-452. https://doi.org/ 10.1038/nature13318, 2014.

[90] Sawka N, Potekhin A, Singh DP, Grevtseva I, Arnaiz O, Penel S, Sperling L, Tarcz S, Duret L, Nekrasova I, Meyer E, "Evolutionary plasticity of mating-type determination mechanisms in Paramecium aurelia sibling species.," Genome Biol Evol 13:evaa258. https://doi.org/10.1093/gbe/evaa258, 2021.

[91] Fokin SI, Brigge T, Brenner J, Görtz HD, "Holospora species infected the nuclei of Paramecium appear to belong into two groups of bacteria.," Eur J Protistol 32:19-24, 1996.

[92] Tsukii Y, "Mating-type inheritance.," 著: *Paramecium*, Verlag, Springer, 1988, p. pp 59-69.

[93] Kodama Y, Fujishima M, "Endosymbiosis of Chlorella species to the ciliate Paramecium bursaria alters the distribution of the host's trichocysts beneath the host cell cortex.," Protoplasma 248:325-337. https://doi.org/10.1007/s00709-010-0175-z, 2011.

[94] McGrath CL, Gout JF, Doak TG, Yanagi A, Lynch M, "Insights into three whole-genome duplications gleaned from the Paramecium caudatum genome sequence.," Genetics 197:1417- 1428. https://doi.org/10.1534/genetics.114.163287, 2014.

[95] Natalia Sawka-Gadek, Alexey Potekhin, Deepankar Pratap Singh, Inessa Grevtseva, Olivier Arnaiz, Simon Penel, Linda Sperling, Sebastian Tarcz, Laurent Duret, Irina Nekrasova, Eric Meyer, "Evolutionary plasticity of mating-type determination mechanisms in Paramecium aurelia sibling species.," Genome biology and evolution. 13 (2). Epub 2020/12/15 https://doi.org/10.1093/gbe/evaa258 PMID:

33313646. PubMed Central PMCID: PMC7900874., 2021.

[96] Skotarczak B, Przyboś E, Wodecka B, Maciejewska A, "Random amplified polymorphic DNA fngerprinting as a marker for Paramecium jenningsi strains.," Folia Biol 52:117-124, 2004.

Acknowledgments

SCADS Inhibitor Kit were provided by Screening Committee of Anticancer Drugs; supported by Grant-in-Aid for Scientific Research on Innovative Areas, Scientific Support Programs for Cancer Research, from The Ministry of Education, Culture, Sports, Science and Technology, Japan.

This work was supported by JST SPRING, Grant Number JPMJSP2111.

I would like to acknowledge the following people for their cooperation in these studies especially.

Dr. Masahisa Watarai, Dr. Takashi Shimizu, Dr. Kenta Watanabe, and Ms. Akiko Imamura, Ms. Hiroko Kiyota, students in Laboratory of Veterinary Public Health, Joint faculty of veterinary medicine, Yamaguchi university.

Dr. Takuya Mizuno in Laboratory of Veterinary Clinical Pathology, Joint

faculty of veterinary medicine, Yamaguchi university.

Dr. Masato Tachibana in the NBRP, Yamaguchi university.

Dr. Akihiko Uda in Department of Veterinary Science, National Institute of Infectious Diseases, Japan.