Long-Term Survival of *Escherichia coli* Lacking the HipBA Toxin-Antitoxin System during Prolonged Cultivation

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**Key words:** HipBA; toxin-antitoxin; *E. coli*.

We designed and constructed six major toxin-antitoxin disruptants (*Δ*chpB1K, *Δ*dinJ-yafQ, *Δ*hipBA, *Δ*mazEF, *Δ*relBE, and *Δ*yefM-yoeB) of *Escherichia coli* K-12 W3110. On prolonged cultivation of these disruptants with minimal M9 medium, the *Δ*hipBA cells exhibited a significantly longer life span than that of the other disruptants and of wild-type cells, as analyzed using a LIVE/DEAD BacLight Kit (Invitrogen, Carlsbad, CA) in combination with flow cytometry analysis. The gene expression level of *hipA* in the wild-type cells was highest at the stationary phase of 40 h. The *Δ*hipBA cells showed higher macromolecular synthesis activity than the wild-type cells at the stationary phase. Stationary phase cells of *Δ*hipBA and the wild-type strain showed a significantly extended life span under anaerobic conditions. Furthermore, the *Δ*hipBA cells showed higher resistance to *H₂O₂* than the wild type. These results suggest that HipBA induces cell death with oxidative stress during prolonged cultivation. This is the first report that an *E. coli* toxin-antitoxin (TA) system affects frequency of survival during the long-term stationary phase.

**Key words:** HipBA; toxin-antitoxin; *Escherichia coli*; prolonged cultivation; oxidative stress

The bacterial life cycle consists mainly of four phases under laboratory and industrial cultivation: the lag phase, the logarithmic phase, the stationary phase, and the death phase. *Escherichia coli* K-12 growing in LB and M9 medium usually enters the death phase after 3 and 4 d respectively. The triggers and the mechanisms of the death phase are not well understood. We have been searching for *E. coli* genes that control the death phase. Long-life mutants are interesting for application uses because the production period can be prolonged using them. Several toxin-antitoxin (TA) cell death systems have been identified and characterized in the *E. coli* K-12 chromosome: *mazEF*, *Δ*chpB1K, *Δ*mazEF, *Δ*yefM-yoeB, *Δ*yafQ, *Δ* dinJ-yafQ, and the newly identified *yhaV-prfI*. The role of these TA gene pairs in the death phase remains an open question.

The most-studied TA is *mazEF*, which has all the characteristics of an addiction module, causing cell death triggered by various stress conditions, including high temperature, DNA damage, oxidative stress, and exposure to chemical inhibitors of transcription and/or translation, such as antibiotics, rifampicin, chloramphenicol, spectinomycin, and so on. Under these conditions, transcription from the promoter of *mazEF* is repressed and unstable antitoxin MazE is degraded by protease. Due to lack of antitoxin, free MazF protein can degrade mRNAs having ACA sequences and trigger subsequent cell death. Almost all the reported toxins show endoribonuclease activity, and overproduction of them induces cell death. In contrast, some researchers have claimed that the toxins have bacteriostasis effects, making cells dormant, but are not a bactericidal system for cell death. The physiological function of TA systems in the chromosome of *Escherichia coli* is still an area of debate.

Mutants of *hip* have been identified and isolated as high-frequency-persistent mutants (*hip*). Persistence refers to the ability of a small fraction of the bacteria within a genetically homogenous population to go dormant. Persistor cells survive lethal concentrations of antibiotics. Recent studies have shown that survival of persisters exposed to antibiotics is caused by a transient dormancy of cells. HipBA is now recognized as a major TA system in *E. coli*. The *hip* operon, on the *E. coli* chromosome, consists of two genes, *hipA* and *hipB*, encoding proteins of 50 and 10 kDa respectively. HipA protein, differently from other RNase toxins, belongs to the phosphatidylinositol 3/4-kinase superfamily. It has been reported that expression of wild-type *hipA*, encoding the toxin, in excess of *hipB*, encoding the antitoxin, inhibits peptidoglycan, DNA, RNA, and protein synthesis and results in persistor cell formation. Low-level expression of *hipA* from a promoter in the presence of glucose has been found to be sufficient to inhibit cell growth and make persisters. Furthermore, Korch and Hill reported that HipA functions as a bacteriostatic system inhibiting macromolecular synthesis and making a majority of cells dormant, but was not a bactericidal system in their study using a LIVE/DEAD BacLight Kit in combination with microscopy observation of dormant cells upon *hipA* induction.

In this study, we designed and constructed six major toxin-antitoxin disruptants (*Δ*chpB1K, *Δ*dinJ-yafQ, *Δ*hipBA, *Δ*mazEF, *Δ*relBE, and *Δ*yefM-yoeB) of an *E. coli* K-12 strain, W3110. We found that only *Δ*hipBA cells exhibited a significantly longer life span than other disruptants and the wild type. Physiological analysis revealed that *hipBA* might function as a bacteriostatic system, resulting in cell death after the stationary phase.

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**Abbreviations:** TA, toxin-antitoxin; cat, chloramphenicol-resistant gene; LB, Luria–Bertani; cfu, colony forming units; ROS, reactive oxygen species
Materials and Methods

**Bacterial strains and strain descriptions.** Bacterial strains used in this study are summarized in Table 1. The *E. coli* strains used were *Escherichia coli* K-12 W3110 and its derivatives: W3110red (W3110ΔspacB::P::Pnu-bet exo kan),26) ΔyeFM-yoeB (W3110ΔyeFM-yoeB), ΔmaeEF (W3110ΔmaeEF), ΔdinK-yafQ (W3110ΔdinK-yafQ), ΔrecBE (W3110ΔrecBE::SacB), ΔchpBIK (W3110ΔchpBIK::SacB), and ΔhipBA (W3110ΔhipBA). These deletion strains were designed and constructed by a two-step markerless deletion method described elsewhere.26 At the first step, target genes were deleted and replaced with a DNA cassette consisting of a chloramphenicol-resistant gene (*cat*) and a negative selection marker (*SacB*). In the second step, markerless deletion mutants were selected as sucrose-resistant colonies that lost the negative selection marker *SacB*. Gene disruption was performed from the coding region of toxIn to toxIn. In the case of the ΔrecBE and ΔchpBIK disruptants, markerless deletions could not be obtained, probably due to a polar effect of neighbor gene transcription. Hence, deletion mutants with the DNA cassette, consisting of *cat* and *SacB*, were used as ΔrecBE and ΔchpBIK disruptants instead of markerless deletion mutants.

**Media and growth conditions.** Luria-Bertani (LB) medium (BD, Franklin Lakes, NJ) and minimal M9 medium (BD) containing 1% glucose, 10 mM MgSO₄, 1 mM CaCl₂, 18 mM FeSO₄, and 2% CaCO₃ were used. Bacteria were cultured first in LB medium for 8 h at 37°C with agitation at 250 rpm. Fifty μl of the fully grown LB culture was inoculated to 5 ml M9 medium and this was incubated at 37°C with agitation at 250 rpm. In anaerobic experiments, bacterial strains were inoculated anaerobically at 37°C in an anaerobic chamber, as described by Dukan and Nystroém.27) 

**Oxidative-stress assay.** Cells were cultured for 62 h at 37°C in M9 medium. The resulting cell suspension was split into two, one incubated aerobically at 37°C in a rotary shaker, and the other incubated anaerobically at 37°C in an anaerobic chamber, as described by Dukan and Nystroém.27)

**Plasmids.** Plasmids used in this study are summarized in Table 1. An expression plasmid containing the *hipBA* gene was constructed for complementation analysis of *HipBA* cells. A chromosomal *hipBA* region including the upstream P2 promoter was amplified by PCR using primer hipBA1 (5'-AAAGATTCTAGCTGCTAAGCC-TATAAGCGCGGATT-3') and primer hipBA2 (5'-AAAGATTCTT-CACTTAACCGGATTCTCGGCTAAACCCG-3'). The resulting fragment was sub-cloned into the pDrive plasmid (Qiagen, Valencia, CA) to generate pDrivehipBA, and transformed into *E. coli* K-12 DH5α competent cells. Plasmid purification was performed with a QIA prep spin Mini prep kit (Qiagen), and the sequence of inserted fragment was confirmed using an ABI3700 sequencer. The insert of pDrivehipBA was digested with EcoRI and then cloned into pMW119 plasmid (Nippon Gene, Tokyo) to generate pMW119hipBA.

**Complementation analysis.** A plasmid with *hipBA* genes was constructed and designated pMW119hipBA. This plasmid was introduced into *ΔhipBA* cells for complementation analysis. An ampicillin-resistant recombinant was cultured in M9 minimal medium for 90 h. At 90 h, live cells of W3110red harboring pMW119, W3110red harboring pMW119hipBA, *ΔhipBA* harboring pMW119, and *ΔhipBA* harboring pMW119hipBA were counted using flow cytometry analysis as described below.

**Cell staining procedure and flow cytometry.** Cultured cells were harvested and diluted (10^4 dilution) in 0.85% NaCl, and then were stained with a LIVE/DEAD BacLight kit (Invitrogen, Carlsbad, CA). Fluorescent cell staining was done using SYTO9 (Invitrogen), and PI (Invitrogen) according to the manufacturer’s instructions. Stained samples were incubated in the dark at room temperature for 15 min. Flow cytometric measurement was done using a BD Biosciences FACSCalibur flow cytometer (BD), with excitation (488 nm) from an argon laser. Optical filters were used for green fluorescence (530 nm, FL1) and red fluorescence (580 nm, FL2). The voltages for FL1 and FL2 were 520 and 550 respectively. Detectors of FL1 and FL2 were set to a logarithmic scale. The parameter for FL1 and FL2 was 1.0. The thresholds of the FL1 and FL2 parameter values were set to 250 and 100 respectively. Compensation of FL2-%FL1 was set to 60. Fluorescence from the samples was detected at a low-speed flow rate (12μl/min) for 2 min, and the numbers of fluorescent cells were counted. Cellular condition, live or dead, was estimated depending on the density-plot distribution of FL1 and FL2, as described by Bernes et al.28) We validated the flow cytometric method for the counts of live cells using ethanol-treated dead cells as a control (Fig. 1B). In this measurement, live cell numbers per culture volume were calculated from live cell counts detected in a 1-min measurement. Total particles counted by forward scatter were different from the cell numbers because bacterial cells are too small to discriminate from dust in the culture media. Hence we used live cell counts per volume instead of live cell counts per particle numbers from forward scatter.

**Standard and real-time RT-PCR.** Total cellular RNA was isolated from cells cultured in M9 medium for 15, 25, 40, and 65 h using TRIZOL Reagent (Invitrogen). The resulting RNA was treated with DNase I to avoid DNA contamination. RNA (0.1μg) was subjected to cDNA synthesis reaction using random primers (Invitrogen) and 200 units of SuperScript II Reverse Transcriptase (Invitrogen). Subsequently, PCR was performed using 1 μl of cDNA in 20 μl of buffer containing 100μl of each primer and ExTag DNA polymerase (Takara Bio, Shiga, Japan). For detection of *hipA* transcript, primers of *hipA1* (5'-CGAAACGCTAACCAGCTC-3') and *hipA2* (5'-CAGTGCTGCTTCTCTGCTG-3') were used. Real-time PCR was also performed using power SYBR green PCR Master Mix (Applied
Biosystems, Foster City, CA) with the above-mentioned primer set for hipA. The resulting intensity was corrected based on the results of dnaA expression level as an internal control.

**Measurement of total RNA and protein contents.** Total cellular RNA and protein were isolated from cells cultured in M9 medium for 40 h. Total cellular RNA was purified using an RNeasy Mini kit (Qiagen), and the resulting RNA was treated with DNase I. Total protein was extracted from the cells by sonication, and the resulting protein content was measured with a Protein Assay kit (Bio-Rad, Hercules, CA). Values of both total RNA and protein were calculated as contents per cell. Cell numbers used for extraction were counted by flow cytometry.

**Results**

A hipBA disruptant extends its life span during prolonged cultivation

To investigate the role of TAs in cell-viability loss during the long-term stationary phase, growth experiments on six TA disruptants were done with minimal M9 medium. Then the time-course of viable-cell counts was observed using the LIVE/DEAD BacLight Kit in combination with flow cytometry. As shown in Fig. 1A, the six disruptants maintained similar viable cell numbers (approximately $10^9$ cells/ml) as did the wild-type cells for 62 h. Subsequently, these strains, except for ΔrelBE, significantly fell in cell numbers at 90 h (to approximately $10^6$ cells/ml). The ΔhipBA disruptant kept a higher live cell number at 90 h (approximately 400-fold higher than the other strains, Fig. 1B), and decreased in viable cell numbers more slowly, to an undetectable level at 120 h, as compared to the other strains. The optical density of the ΔhipBA disruptant was slightly higher (OD$_{660}$, 2.7) than the other strains (OD$_{660}$, 2.2) at 90 h (data not shown). These results indicate that HipBA regulates cell viability during prolonged cultivation of E. coli.

The chromosome locus of hipBA (34.3 min) is close to dif (34.5 min), which is characteristic of the replication terminus region. In order to determine whether hipBA function as a trans- or a cis-acting element, complementation analysis of ΔhipBA cells with plasmid
Affecting cell viability in the death phase. HipBA proteins function as regulators of cell viability via cis-action during prolonged cultivation. Hence we investigated the function of HipBA in the wild-type strain. Deletion of the HipBA gene resulted in a wild-type-life-span phenotype (Fig. 2).

The results for plasmid pMhipBA were performed. The resulting recombinant strain showed a wild-type-life-span phenotype (Fig. 2). It has been found that low-level expression of HipA is bacteriostatic.6,7,20,21,23,24) LIVE/DEAD analysis indicated that the time point of the start of cell-viability loss is determined by HipA-dependent inhibition of macromolecular synthesis from about 40 h. It is probable that the time point of the start of cell-viability loss is determined by HipA-dependent inhibition of macromolecular synthesis, but the effect of HipA is thought to be bacteriostatic.6,7,20,21,23,24) LIVE/DEAD analysis indicated that both HipBA and the wild-type cells were killing during prolonged cultivation (Fig. 1). It is probable that cells were killed not by HipA, but by other stresses, and that HipA only hastened cell death via early inhibition of macromolecular synthesis during the long-term stationary phase.

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**Fig. 2.** Complementation Analysis of the ΔhipBA Disruptant. Plasmid pMhipBA was introduced into ΔhipBA cells. Bacterial growth with minimal M9 medium and comparison of live cell numbers were performed as described in the legend to Fig. 1.

**Fig. 3.** Gene Expression Level of hipA during Cultivation. Gene expression of hipA at 15, 25, 40, and 65 h was analyzed by standard and real-time RT-PCR. A, Results of standard RT-PCR. The results for dnaA expression as an internal control are shown below. B, Results of real-time RT-PCR. The hipA product intensity was normalized using that of dnaA as an internal control. Error bars indicate standard deviations for three independent experiments.

**Fig. 4.** HipA Inhibited Macromolecular Synthesis.

A, Comparison of total RNA content between wild-type and ΔhipBA cells at the stationary phase. B, Comparison of total protein content between wild-type and ΔhipBA cells at the stationary phase.

Total RNA and protein were extracted from cells of 40 h-cultured cells with an RNeasy Kit and by sonication respectively. Both total RNA and protein content are expressed as fg per cell based on cell numbers counted by flow cytometry. Error bars indicate standard deviations for three independent experiments.

**Comparison of macromolecular synthesis activity**

Pulse chase experiments showed that ectopic expression of hipA inhibits synthesis of macromolecules, including RNA and protein.7) As shown in Fig. 3, the highest hipA expression was at 40 h. The total RNA and protein contents of wild-type and ΔhipBA cells at 40 h were compared (Fig. 4). As shown in Fig. 4, both total RNA and protein contents at 40 h were higher in the ΔhipBA cells. These results indicate that HipA in the wild-type strain is activated and starts to inhibit macromolecular synthesis from about 40 h. It is probable that the time point of the start of cell-viability loss is determined by HipA-dependent inhibition of macromolecular synthesis, but the effect of HipA is thought to be bacteriostatic.6,7,20,21,23,24) LIVE/DEAD analysis indicated that both HipBA and the wild-type cells were killing during prolonged cultivation (Fig. 1). It is probable that cells were killed not by HipA, but by other stresses, and that HipA only hastened cell death via early inhibition of macromolecular synthesis during the long-term stationary phase.

**Rates of hipA expression during cultivation**

It has been found that low-level expression of hipA is sufficiently toxic to inhibit cell growth or to kill cells.24) Hence we investigated the hipA expression of wild-type cells during cultivation. As shown in Fig. 3, hipA expression was detected at 15 h (log phase). Expression increased from 15 h to 40 h (stationary phase), then decreased to 65 h. Though hipA was expressed at 15 h, there was no difference in growth profiles between the wild-type and ΔhipBA strains during the log phase. At the late log phase, it is thought that HipA is inactivated by HipB antitoxin, which is co-expressed with HipA.7) HipA might be activated later when loss of cell viability is observed. These results suggest that HipA is activated when the transcription level of hipA reaches the maximum via HipB degradation or inactivation. HipB has also been reported to be a repressor based on findings that hipB in trans can repress hipA expression, and that HipB binds to four operator sites of the hipBA promoter region, as demonstrated by gel-shift assay.29)

Therefore, HipB should be fully inactivated at 40 h when the expression of hipA is maximized. This timing of HipA activation is further discussed in the next section.
To confirm the strains’ differences in susceptibility to oxidative stress, we performed an oxidative-stress assay by the addition of an oxidant, H$_2$O$_2$. The effect of 25 mM H$_2$O$_2$ treatment on cfu is shown in Fig. 5B. The two cell groups maintained approximately 60% of their viability over 30 min of treatment. The wild-type cells showed reduced viability of 45% and 30% at 60 and 120 min respectively, whereas the ΔhipBA cells retained their viability, of 75% and 70% respectively, at the same treatment times. These results, together with the fact that the life span of cells is extended by omitting oxygen, indicate that ΔhipBA cells are more tolerant than wild-type cells of oxidative stress during prolonged cultivation.

Discussion

In the present study, we identified new phenotypes of a hipBA disruption that confers long-term survival in the stationary phase. The survival phenotype was characteristic only of ΔhipBA cells among the six TA disruptants. This is the first report to the effect that an E. coli TA system affects frequency of survival during the death phase.

Several TA systems exist in the E. coli chromosome, but their physiological roles are still under discussion. The most characterized TA, MazEF, triggers cell death only under stressful conditions, using chemical inhibitors for growth. These conditions are thought to be artificial compared to natural stresses. Recently Tsilibaris and colleagues compared the behavior of a wild-type strain and its derivative, devoid of all five proteic TA systems (ΔchpBIIK, ΔdinJ-yafQ, ΔmazEF, ΔrelBE, and ΔyefM-yoeB), under various stress conditions, and found no differences. It has been reported that MazEF mediates cell death under oxidative-stress conditions, but in this study, ΔmazEF cells did not show an extended life span (Fig. 1), indicating that MazEF-mediated cell death was not very effective under our experimental conditions. On the other hand, we identified a new hipA phenotype of long-term survival during the stationary phase without using chemical inhibitors (Fig. 1). The survival phenotype of the ΔhipBA cells suggests that among the tested 6 TAs, hipBA was the only functional TA that affects cell viability under physiological conditions.

Recently, it has been concluded that the function of HipA is not bactericidal but bacteriostatic. Hence, HipA is not expected to kill cells during prolonged cultivation. As described above in “Results,” oxidative stress is one of the conceivable stresses resulting in cell death during prolonged cultivation. Production of reactive oxygen species (ROS), including H$_2$O$_2$, and subsequent oxidation of the proteins, and non-native cystein disulfide bonds and the introduction of carbonyl groups at the lysine, arginine, proline, and threonine residues, promote cell death. Stationary-phase cells accumulate these oxidized proteins due to a low translation level. The oxidized proteins lose their structural integrity and functions, and this affects cell viability. As explained in Results, both ΔhipBA and wild-type cells showed significantly extended life spans under anaerobic conditions as compared with aerobic conditions (Fig. 5A). Under anaerobic conditions, no remarkable cell death was detected as far as we tested. This result indicates that oxidative stress does cause cell death during prolonged cultivation.
The HipBA cells showed higher resistance to the oxidant than the wild-type cells (Fig. 5B). Furthermore, we also measured and compared the carbonyl content of total proteins in the wild-type and HipBA cells during prolonged cultivation. Unexpectedly, no significant difference in the carbonyl content of total proteins was observed between the two strains (data not shown). These results indicate that the hipA expressing wild-type cells are susceptible to oxidative stress, probably due to macromolecular synthesis inhibition, but do not depend on ROS defensive mechanisms via antioxidant enzymes. We propose that active macromolecular synthesis of HipBA cells functions as an additional antioxidant mechanism for higher resistance to H2O2 and survival in the long-term stationary phase.

We conclude that HipA regulates the starting point of the process wherein stationary cells lose viability via inhibition of macromolecular synthesis. However, in long-term cultivation, oxidative stress accumulates and finally kills cells with or without HipBA. Oxidative stress is the main reason for cell death in the stationary phase.

In this study, we could not identify the signal that induces hipA expression in the stationary phase or the downstream cascade of HipA toxin action. Microarray analysis of wild-type and HipBA cells during prolonged cultivation might answer these questions in the future.

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