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Mutual genetic alterations in the E. coli-T4 system

Seiko Hara

shara.laofu@gmail.com

Miyazaki International University

Isao Koike

The University of Tokyo

Research Article

Keywords: halo, nutrient decrease, competence, infection resistance, high-frequency genetic alteration

Posted Date: September 17th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-10781460/v1

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Additional Declarations: No competing interests reported.

Abstract

Turbid halos around the transparent plaques in *E. coli*-T4 plates are well known, while characteristics and mechanisms of the turbid halo formation are not well known. We showed that the original *E. coli* (oEc) was genetically altered to turbid halo-*E. coli* (hEc) to resist the original T4 (oT4) infection. A genetic agent included in the lysate of the oEc-oT4 infection caused this genetic alteration. Equally, a sub-strain of T4 which could infect hEc; anti-halo T4 (ahT4); was produced by incubations of stationary phase hEc, oT4 and specific fractions of oEc-oT4 lysate ultracentrifugation; another genetic agent. In batch cultures, the infectious characteristics of *E. coli* and T4 were genetically altered continuously and mutually. Up to halves of *E. coli* cells and T4 virions were genetically altered. The high frequency of genetic alteration in *E. coli*-T4 infection system may be incorporated in the phage-therapy and HGT.

Introduction

Bacteriophage T4 and *Escherichia coli* are the most well examined model of the pair of a virulent phage and its host, respectively (Birge 2010). As the definition of virulent phage, *E. coli* host cells are lysed by the infection of T4, this will produce a transparent plaque on the lawn of *E. coli* in the plaque formation process (Jurczak-Kurek et al. 2016, Ramesh et al. 2019). The margin between the lawn and the plaque forms a clear line at the early stages of plaque formation. However, it is well known that a turbid halo zone will appear around the initial clear plaque after a certain period (Cornelissen et al. 2011, Harper et al. 2014, Yan et al. 2014, Pires et al. 2016). This phenomenon itself has been described repeatedly, while the mechanisms of the turbid halo formation and the characteristics of the *E. coli* cells forming turbid halo have not been well reported. The phage derived enzymatic degradation of the bacterial cell wall was hitherto proposed as a possible mechanism for turbid halo formation (Cornelissen et al. 2011, Yan et al. 2014, Harper et al. 2014, Pires et al. 2016). If this is the case, the central clear plaque zone will increase its size continuously outward. The clear plaque, however, does not increase its size after the turbid halo formation (Cornelissen et al. 2011, Pires et al. 2016). Here, studies of plaque-forming plates and time series studies of batch cultures of *E. coli*-T4 systems indicated that the cells forming the turbid halo surrounding the clear plaque consisted of *E. coli* cells genetically modified from the original *E. coli* (abbreviated as oEc, hereafter) to a new strain which was resistant to the infection of the original T4 (abbreviated as oT4, hereafter); turbid halo-*E. coli* (abbreviated as hEc, hereafter). A genetic agent (abbreviated as ga, hereafter) included in the lysate of the *E. coli*-T4 infection produced this genetic reformation. Equally, a new sub-strain of T4 which could infect hEc; anti-halo T4 (abbreviated as ahT4, hereafter); was produced by the incubation of the stationary phase hEc, oT4 and another genetic agent (abbreviated as ga', hereafter) constituted of the specific fractions of the ultracentrifugation of lysate of the *E. coli*-T4 infection. Homologous processes repeated successively in batch cultures. The extremely high frequency of these genetic modifications and the simple required conditions are unique, and they may influence the processes of phage therapy (Kortright et al. 2019, nature microbiology editorial 2024) and of HGT (Hasegawa et al. 2018, de Sousa et al. 2023, Al-Gashgari et al. 2023).

Materials and methods

Strains:

The host bacteria studied were *Escherichia coli* (ATCC 11303) and the infective bacteriophage to the host was T4 (ATCC 11303-B4).

The preparation of E. coli cells and T4 virions:

The host bacteria, *E. coli*, was cultured with DifcoTM Nutrient Broth (NB) (peptone 5g, beef extract 3g, NaCl 3g, in 1l solution, pH 8). T4 suspension was obtained by the plate lysate method and the small-scale liquid culture (Sambrook and Russell 2001). In the plate lysate method, the virions were extracted by adding 2–3 ml of an electrolyte solution (TB+) including 100 mM NaCl, 5 mM $MgSO_4$ and 0.1 mM phosphate buffer (pH 7.5), incubated several hours to elute virions and finally filtrate the extract with 0.2 μm filter (Advantec AS020) to remove bacteria and bacterial debris. The suspensions of T4 virions were purified with ultracentrifugation and dialysis. For ultracentrifugation, crude bacteriophage particles were purified by isopycnic centrifugation through CsCl gradients (Sambrook and Russell 2001; Beckman XPN-90, SW32 rotor, 4 ℃, 24 h). Following ultracentrifugation, fractions of the specimens were collected from the surface to the bottom of the specimens to prepare ca. 20 fractions labeled numerically from the surface fractions, e.g. the fraction 1 (fr1), the fraction 2 (fr2), etc. The T4 virions were concentrated into fr14 and fr15. The chemicals used were special grade products from FUJIFILM Wako Pure Chemical Corp., Tokyo, Japan.

undefined

Plaque-forming unit (PFU) count, colony-forming unit (CFU) count and spot formation:
In PFU count, spot formation and colony count, Nutrient Broth with 1% agar for plates and 0.5% agar for top agars were used. Relevant dilutions of T4 and *E. coli* suspensions were spread on the agar plates for PFU and CFU counts, respectively. In the progress of T4 infection on *E. coli* lawn on an agar plate, the turbid halo developed around the transparent plaque every time. Accordingly, in figures of plaques and spots the development of the halo was not mentioned each time except for the case when the development of the halo was in question. The PFU values of T4 suspensions were enumerated frequently to confirm the activity of the T4 virion during the experiments.

The production of hEc and ahT4 in different concentrations of NB between the fresh and the stationary phase oEc:

In the genetic alterations of *E. coli* cells from oEc to hEc, specimens collected from close outside of a halo margin were examined. Specimens were collected during the development of the turbid halo and cultured in 100%, 10% and 1% strengths of nutrient broth, plated and the suspensions of oT4 and ahT4 phages were spotted.

For the production of hEc and ahT4 in liquid, each one % of the oT4 and oEc suspensions were inoculated in 100%, 10%, 1% and 0% of NB in TB+ solutions. At the case of original *E. coli* suspension, one % of oT4 was directly inoculated. The PFU values of these suspension mixtures were counted against oEc for total T4 production and against hEc for ahT4 production after being incubated for 6 hr in 36℃. For the oEc suspension, the stationary phase and the freshly growing *E. coli* suspensions were compared.

Mutual alterations of infectivities between E. coli and T4 in the time series specimens

In one ml of NB solution, 0.1 ml of the stationary phase *E. coli* suspension, ca. 10^8 CFU, and 10 μl of oT4 suspension, ca. 10^5 PFU, were inoculated and blended with a vortex mixer for ca. 10 seconds. To prepare the time series T4 specimens, each 10 μl of sub-sample was diluted in one ml of TB + at each 12 hours interval from 0h to 60h. About 0.5 ml of this time series specimens were filtrated with 0.2 μm filter (Advantec AS020). The time series *E. coli* specimens were prepared as the original time series suspension at each time was streaked on NB agar plate, after incubation specimens were isolated and cultured. The time series *E. coli* and T4 specimens were mutually spotted with oEc, hEc, oT4 and ahT4 as the controls. The sub-strains of *E. coli* and T4 collected at t hours in the time series specimens were expressed as nEc and nT4, respectively.

Genetic agents in the fractions of the lysates of the oEc-oT4 infection system

To identify the presence of the genetic agent (ga) in the oEc-oT4 lysate which could alter the genetic characteristics of *E. coli* from oEc to hEc, first, the oEc-oT4 lysates were filtrated with the 20 nm pore sized syringe top Anodisc to remove the T4 virions, second, 20 μl of diluted < 20 nm fraction of the lysates were spotted on the lawn of oEc in halo-forming stage, which was determined by the development of plaque-formation of a small spot of oT4 suspension on the plate, third, after one hour incubation, *E. coli* strains were collected with a tiny loop of a Nichrome-wire from the spot and streaked on the surface of NB plate. Developed colonies were cultured in NB. The infectivity of *E. coli* cells was examined with spotting oT4 and ahT4 suspensions.

The presence of a genetic agent (ga'), which altered the genetic characteristics of T4 from oT4 to ahT4, was identified among the fractions of the ultracentrifugation specimens of the oEc-oT4 lysate (fr) lighter than T4 virions, which were concentrated in fr14 and fr15. The fractions from fr1 to fr12 were divided into four groups. Each group included three fractions, e.g. the fraction group 1 (fg1) included fr1, fr2 and fr3; the fraction group 4 (fg4) included fr4, fr5 and r6, and so forth. To identify the fraction groups (fg) which composed ga', suspensions of stationary phase hEc were incubated with one or two fg for one hour, followed by the addition of NB and the mixtures were incubated for one hour. Then, add aliquots of oT4 suspensions, which were the time zero specimens of the time series samplings. Samples were taken periodically and plated on oEc and hEc plates to enumerate the PFU abundances of oT4 and ahT4.

Results

Characterization of turbid halo type *E. coli* cells (hEc) and a counter strain of T4 (ahT4)

Infection of T4 on the lawn of *E. coli* formed turbid halo rings around the central transparent plaques (Fig. 1). At the beginning of the infection process, all the original *E. coli* cells (oEc) were sensitive to the infection of the original T4 (oT4) and they were totally attacked and lysed by the infection, with the result that transparent central plaques formed had discrete lawn-plaque margins (Fig. 1a). However, after a certain period of the infection process, turbid halo rings were formed outside of the transparent plaques (Fig. 1b). The density of the turbid halo cells was uniform, and the gray value of the photograph of turbid halo ring was more than a half of the surrounding lawn area (Fig. 1b, c). The colony counts of *E. coli* extracted from nearly equal sized discs of the agar plate from the background bacterial lawn and the turbid halo zones revealed that the turbid halo zone harbored ca. a half of viable cells (46.1±1.9%, av. ±SE%, n=3). The margins between the lawn-halo and the halo-plaque were discrete (Fig. 1b, c). Based on the results mentioned above, to explain the formation of the turbid halo, we hypothesized that along with the lysis of oEc with oT4 infection and expansion of plaque area outward, a part of cells outside of the plaques were avoided from the infection of oT4 and remained without lysis, which formed the turbid halo ring (Fig. 1b).

The *E. coli* cells in stationary phase were not dead, but just dormant by the depletion of nutrients in the agar and showed the avoidance of T4 infection (Bryan et al. 2016) (Fig. 2a). This was a physiological and temporal avoidance of T4 infection. They started to multiply again once they were inoculated into a nutrient rich broth which recovered the original sensitivity to T4 (Fig. 2b left column spots). Without mentioning, oEc cells were sensitive to oT4 infection (Fig. 3a left column spots). However, the turbid halo forming *E. coli* cells, which were collected from a turbid halo, cultured, plated and spotted with T4 suspensions, showed the resistance against oT4 infections in physiologically active conditions (Fig. 3b left column spots). So, the physiological dormancy was not the cause of this avoidance of oT4 infection in *E. coli* cell forming turbid halo. This avoidance of oT4 infection was inherited to the descendant daughter cells; therefore it might be a genetically acquired resistance. This new strain of *E. coli* which formed the turbid halo and was resistant to oT4 infection was abbreviated as hEc.

To confirm the resistance of hEc was a genetically altered resistance to oT4, and not a genetic alteration to acquire a complete avoidance of T4 infection, a T4 strain which could infect hEc was searched. As hEc was formed in the process of plaque formation, the T4 strain which infected hEc also could be produced on the processes of *E. coli*-T4 infections. To find out a T4 strain infective to hEc, suspensions of multiple isolates of T4 were spotted on the plates of oEc and hEc (Fig. 3). Among the multiple isolates of T4 produced, an isolate showed the ability to infect both oEc and hEc (Fig. 3a, b right column spots). This isolate of T4 which could infect both oEc and hEc was abbreviated as ahT4. Accordingly, oEc was characterized as a sub-strain of *E. coli* which was susceptible to both oT4 and ahT4, while hEc was a sub-strain of oEc which was genetically altered to avoid the infection of oT4 but susceptible to the

infection of ahT4. Equally, oT4 was a sub-strain of T4 which could infect oEc, but could not hEc, while ahT4 could infect both oEc and hEc (Fig. 3). The oT4 and ahT4 virions used were isolated as monoclonal populations.

A genetic alteration of *E. coli* cells from oEc to hEc

The turbid halo type *E. coli* cells (hEc) were not formed at the beginning of the plaque formation processes, while after certain period of development of the transparent plaque, abruptly hEc appeared as the expansion of the T4-infection zone. These implied exudates from the transparent plaque might alter a part of oEc at the outside of the transparent plaque to hEc in the decreased nutrient conditions formed by the multiplication of the lawn bacteria. So, specimens were collected from a series of three points close outside of a halo margin, i.e. less than 1 mm from the margin (N), ca. 1 mm from the margin (M), and ca. 2 mm from the margin (A), during the turbid halos were developing (Fig. 4, left end). Collected *E. coli* cells were cultured in 100%, 10% and 1% strengths of NB overnight (vid. the section after next). Cell suspensions were plated and spotted with suspensions of oT4 and ahT4 on the left and the right columns, respectively (Fig. 4). The labels of photos indicate, e.g. M1: cells were collected from point M in the left end photo, cultured in 1% strength NB, plated, and spotted with suspensions of oT4 and ahT4. N100 and M100 showed confluent lysis (Fig. 4, N100). The *E. coli* cells in N10, N1 and M10 were resistant to both oT4 and ahT4 (Fig. 4, M10). The *E. coli* cells in M1 showed resistance to oT4, while they were susceptible to ahT4, or they showed the same infectivity as hEc (Fig. 4, M1). All the specimens collected from point A were susceptible to both oT4 and ahT4, or they were homologous to oEc (Fig. 4, A1). These results indicated that oT4 diffused from clear plaque to N and M (N100, M100), genetic agents (ga) which altered oEc to resistant to oT4 or resistant to both oT4 and ahT4 also diffused from clear plaque to N and M (N10, N1, M10, M1), this ga did not work when the concentration of NB was high (N100, M100), while they altered the susceptibility of oEc in low concentration of NB (N10, N1, M10, M1), the ga altered oEc to resistant to both oT4 and ahT4 in samples at N or higher concentrations of or longer exposure to the ga (N10, N1), the ga altered oEc to hEc in samples at M or lower concentration of or shorter exposure to ga in low concentration of NB (M1 vs. M10), and both oT4 and the ga did not diffused to A, yet (A100, A10, A1).

E. coli cells collected near but outside of the turbid halo margin after the saturation of turbid halo development were resistant to the infections of both oT4 and ahT4. The populations of oEc were cultured in 10% and 1% strength of NB for overnight. These populations were sensitive to the infection by oT4, which indicated that the simple incubations of oEc in the diluted NB did not produce hEc population and the infectivity of oEc were not altered.

These abovementioned results indicated that a genetic agent (ga) which altered *E. coli* cells from oEc to hEc in low nutrient condition was included in the exudates from the full-lysed plaque. To confirm the presence of ga in the oEc-oT4 lysate, 20 µl of 1% or 10% dilutions of <20 nm oEc-oT4 lysate were spotted on the oEc lawns at halo-forming stage and incubated for one hour. Bacterial strains were collected from the spot areas, streaked on the surface of the NB plates and isolated colonies were cultured. The

infectivity of the cultured cells was examined with spotting of oT4 and ahT4 suspensions on the culture plates (Table 1). The *E. coli* populations in the spots, which were originally susceptible to oT4 infection, were genetically modified to resistant to oT4, i.e. hEc. At the case of 1% lysate 2, the *E. coli* cells in the spot were still susceptible to oT4, however, these cells were altered to hEc after four hours incubation. These results indicated that ga was included in <20 nm fractions of oEc-oT4 lysate.

The resistance of hEc against oT4 can take place pre- or post-infection. To identify the timing of the resistant process, aliquots of oT4 suspensions were incubated with hEc suspension. PFU counts of oT4 did not change for three days. The original abundances of oT4 and hEc were ca. $4x10^8$ and ca. $2x10^9$, respectively. Based on the particle-particle encounter model, when T4 virions and *E. coli* cells were treated as balls of diameters as 0.1 μm and 1 μm, respectively, all the oT4 virions encountered with *E. coli* cells within one minute. The constant abundance of oT4 for three days indicated that the oT4 populations encountered with hEc while they did not adhere with hEc. This indicated the resistance of hEc against the infection of oT4 occurred at the pre-infection process or the repulsion of the infection.

A genetic alteration of T4 virions from oT4 to ahT4

As described above, populations of hEc were producedin suspensions of samples collected just near the outside of developing halo regions and incubated in diluted NB (Fig. 4). On some of these hEc plates with longer incubations, plaques of individual phages, which could attack hEc, were formed (see also the next section). So, the conditions to form the anti-hEc T4 (ahT4) in diluted NB suspensions were examined.

Small pieces of agar were collected from close outer margin of turbid halos of oEc-oT4 pairs during their development and incubated in full, 10%, 1% and 0% strengths of NB for one day (vid. next section). These suspensions were spotted on oEc and hEc (Fig. 5). Spots of suspensions in all the concentrations of NB except for the incubation in 0% NB formed clear plaques on the lawns of oEc (Fig. 5 left). While, on the lawns of hEc, plaques were formed only at spots of incubations with 10% and 1% NB (Fig. 5 right). The mixed suspension of the oEc, diffused oT4 and the lysate of oEc-oT4 system collected beside the turbid halo developing stage and incubated in the diluted nutrient conditions for one day produced ahT4 virions. To confirm the growth stage of *E. coli* and nutrient concentrations sensitive for the formation of ahT4, oEc suspensions in the freshly prepared and the stationary phase cultures were inoculated with oT4 suspensions in different nutrient concentrations (Fig. 6). In general, the production of ahT4 with the freshly prepared oEc inoculations decreased with a decrease of NB concentrations (Fig. 6a), while that with the stationary phase oEc inoculations ahT4 production increased with the decrease of NB concentrations and in the case of 1% NB, the productions of oT4 and ahT4 were a half-and-half mixture (Fig. 6b). The productions of ahT4 within the freshly prepared oEc were several orders lower than that of stationary phase oEc suspensions in low nutrient concentrations. These results indicated that the lysate of oEc-oT4 system included genetic agents (ga') which alteredT4 virions from oT4 to ahT4 under the stationary phase oEc-oT4 system.

To identify the components of ga' among the fractions of the ultracentrifugation specimens of the oEc-oT4 lysate lighter than T4 virions, these fractions were divided into three fraction groups (fg) of fg1, fg4 and fg7 arranged from lower to higher densities. One or two fg were incubated with suspensions of the stationary phase hEc for one hour, followed by the addition of NB, incubated for one hour, then, added aliquots of oT4 suspensions. In inoculations of sole fg or fg4-fg7 pair the abundances of oT4 were constant as the initial inoculation for over one day and no ahT4 appeared. In inoculations of fg1-fg4 or fg1-fg7 pairs, abundances of oT4 were constant at the initial several hours. After this, hEc was altered to susceptible to oT4, hereafter expressed as hEc', and oT4 started to multiply. Synchronous with the multiplication of oT4, ahT4 was produced and increased its abundance which replaced oT4 (Table 2). When the hEc populations in the above processes were replaced with oEc populations, no ahT4 was produced. Accordingly, hEc' was not oEc, and the essential components of ga' were included in fg1-fg4 and fg1-fg7 pairs. The target cell of ga' was hEc, not oEc. The longer, e.g. one day, incubation of the stationary phase oEc populations with fg1-fg4 or fg1-fg7 pairs altered oEc populations to be resistant to both oT4 and ahT4.

Mutual genetic alterations of *E. coli* and T4 in time series batch cultures

The productions of hEc and ahT4 at certain moments of incubations or pre-determined combinations were examined in the previous sections. Here, the successive genetic alterations of *E. coli* and T4 in the liquid culture conditions were described (Table 3, Fig. 7).

Aliquots of oEc and oT4 suspensions were inoculated into a NB batch culture. Subsamples of *E. coli* and T4 were collected every 12-hour intervals till 60 hours. The sub-strains of *E. coli* and T4 obtained after *n* hours incubations are expressed as nEc and nT4, respectively. These subsamples, and oEc, hEc, oT4 and ahT4 as controls, were mutually spotted (Table 3, Fig. 7). The oT4 spot on oEc plate was smaller than other spots because the density of oT4 was diluted one hundredth of the original batch culture of oEc and oT4. In Table 3, the dark gray square, the light gray square and the white square indicated confluent lysis, partial infection and no infection, respectively (Fig. 7). The partial infection in Fig. 7 or the light gray squares in Table 3 did not appear at the beginnings of the plaque developments, while they gradually showed according to the elongation of the incubations.

The infection pattern of Table 3 could be divided into three sections; controls and time series specimens, and the latter was divided into pre-24h and post-24h specimens which were corresponded with before and after the genetic alteration of T4, respectively. The 0Ec and 12Ec populations accorded with oEc and hEc populations, respectively. Both 0T4 and 12T4 populations accorded with oT4 population. After 12h incubation, oEc altered to hEc, while, oT4 did not alter its genetic characteristics, yet. From 24h incubation, *E. coli* and T4 altered their infective characteristics mutually. 24Ec able to be attacked and lysed only by the 24T4 and 24T4 showed infectivity to all the sub-strains of *E. coli* examined (Table 3, Fig. 7e). *E. coli* sub-strains after 24h incubation; 24Ec, 36Ec, 48Ec and 60Ec; were susceptible to the T4 sub-

strains from 24h to the time in question, while they were resistant to the T4 sub-strains after the time in question. On the other hand, the T4 population after 24h incubation showed the infectivity against the *E. coli* sub-strains at the time in question and after, while they were un-infective to the *E. coli* sub-strains before the time in question (Table 3, Fig. 7). The genetic alterations of T4 in batch cultures continued afterward and the composition of T4 populations became stabilized with dominant, over 99%, oT4 and minor, less than 1%, ahT4 mixtures.

In some cases, 24Ec acquired resistance against 24T4 which resulted in resistance against all the sub-strains of T4. This *E. coli* population maintained total resistance thereafter continuously.

The succession of viral abundance (PFU) supported the spot pattern results (Table 3). PFU ofoT4 in the batch culture increased rapidly after the inoculation. The abundances were saturated after 12h and maintained nearly the same abundance thereafter. Contrarily, PFU of ahT4 did not increase until 12h incubation, while it rapidly increased after 12h and maintained a similar abundance afterward. The PFU of sub-strain ahT4 includes the sub-strains of 24T4, 36T4, 48T4 and 60T4 (Table 3, Fig. 7).

In the *E. coli* suspensions without T4 inoculations, the abundances rapidly increased by around 24h and thereafter the abundances stabilized (Fig. 8, black circles). The *E. coli* suspensions with T4 inoculations decreased their abundance in the specimens collected after 6h (Fig. 8). After that, the abundance increased to more than three times the initial inoculation at 12h, followed to decrease its abundance at 24h, and the abundance stabilized at the level of twice the initial inoculation after that (Fig. 8).

Discussion

Turbid halo type E. coli cells

Development of hazy rings, turbid or opaque halos surrounding a clear plaque, is well known (Fig. 1) (Yan et al. 2014, Harper et al. 2014, Pires et al. 2016). Besides the formation of the turbid halo, the mechanism of turbid halo formation and the characteristics of the cells forming turbid halo were not well understood (Cornelissen et al. 2011, Yan et al. 2014, Harper et al. 2014, Pires et al. 2016). The mechanism hitherto proposed was the degradation of the bacterial cell wall by phage derived enzymes (Cornelissen et al. 2011, Yan et al. 2014, Harper et al. 2014, Pires et al. 2016). If this is the sole mechanism, halo will be formed with a continuous gradation around the central clear spot which will increase its size continuously outward. While the bacterial densities in halo area are uniform (Fig. 1b, c), borders between clear plaque-halo and halo-lawn are discontinuous and the size of the clear plaque does not increase once the halo starts developing (Fig. 1) (Cornelissen et al. 2011, Pires et al. 2016). We found that transparent plaque was formed by the lysis of oEc by oT4 at the beginning of plaque formation, while after a certain period of incubation; oEc-oT4 infection system produced oT4-resistant hEc in the outside oEc lawn of the transparent plaque where the nutrients were decreased by the growth of oEc population (Fig. 4). oEc altered to hEc by the genetic agent (ga) produced with *E. coli*-T4 infection system in low nutrient environment (Fig. 4, Table 1). These results implicated depletion of nutrient induced competency in *E. coli* cells (Baur et al. 1996, Chen and Dubnau 2004, Hasegawa et al. 2018, Sun

2018, Haudiquet et al. 2022) which triggered the intake of ga into *E. coli* cells, and oEc was genetically altered to hEc (Fig. 4, Table 1).

Successive genetic alterations of E. coli and T4

The genetic alteration of *E. coli*-T4 system was not stopped at the formation of hEc (Figs. 1, 3, 4, Table 1) but continuously forming the sub-strains with mutual resistant-infection series (Tables 1, 3, Figs. 5–7, 9). This means that the infection between *E. coli* and T4 is not a simple one side attack, but a highly dynamic process to change their genetic offence-defense system continuously (Table 3, Fig. 7).

In the time series specimens of mutual infections after 24h incubations (Table 3, Fig. 8), at the beginning of the plaque formations, confluent plaques, dark gray squares in Table 3, appeared. In the plates of elongated incubation, partial infection plaques, light gray squares in Table 3, gradually appeared from the spots of next previous specimen to the spots of one more previous specimen. The genetic alterations of *E. coli* cells might keep proceeding in the spots of a plate, and these partial infections might be the a posteriori results of the continuous genetic alterations in the agar of the plates.

At 24h incubation, *E. coli* produced a sub-strain 24Ec with a wide resistance. In response to that, 12Ec produced a new sub-strain 24T4 which could infect 24Ec. Then, 24Ec produced a new sub-strain of T4, 36T4 which had lost infectivity to 24Ec, which induced a new sub-strain of *E. coli*, 36Ec which was susceptible to 36T4, and so on. In this process, in general, *E. coli* altered new sub-strain of T4 which lost infectivity to the previous strain of *E. coli*, while *E. coli* itself did not change its susceptibility to being resistant to the previous T4. Accordingly, the susceptibility of *E. coli* altered wider; on the other hand, the infectivity of T4 turned out to be narrower (Table 3, Fig. 7). Because the sampling interval was 12 hours, the genetic alteration at each 12h segment was described here, while this did not exclude the possibility that the genetic alterations were continuous or more segmentalized.

Based on the pattern of the infections between *E. coli* and T4 (Table 3), ahT4 was equivalent to 36T4. The derivation of ahT4 could be an isolate taken after ca. 36h incubation of a batch culture of *E. coli* and T4. At the formation of ahT4 from oEc-oT4 pair, the required process was first, the alteration of oEc to hEc by the intake of ga, second, the alteration of hEc to hEc' by ga' composed of components in fg1 and fg4 or fg1 and fg7, third, infection of oT4 to hEc', and last, the production of ahT4 by hEc' (Table 2, Fig. 9). According to the genetic alterations of *E. coli* and T4 in Table 3 after 24h, the ahT4 in Table 2 corresponded with 24T4 in Table 3. This process indicated the reason why ahT4 appeared after the production of hEc in a batch culture (Table 3, Figs. 7, 9). Kuo et al. 1971 indicated that the fractions of the ultracentrifugation specimen of *E. coli*-T4 lysate lighter than T4 virions included the free DNA at the lighter fractions and the tails at the heavier fractions. Accordingly, fg1 might include DNA fractions and fg4 and fg7 might include tails of T4. If this is the case, free DNA and tails are the essential components of ga'.

The difference between the cultures of the infection pair *E coli*-T4 in agar plates and in batch culture media was the migration abilities of the components. In the liquid media, all the components could move

freely, and reactions proceeded homogenously in the media. Contrarily, in an agar plate, the diffusion of *E. coli* cell was restricted by the network of agar molecules, and the positions of *E. coli* cells were nearly fixed, while T4 virions, ga and ga' could move in the agar (Fig. 4). The formations of plaques and turbid halos depended on these differences of diffusion speeds, e.g. in the turbid halo formation, first ga diffused outward and gradually altered oEc to hEc, then, oT4 diffused and lysed the un-altered oEc. The difference of diffusion rates between ga and oT4 was constant, so, the ratio of lysed oEc and hEc might also be constant. This could be a mechanism to produce the uniform turbid halo area (Fig. 1). In case, T4 virions had higher diffusion speeds than ga, the turbid halo could not produce, only the transparent plaques expanded.

Occasionally, *E. coli* populations which acquired wide-range resistance against the infection of multiple sub-strains of T4 appeared, e.g. near the outer margin of halo (Fig. 4) and stationary phase oEc cells incubated with fg1 and fg4 or fg1 and fg7 longer, e.g. one day. *E. coli* cells passed relatively long exposure to the higher concentration of lysate might acquire wide resistance. Exposure to different components of the lysate for different periods determined the fates of *E. coli* in different genetic traits, which also produced T4 with different genetic characters.

In the cultures of *E. coli* with and without T4, the abundance of the stationary phase *E. coli* with T4 was lower than but ca. 10% of that without T4 (Fig. 8). The attacks of T4 did not wipe out the *E. coli* populations. This can be explained as, oEc was modified to hEc (Fig. 7), which escaped from oT4 attack and increased the abundance (Fig. 8, white circles, 12h). Then, the mutual genetic alterations of both *E. coli* and T4 continued (Table 3, Fig. 7), and the abundances of *E. coli* and T4 were maintained relatively constant levels even though the sub-strains of both *E. coli* and T4 were continuously altered (Table 3, Fig. 8).

The offense and defense systems between bacteria and phages can be divided into pre- and post-infection systems. Previous studies are classified in the post-infection systems, e.g. CRISPR-Cas (Makarova et al. 2020), anti-CRISPR process (Camara-Wilpert et al. 2023) and restriction system (Kang et al. 2025). The constant PFU values of oT4 in suspensions of hEc for considerable period (section of "A genetic alteration of *E. coli* cells from oEc to hEc", Table 2) indicated that oT4 did not conjugate with hEc and the resistance of hEc against oT4 infection was established by blocking the conjunction of oT4. The present anti-phage defense system was counted in a pre-infection defense which is an autonomic genetic alteration of *E. coli* induced by the acquisition of the genetic agent produced by own *E. coli*-T4 infection system.

Equal with oEc-oT4 pair, the infection of ahT4-hEc and ahT4-oEc pairs formed the transparent central plaques and surrounding turbid halo rings. Not only *E. coli*-T4 pair, but the turbid halo forming type plaque formation is widely known among host bacteria-phage pairs (Cornelissen et al. 2011, Harper et al. 2014, Yan et al. 2014, Pires et al. 2016). If these turbid halos are formed by equal mechanism of genetic alteration as oEc-oT4 pair, homologous but different series of genetic alterations between the host bacteria and phage might widely distribute among the turbid halo forming host bacteria-phage pairs.

The large-scale and high-rate genetic alterations of *E. coli* and T4 in a short time described above could not be attributed to the results of the genetic mutations (Drake et al. 1998). Apparently, these genetic alterations might be associated with the processes of the horizontal gene transfer (HGT) (Sun et al. 2013, Hasegawa et al. 2018, Al-Gashgari et al. 2023, de Sousa et al. 2023). The cellular DNA released by the phage attack could contribute to natural transformation (Sun 2018). However, the plain difference between the conventional HGT and the present genetic alteration was the genetic agent causing the genetic modification being derived from outside of the systems in question in the former case (Sun et al. 2013, Hasegawa et al. 2018, Al-Gashgari et al. 2023, de Sousa et al. 2023), while it was derived within the same organisms in question in the latter case. In addition, the stationary phase (Fig. 6), the low nutrient condition (Figs. 4–6) plus the lysate of *E. coli*-T4 infection system (Figs. 4–6) are a new combination of the key factors for the acquisition of competence and genetic alteration (Table 3, Figs. 4–7). Accordingly, the large-scale and high-rate genetic alteration described here can be new system details of which need to be elucidated. Also, the processes may have potential for the efficient HGT in bacteria (Hasegawa et al. 2018, de Sousa et al. 2023, Al-Gashgari et al. 2023).

Competition might apply to explain the motive force for the mutual genetic alterations between *E. coli* and T4 described here. In the time series examinations (Table 3, Fig. 7), this might be the case at the productions of the sub-strains of *E. coli* and T4 in 24h incubation which could escape from the attack from and could attack all the other sub-strains of T4 and *E. coli* described here except for each of the 24h sub-strains, respectively. If competition is the rule of the genetic alterations of *E. coli* and T4 described here, the populations of *E. coli* might produce a sub-strain which could escape from the 24h sub-strain of T4. However, the real genetic alterations showed opposite direction, i.e. sub-strains of *E. coli* became wider sensitivity ranges, and sub-strains of T4 reformed to narrower infectivity ranges. These genetic alterations did not indicate competition to exclude each other; rather it could be benefit for each other to seek a condition to escape the extinction of one or both to maintain the abundances of the populations (Table 3, Figs. 7, 8).

In recent years, phage therapy has attracted more attention as a renewed approach to combat antibiotic-resistant bacteria (Kortright et al. 2019, Pirnay et al. 2024, Cui et al. 2024, Sawa et al. 2024, Arer and Kar 2025). There are still yet to be solved difficulties like bacterial phage resistance (nature microbiology editorial: https://doi.org/10.1038/s41564-024-01733-7). The present results about the control of the production of anti-counterpart genetic alterations with the growth phases and the nutrient conditions may be incorporated into the design of a phage therapy to increase its effectiveness. Here, we showed that an extremely high frequent genetic alteration occurred under ordinary culture conditions with ordinary culture stuff. This implicates homologous genetic alteration processes might take place with other host-phage pairs and other genetic traits, also might proceed in natural environments. The mechanism and the scope of the present pre-infection anti-phage defense system and following genetic alteration system have yet to be elucidated.

Declarations

Compliance with Ethical Standards

Ethics approval: Not applicable

Consent of publication: Not applicable

Availability of data and materials: Data and materials can be shared.

Competing Interest: The authors declare that they have no competing financial and non-financial interests.

Funding: The research was supported by the Cooperative Program (No. 119, 134, 2015) of Atmosphere and Ocean Research Institute, The University of Tokyo to S.H.

Author Contributions: S.H., Conceptualization, analysis and interpretation of data and writing original draft preparation; I.K., Analysis, interpretation of data, preparation of manuscript and funding.

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Tables

Tables 1 to 3 are available in the supplementary files section.

Figures

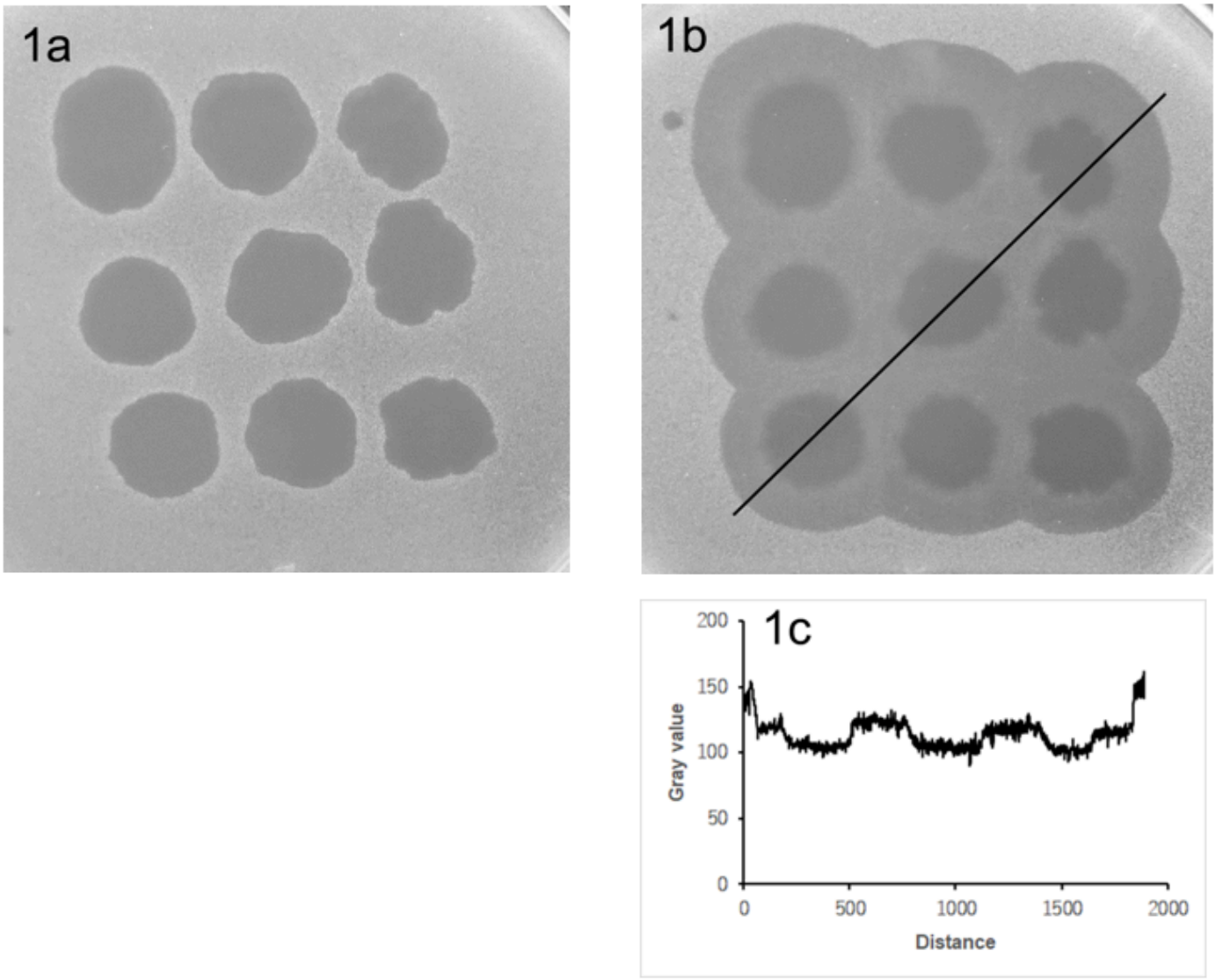


Figure 1

Plaques of bacteriophage T4 on a lawn of *E. coli*. After the development of transparent plaques (Fig. 1a), turbid halos expand surround the central transparent plaques (Fig. 1b). The line plot of the gray value along a line shown in Fig. 1b indicates a constant gray value in the halo zone (Fig. 1c).

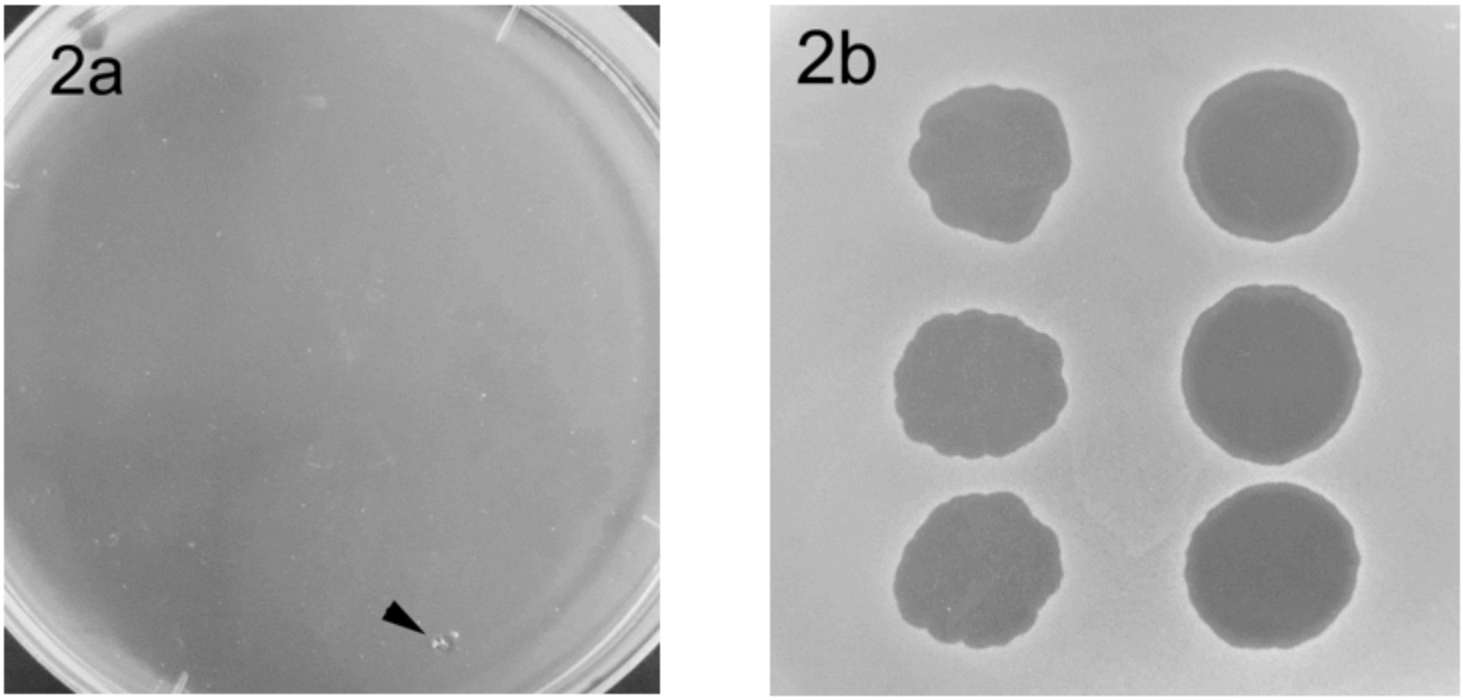


Figure 2

Infectivity of the stationary phase *E. coli*against oT4 and ahT4. After incubation of a lawn of oEc for 18 hrs, oT4 and ahT4 strains of T4 phages were spotted on the left and the right columns, respectively (Fig. 2a). No plaque was formed. Following the spotting, a specimen of *E. coli* (arrowhead) was collected, incubated in the full-strength NB, plated and oT4 and ahT4 strains of T4 phages were spotted on the left and the right columns, respectively (Fig. 2b). Both strains formed clear plaques.

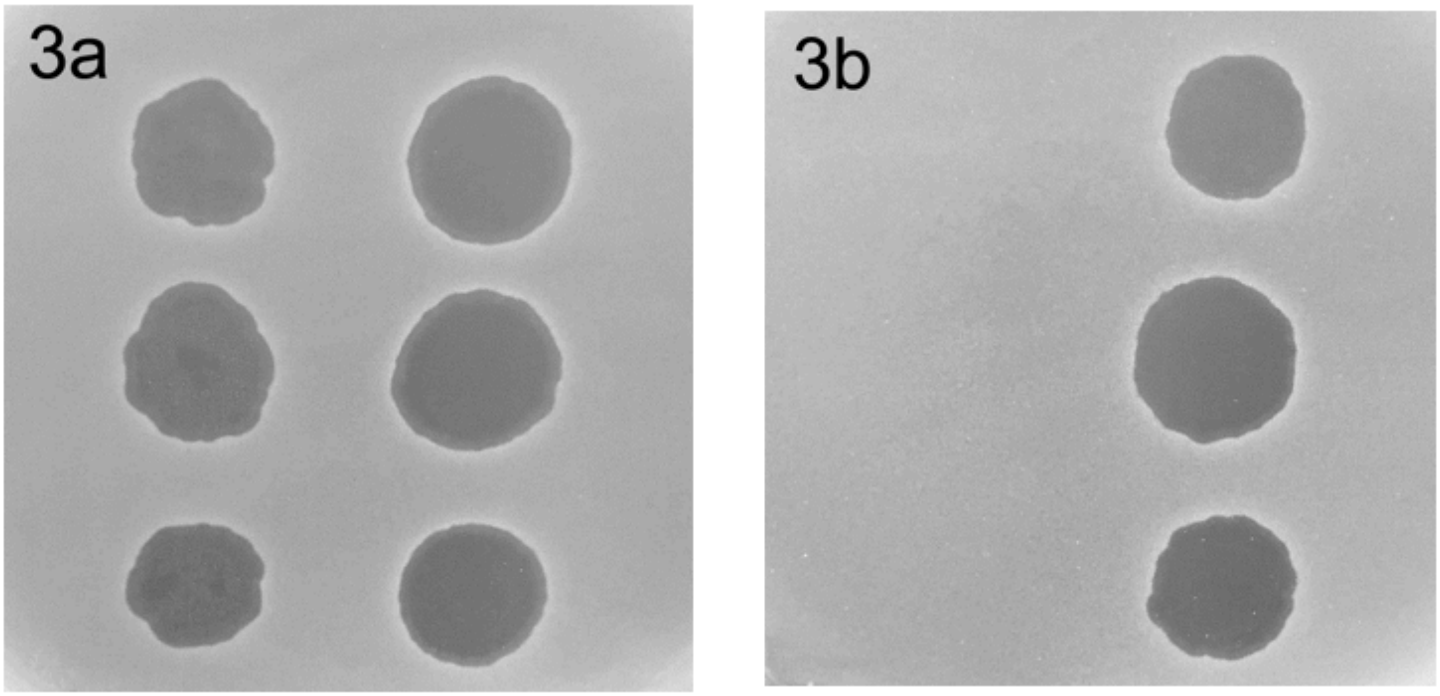


Figure 3

Comparison of the susceptibilities of the original *E. coli*, oEc (Fig. 3a) and the turbid halo *E. coli*, hEc (Fig. 3b) against oT4 and ahT4. oT4 and ahT4 strains of T4 phages were spotted on the left and the right columns, respectively. oT4 did not form plaques on the lawn of the hEc, while ahT4 could do so on the lawn of hEc (Fig. 3b).

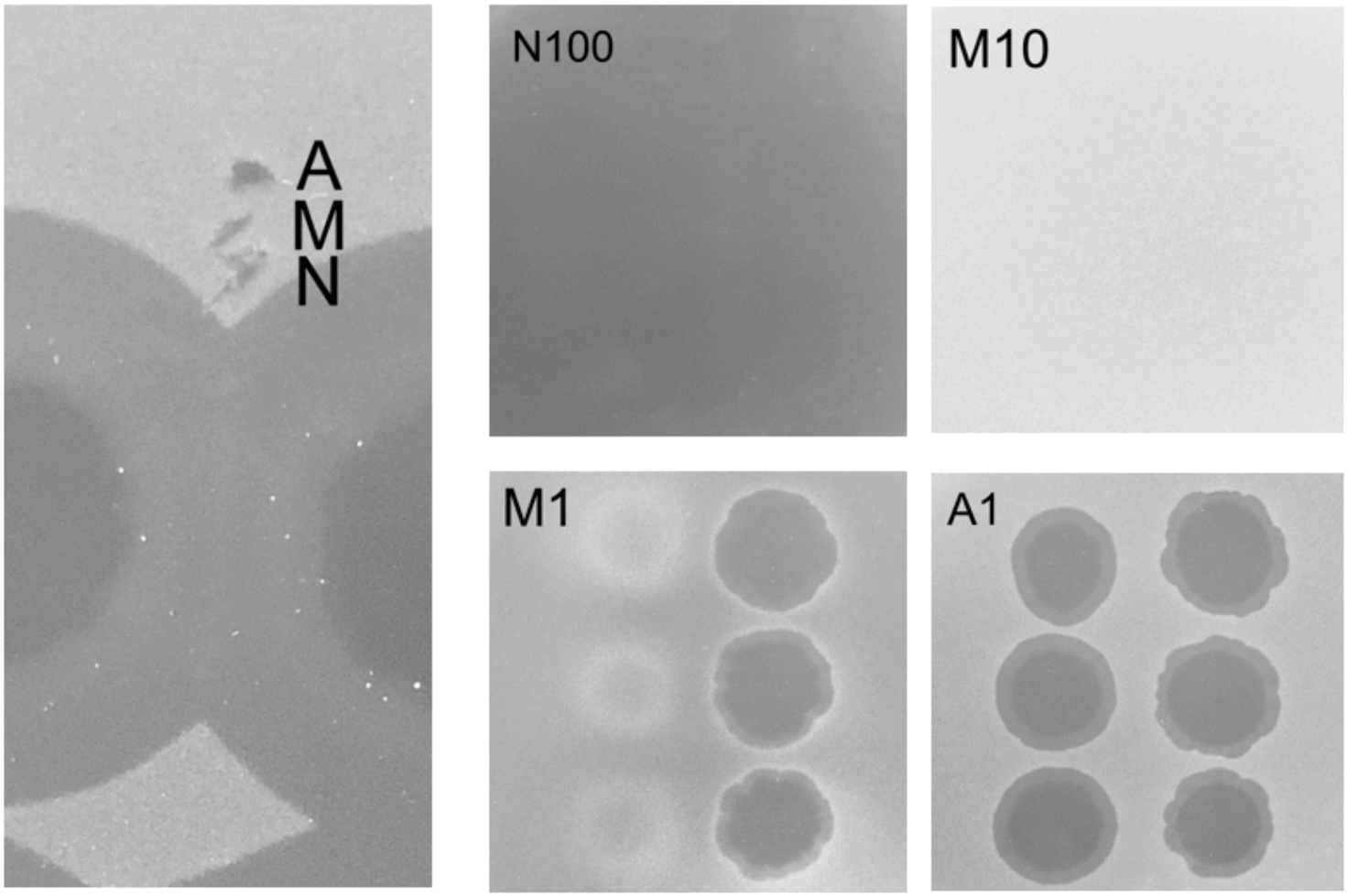


Figure 4

Infectivity of *E. coli* cells collected from close outside of a halo margin; N: less than 1 mm from the margin, M: ca. 1 mm from the margin, A: ca. 2 mm from the margin. Cells were collected during the turbid halo increasing its size, and cultured in 100%, 10% and 1% strengths of nutrient broth. Cultured cell suspensions were plated and oT4 and ahT4 phages were spotted on the left and the right columns, respectively. The label of a photo indicates, e.g. M1: cells were collected from M point in the left end picture, cultured in 1% strength nutrient broth, plated, and spotted suspensions of oT4 and ahT4.

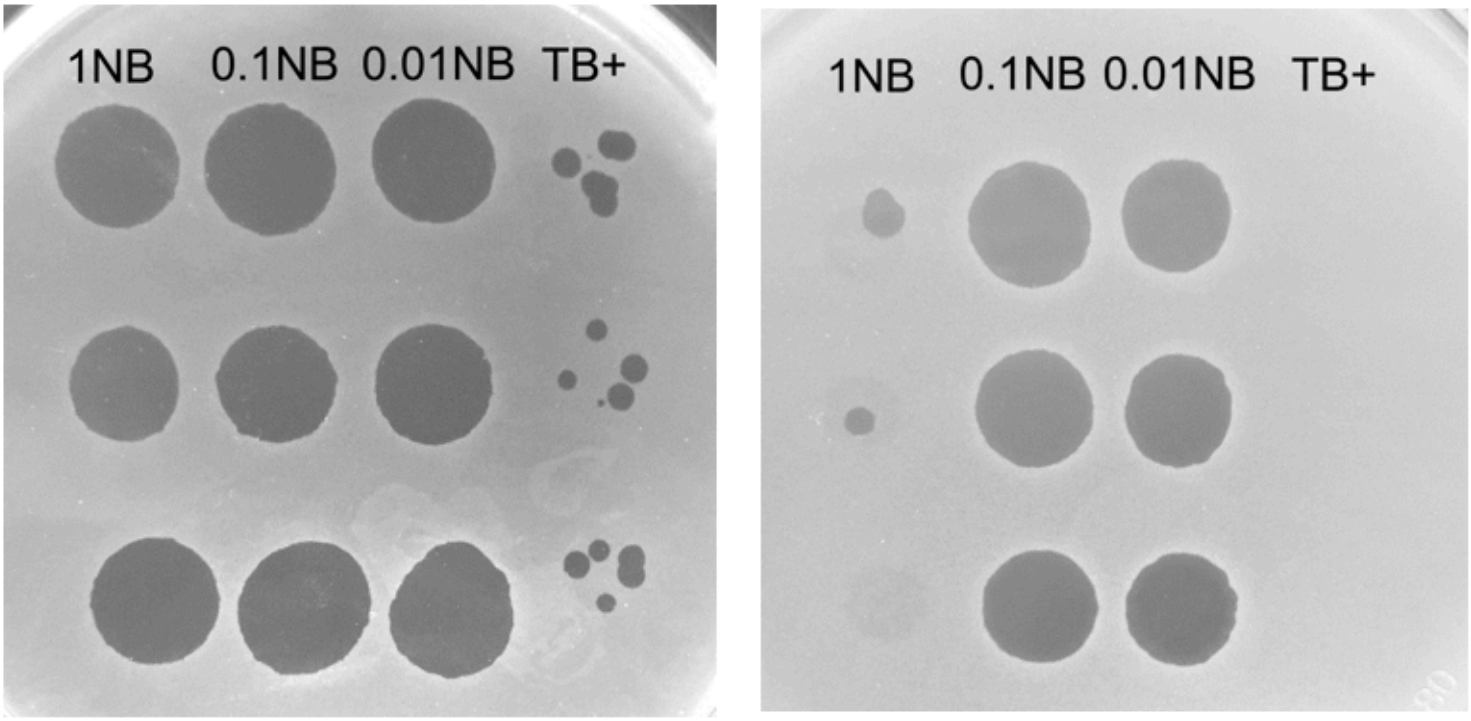


Figure 5

Infectivities of T4 virions in suspensions of samples collected from close outside of an oT4-halo margin and incubated in full, 10% and 1% strengths of nutrient brothand TB+. Samples were collected during the halo increasing its size. Phagesuspensions werespotted on oEc (left) and hEc (right) lawns.

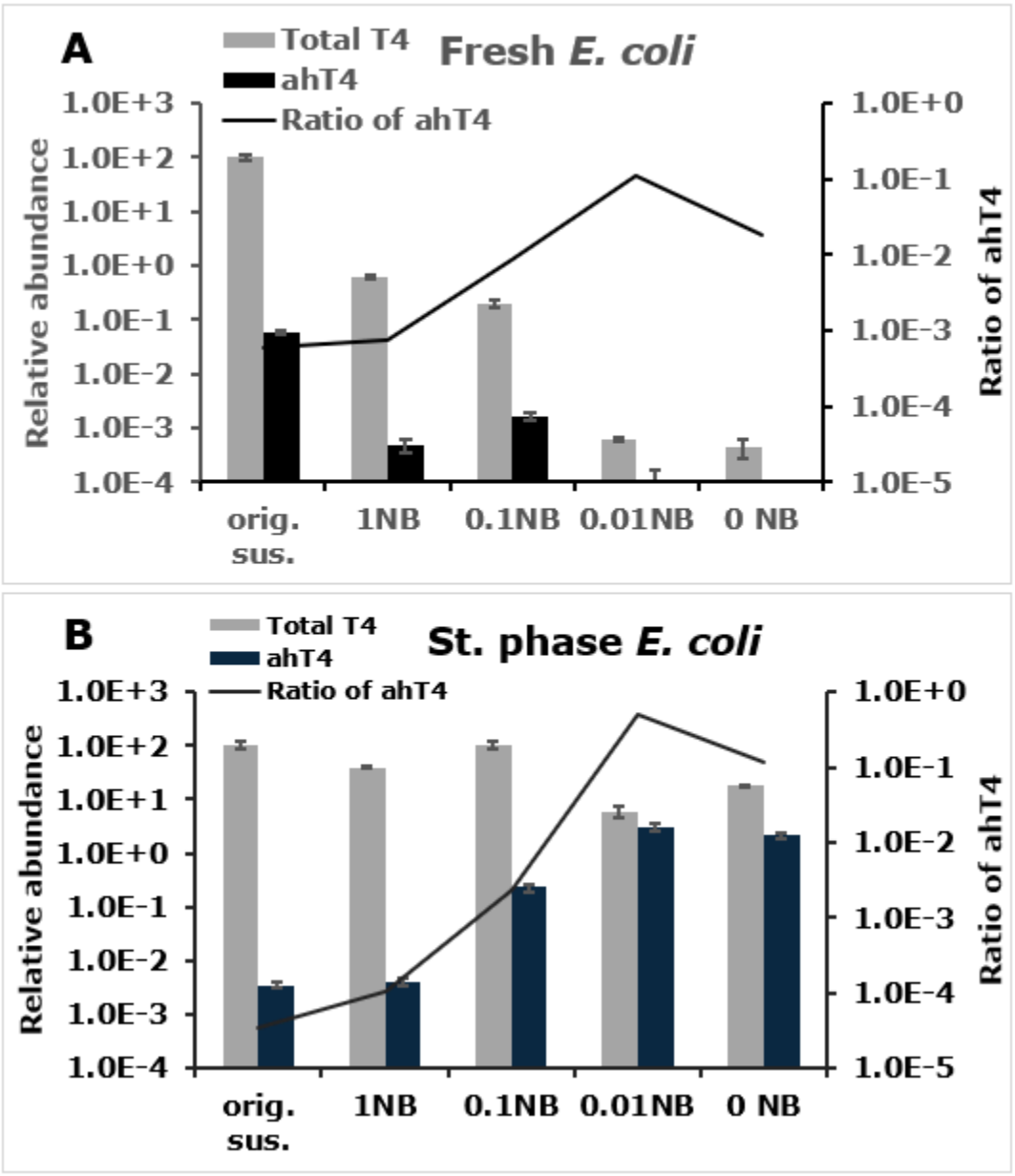


Figure 6

The production of ahT4 in liquid cultures of oEc-oT4 in a set of NB dilutions with the fresh (A) and the stationary phase (B) oEc. The PFU values of the original oT4 were adjusted as 100.

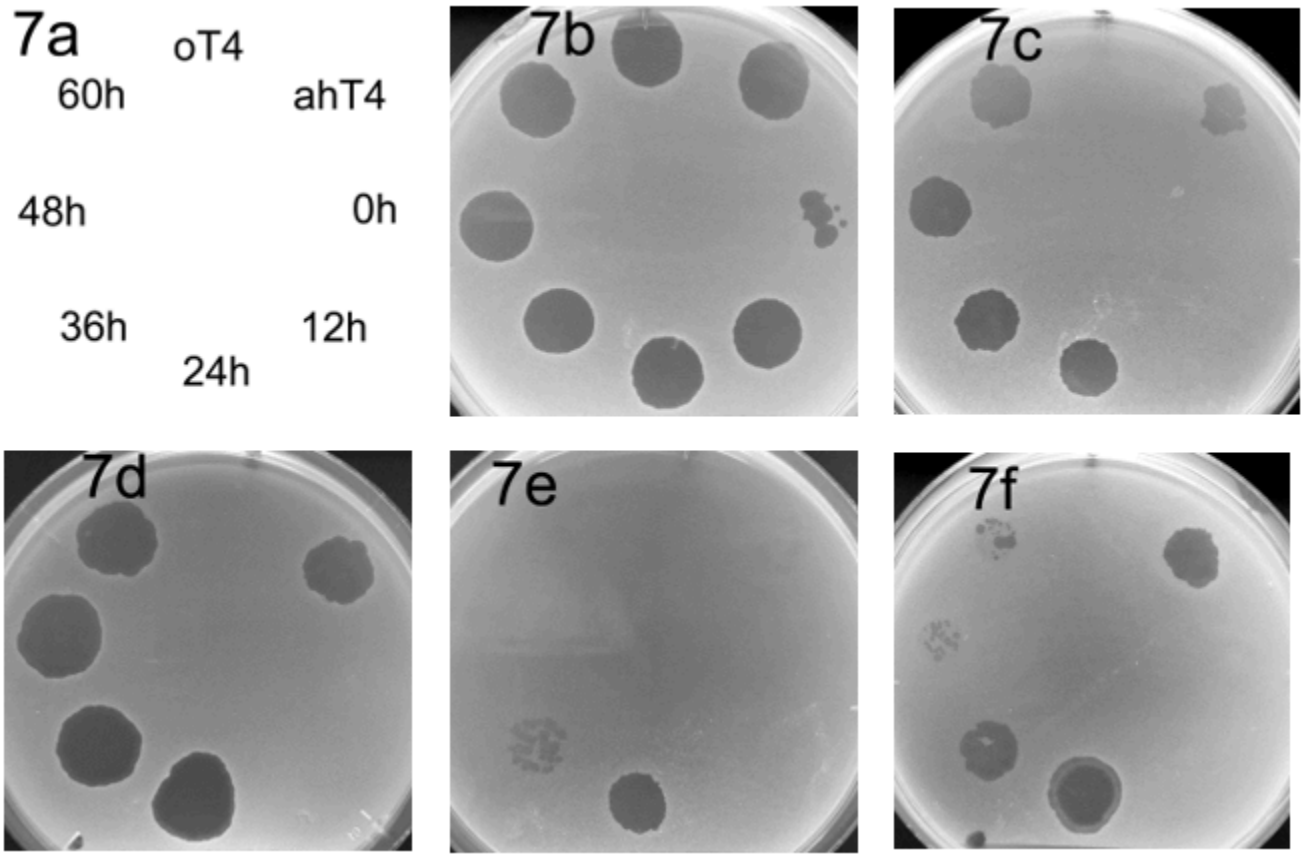


Figure 7

The mutual spotting of T4 suspensions on the lawns of *E. coli* of the time series specimens. Kinds of T4 spotted are shown in Fig. 7a. The types of *E. coli* in the lawns of the agar plates are oEc (Fig. 7b), hEc (Fig. 7c), 12 hours incubation (Fig. 7d), 24 hours incubation (Fig. 7e) and 36 hours incubation (Fig. 7f).

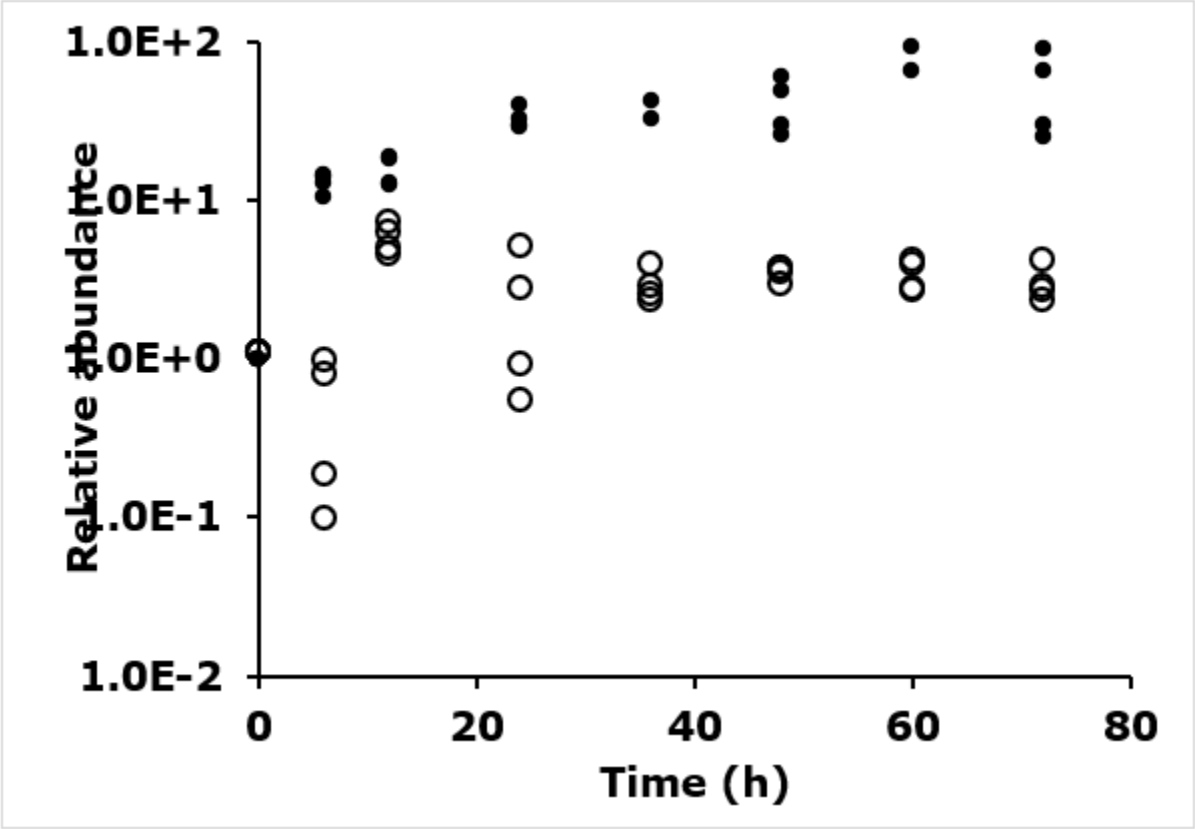


Figure 8

Growth of *E. coli* without T4 (black circles) and with T4 (white circles).Results of two times of pair experiments were plotted.

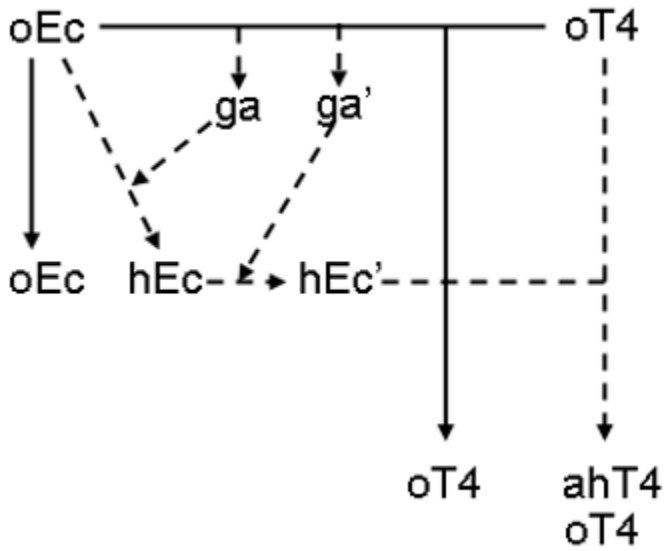


Figure 9

A scheme of the oEc-oT4 infection processes including the genetic alteration events. Solid line: conventional infection processes, dashed line: processes of the genetic alterations.

Supplementary Files

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- Tables.docx