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AUTHORS: Chukwudia, C U; Good, L

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PHENOTYPIC INDICATIONS OF FtsZ INHIBITION IN HOK/SOK-*INDUCED BACTERIAL GROWTH CHANGES AND STRESS RESPONSE*

Chukwudi, Chinwe. U[a,b*1]; Good, Liam[a]

[a]Department of Pathology and Infectious Diseases, Royal Veterinary College, University of London.

[b]Department of Veterinary Pathology and Microbiology, University of Nigeria, Nsukka. Enugu State, Nigeria. chinwe.chukwudi@unn.edu.ng, +234 8020349928, +234 7064465858.

*Corresponding author

Abstract

The *hok*/*sok* locus has been shown to enhance the growth of bacteria in adverse growth conditions such as high temperature, low starting-culture densities and antibiotic treatment. This is in addition to their well-established plasmid-stabilization effect via post-segregational killing of plasmid-free daughter cells. It delays the onset of growth by prolonging the lag phase of bacterial culture, and increases the rate of exponential growth when growth eventually begins. This enables the cells adapt to the prevailing growth conditions and enhance their survival in stressful conditions. These effects functionally complement defective SOS response mechanism, and appear analogous to the growth effects of FtsZ in the SOS pathway. In this study, the role of FtsZ in the hok/*sok*-induced changes in bacterial growth and cell division was investigated. Morphologic studies of early growth-phase cultures and cells growing under temperature stress showed elongated cells typical of FtsZ inhibition/deficiency. Both *ftsZ* silencing and over-expression produced comparable growth effects in control cells, and altered the growth changes observed otherwise in the *hok*/*sok*$^{+}$ cells. These changes were diminished in SOS-deficient strain containing mutant FtsZ. The

[1] Permanent address: Department of Veterinary Pathology and Microbiology, University of Nigeria, Nsukka. Enugu State, Nigeria. chyuzochuks@yahoo.com.

involvement of FtsZ in the *hok*/*sok*-induced growth changes may be exploited as drug target in host bacteria, which often propagate antibiotic resistance elements.

Keywords: *hok*/*sok*, cell division, FtsZ, bacterial growth, stress response

1. Introduction

The *hok*/*sok* locus is a well characterized type I toxin/antitoxin system frequently associated with multi-drug resistance plasmids in bacteria. It is often present in plasmids encoding extended spectrum beta-lactamases (ESBLs), especially CTX-M [1]. Other than plasmids, the *hok*/*sok* locus also occurs in the chromosomes of enterobacteria and closely related bacteria, especially pathogenic strains [2]. This is suggestive of additional functions other than the established plasmid-stabilization function by post-segregational killing of plasmid-free daughter cells [3, 4]. Further investigations have shown that the *hok*/*sok* locus also function as a stress response element in bacteria. It prolongs the lag phase of bacterial cultures to enable the cells adapt to the prevailing growth conditions before growth resumes [5]. Thus, the *hok*/*sok* locus inhibits bacterial growth during the lag phase and functionally complements existing or defective SOS response.

Growth arrest and inhibition of cell division leading to filamentation is often associated with bacterial response to stressful growth conditions [6, 7]; especially in bacterial SOS response to DNA damage [8], and as a mechanism of evasion and resistance to certain antibiotics such as the beta-lactam antibiotics [9]. In many of these cases, inhibition of the cell division protein, FtsZ, is believed to inhibit cell division and induce filamentation [10]. During cell division, FtsZ proteins localize to the cell membrane at the midcell, which is the future site of the septum. They are assembled to form the Z-ring, which consists of overlapping

protofilaments of polymerized FtsZ [11]. FtsZ also recruits other proteins which are necessary for cell division (e.g. FtsA, FtsI, FtsQ, FtsW, FtsK, and ZipA) to the cell division site [12, 13]. Hence, inhibition of growth and cell division in bacteria cells are often accompanied by changes in the morphology of the cells due to inhibition of FtsZ action. In this study, the morphology of *E. coli* cells with plasmids expressing the *hok*/*sok* locus was examined, and the role of FtsZ in the *hok*/*sok*-induced growth arrest and prolonged lag phase was investigated using *ftsZ*-antisense PNA (peptide nucleic acid) and expressed *ftsZ*-antisense silencing or over-expression.

2. Materials and Methods

2.1. Bacteria cells and cultures

E. coli strains and plasmids used in this work are listed in Table 1. All the strains of *E. coli* used are derivatives of K-12. The peptide nucleic acids (PNA), *ftsZ* antisense PNA (Ec326) and *acpP* antisense PNA (sp4), were synthesized by Cambridge Research Biochemicals, reconstituted to 100µM solution in distilled water and stored at -20^{o}C. Host *E. coli* cells were made competent chemically ($CaCl_2$) for subsequent transformation with indicated plasmids. Bacterial cell stocks were stored in LB broth containing 15% glycerol at -80^{o}C (for long term storage) or -20^{o}C (for short term). Overnight cultures were diluted to the required cell concentration (1000x dilution ≈10^6 CFU ml^{-1}) in MH broth containing appropriate antibiotics (100µg ml^{-1} of ampicillin (Amp) and/or 30µg ml^{-1} of chloramphenicol (Chlr) as appropriate) for growth experiments. Cell size was assessed by fluorescence microscopy and culture growth by spectrophotometry. For spectrophotometry, 200µl of the diluted culture were incubated in 96 well plates for 18-22hrs using Biotek Powerwave XS universal spectrometer

and Gen 5 software to monitor culture growth. Optical density (OD) of cultures was measured at 550nm, and growth kinetic curves were plotted with Microsoft® Office Excel.

Table 1: Plasmids and bacterial strains.

Plasmid/strain	Relevant features/genotype	Reference/source
pUC19	*hok/sok*$^-$, Amp^R, high copy number	Invitrogen
pCCB1	*hok/sok*$^+$, Amp^R, high copy number	[5]
pOU82	*hok/sok*$^-$, Amp^R, low copy number	[14]
pPR95	*hok/sok*$^+$, Amp^R, low copy number	[14]
pHNZ	*hok/sok*$^-$, *ftsZ*-antisense, Chlr^R, IPTG inducible	[15]
pCCB3	*hok/sok*$^+$, *ftsZ*-antisense, Chlr^R, IPTG inducible	[5]
ASKA-(pCA24 N-*ftsZ*)	*hok/sok*$^-$, *ftsZ*-over-expression, Chlr^R, IPTG inducible	NBRP (ID-JW0093)*
plAU80	*hok/sok*$^-$, *ftsZ-yfp*, Amp^R, Arabinose inducible	[16]
pCCB2	*hok/sok*$^+$, *ftsZ-yfp*, Amp^R, Arabinose inducible	[5]
CSH50	*araBAD-0 Δ(pro-lac) λ*$^-$ *rpsL-(strR) thi- fimE1::IS1-*	[14]
Top 10	*F– mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara leu) 7697 galU galK rpsL (Str^R) endA1 nupG*	Invitrogen
SS996	*Ωgfp {Δ(attλ)::sulApWgfp-mut2} sulA*$^+$ *sulB103 recA*$^+$	[17, 18]

* http://ecoli.naist.jp/GB/index.php/aska-library/aska-library-detail

2.2. Fluorescence microscopy

Bacteria samples for microscopy were collected from cell cultures incubated for 2hrs at 37^{o}C. For temperature stress experiments, all cultures were further incubated at 42^{o}C for another 2hrs, and samples collected thereafter**.** 200µl of samples collected were centrifuged to pellet cells. Cell pellets were re-suspended and washed twice in 2x volume of 1X PBS. Cells were then stained by incubating in an equal volume of 1µM DAPI in the dark for about

5mins and fixed onto glass slides with cover slips. Cell morphology was examined with Leica DM4000B fluorescence microscope using the DAPI filter. Images were captured with DC500 camera using Leica IM500 software programme.

2.3. FtsZ silencing and over-expression

To assess the role of FtsZ in the growth changes associated with the *hok*/*sok* locus, *ftsZ* was selectively silenced or over-expressed using antisense PNA and plasmids. For *ftsZ* silencing using PNA, sub-inhibitory amounts (0.5-1µM) of synthetic anti-*ftsZ* PNA were added to the growth media (MH broth containing appropriate antibiotics) in order to inhibit *ftsZ* expression. Anti-*acpP* PNA, which targets the essential fatty acid biosynthesis protein ACP, was used as a control PNA to check for non-specific PNA effects [15]. The growth pattern of the cultures was then monitored to assess the effect of these antisense agents on the growth of bacteria cells. For *ftsZ* silencing using antisense plasmid, *E. coli* cells (Top 10/CSH50 and SS996) were transformed with *hok*/*sok*$^{+}$ anti-*ftsZ* plasmids (pCCB3 or pPR95+pHNZ) and their respective *hok/sok*$^{-}$ control plasmids (pHNZ or pOU82+pHNZ). Expression of the antisense plasmid was induced by adding 50-100µM IPTG in the culture media (LB broth containing appropriate antibiotics), and growth curve monitored. For *ftsZ* over-expression, cells were transformed with *hok*/*sok*$^{+}$ *ftsZ* plasmids (pCCB2 or pPR95+ASKA-) and their respective *hok/sok*$^{-}$ control plasmids (pLAU80 or pOU82+ASKA-).

3. Results

3.1. Effect of the hok/sok locus on the morphology of host bacteria cells in the lag phase of growth

The *hok*/*sok* locus has previously been reported to prolong the lag phase of host bacteria cell cultures [5]. To investigate how the *hok*/*sok* locus achieves this growth inhibitory effect in the host bacteria cell cultures, we examined the morphology of cells from cultures in the lag phase of growth by fluorescence microscopy. Top 10 cells (which contain the high copy number *hok/sok*$^+$ plasmid, pCCB1) were bigger and elongated at normal growth temperature (37^{o}C) compared to the *hok/sok*$^-$ cells (Figure 1). The elongated cells have a smooth appearance, indicating that cell division is inhibited in the *hok/sok*$^+$ cells. Cells of the CSH50 strain (which contain the low copy number *hok/sok*$^+$ plasmid, pPR95) were also elongated in contrast to the cells with the control plasmid, but with many of the cells showing nuclear constriction. This indicates some degree of cell division activity; though the cells failed to completely separate into two daughter cells. Taken together, these results suggest that the presence of the *hok*/*sok* locus inhibits cell division in the lag phase of bacterial growth, leading to elongation of the cells. Although the genetic backgrounds of these *E. coli* K-12 derivatives (Top 10 and CSH50 strains) vary slightly (especially in sugar metabolism genes; see Table 1), the effects on cell division are only observed in the *hok/sok*$^+$ cells, but not in the control cells. These changes in cell division in *hok/sok*$^+$ cells are indicative of inhibition of FtsZ activity. These results also indicate that the level of inhibition of cell division may be directly proportional to the presumed level of expression of the *hok*/*sok* locus in the host bacteria cells (inhibition in cells with low copy number plasmid is less severe than in the cells with high copy number plasmid).

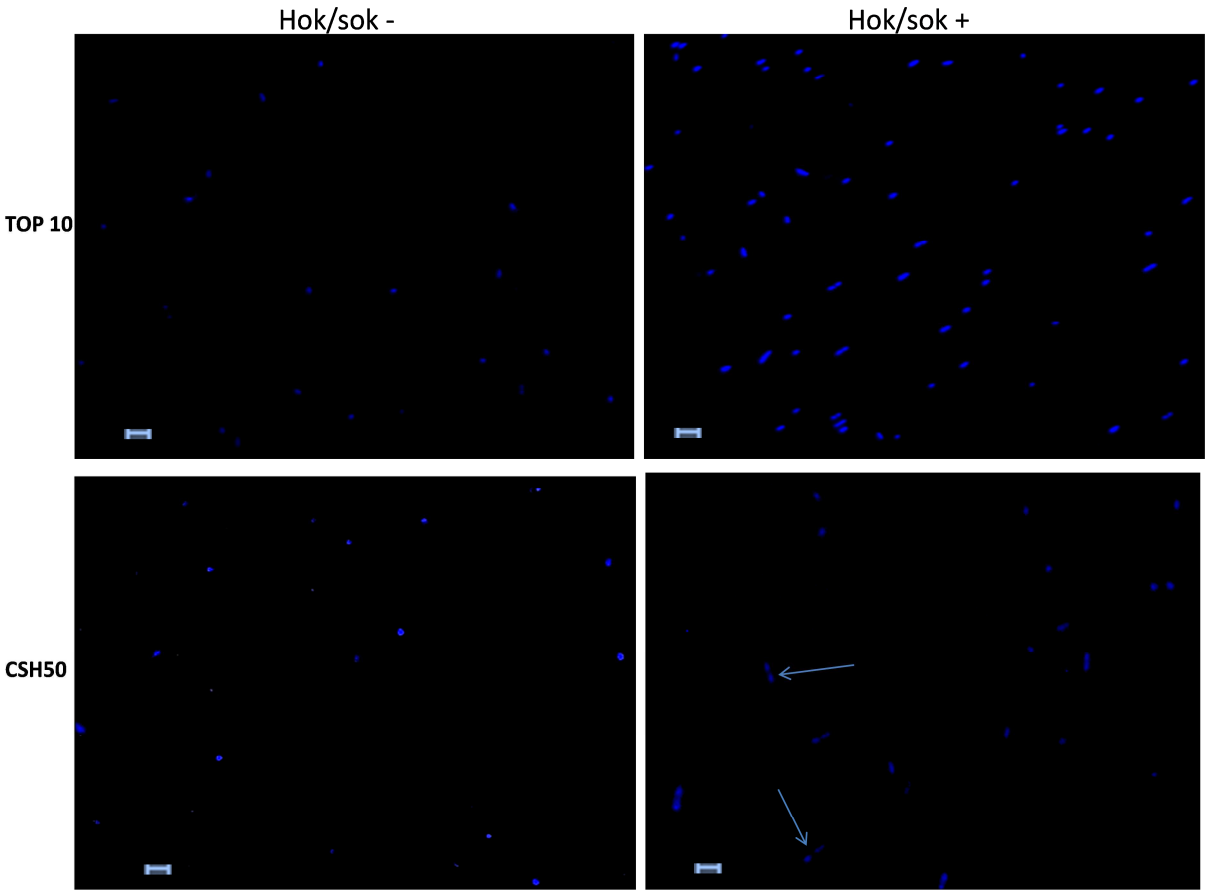


Figure 1: **Effect of the *hok/sok* locus on the morphology of host bacteria cells in the lag phase of growth.**

Fluorescent microscopic images of Top 10 and CSH50 *hok/sok*$^{+}$ (pCCB1 and pPR95) cells and their respective *hok/sok*$^{-}$ (pUC19 and pOU82) cells during the lag phase (2hr) at normal growth temperature (37^{o}C), showing bigger and elongated *hok/sok*$^{+}$ cells than the control. Arrows indicate dividing cells with nuclear constrictions/segmentations. Images were acquired at x630 magnification. Scale bar= 2µm. Data is representative of four repeat experiments.

3.2. Effect of the hok/sok locus on the morphology of bacteria cells growing under temperature stress

The *hok*/*sok* locus has also been reported to shorten the lag phase of host bacteria cultures and increase the rate of exponential growth when the cells are grown under temperature

stress at 42^{o}C [5]. To investigate how this effect is achieved, the morphology of bacteria cells growing under temperature stress was examined. Both Top 10 *hok/sok*$^{+}$ and *hok/sok*$^{-}$ cells appeared elongated under temperature stress. However, the *hok/sok*$^{+}$ cultures showed many elongated cells with nuclear constriction (Figure 2), which indicates some degree of cell division activity. In contrast, the control cells (the *hok/sok*$^{-}$ cells) showed a smooth appearance of the elongated cells. This suggests that the presence of the *hok*/*sok* locus may stimulate cell division in growth conditions that would otherwise inhibit cell division. In addition, the *hok*/*sok*-induced elongation of Top 10 cells at normal growth temperature (*hok/sok*$^{+}$ cells at 37^{o}C; Figure 1) appears similar to the temperature-induced elongation observed in the control cells (*hok/sok*$^{-}$ cells at 42^{o}C). This suggests that the *hok*/*sok* locus may impair cell division via a mechanism that is (at least partly) similar to the thermo-induced inhibition of cell division (via the SOS response pathway). In the CSH50 strain, the *hok/sok*$^{-}$ cells showed filamented cells with few nuclear constrictions and some normal-sized cells, whereas the *hok/sok*$^{+}$ cells showed long strings of cells that are almost completely separated in combination with some normal-sized cells. This shows that cells of the CSH50 strain are still able to divide successfully to a limited extent under temperature stress, even though cell division is also appreciably inhibited. However, despite enhanced nuclear segregation in the *hok/sok*$^{+}$ cells, the cells still fail to separate fully into daughter cells. This further indicates that although the *hok*/*sok* locus may inhibit cell division/segregation into daughter cells, it could also enhance cell division in growth conditions that would otherwise impair cell division. Taken together, these observations indicate that the *hok*/*sok* locus may affect or alter the normal process of cell division depending on the prevailing growth conditions, and is suggestive of an effect via the activity of cell division proteins.

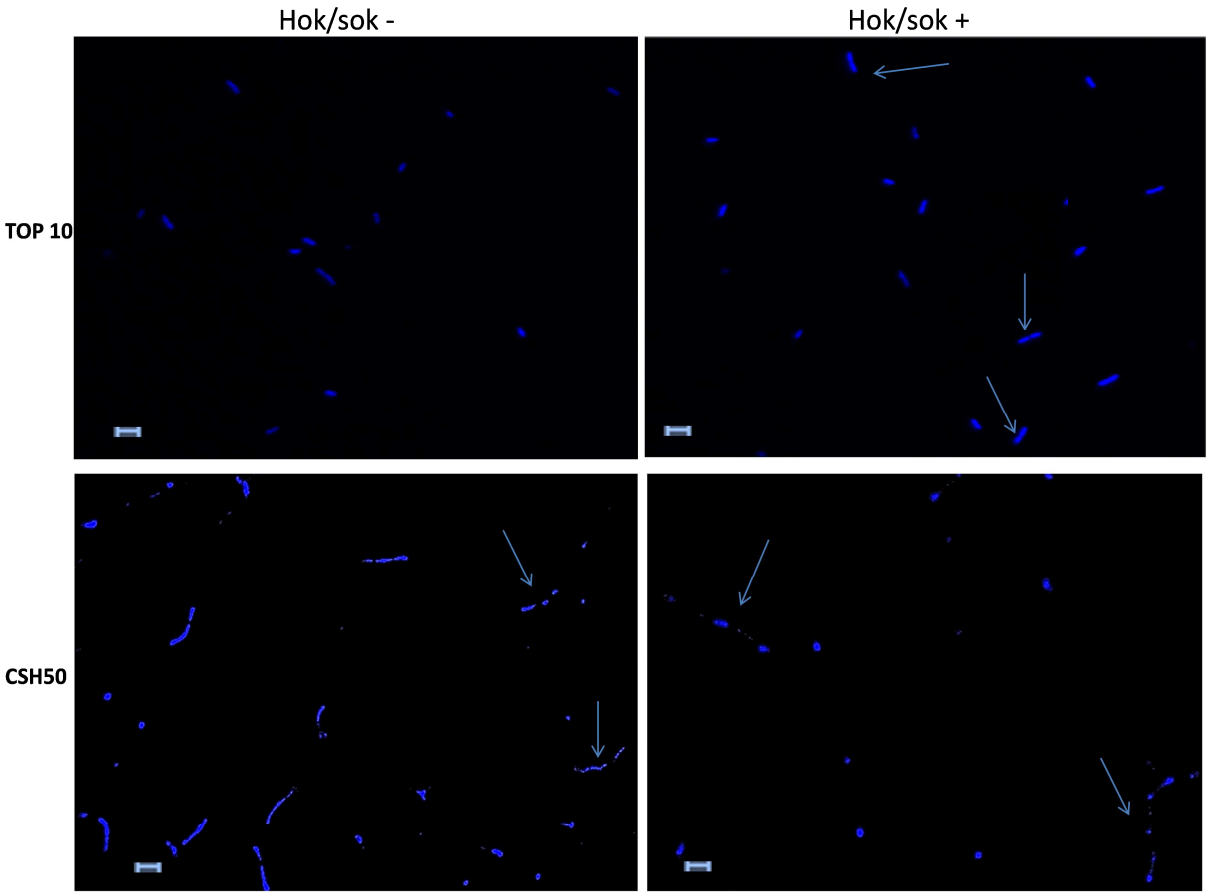


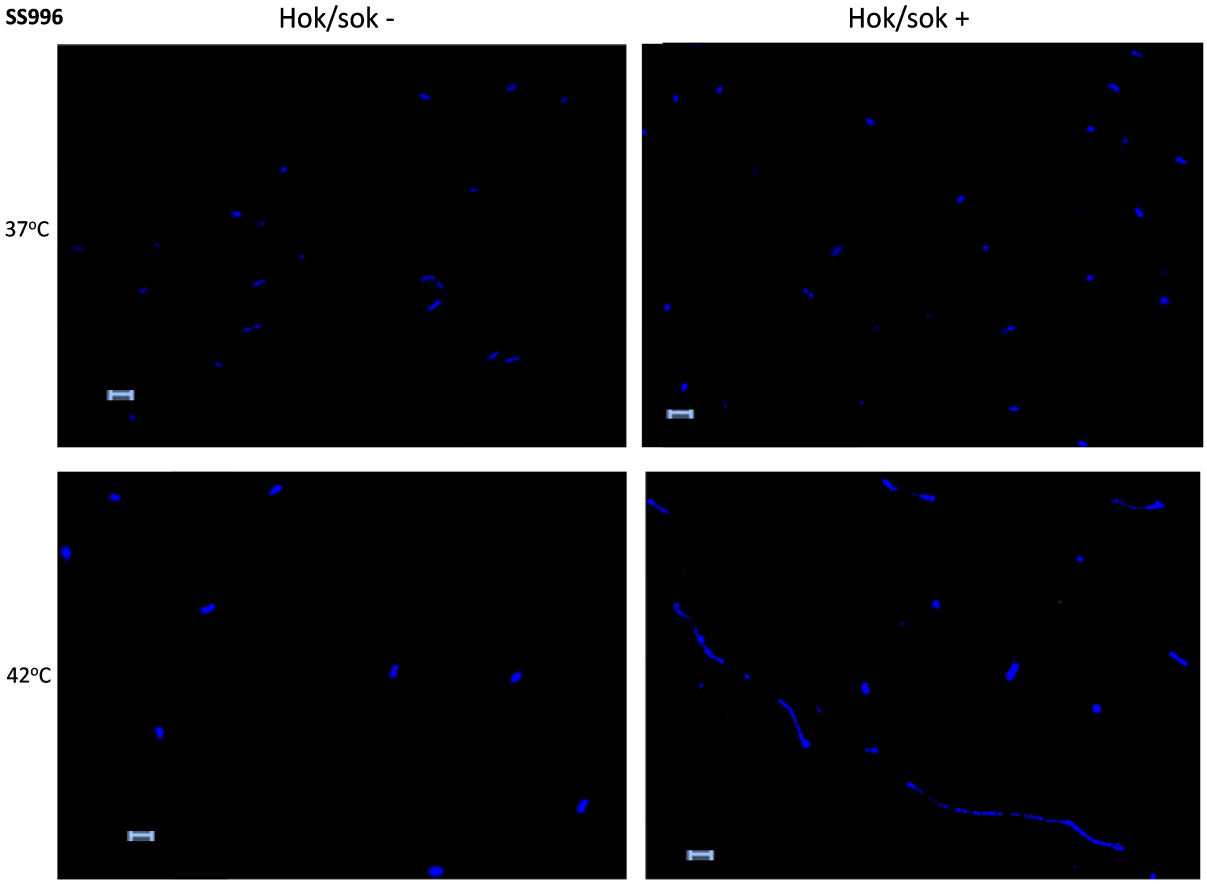
Figure 2: **Effect of the *hok/sok* locus on the morphology of host bacteria cells growing under temperature stress.**

Fluorescent microscopic images of Top 10 and CSH50 *hok/sok*$^{+}$ (pCCB1 and pPR95) cells and their respective *hok/sok*$^{-}$ (pUC19 and pOU82) cells growing under temperature stress (42^{o}C) showing elongated *hok/sok*$^{+}$ cells with nuclear constrictions in Top 10 and long strings of cells with multiple nuclear segmentations in CSH50 strain (indicated by arrows). Control cells of both strains are elongated with little or no nuclear segmentation. Images were acquired at x630 magnification. Scale bar= 2µm. Data is representative of four repeat experiments.

3.3. Effect of the hok/sok locus on the morphology of SOS-negative (SulA-insensitive) E. coli strain

The bacterial cell division protein most commonly associated with cell elongation and growth arrest is FtsZ. To assess the possibility that the *hok*/*sok* growth effects may be mediated via alterations of FtsZ activity, we examined the morphology of an *E. coli* strain that contains a mutant form of FtsZ (SS996). This mutation in FtsZ makes the SS996 strain resistant to the cell division inhibitor, SulA produced during the SOS response. At 37^{o}C, the SS996 *hok/sok*$^{-}$ (control) cells appeared elongated with some nuclear constrictions (Figure 3), whereas the hok*/sok*$^{+}$ cells were not elongated. Similar pattern was observed for both pCCB1 (high copy number) and pPR95 (low copy number) plasmids. The morphology of the control cells appeared similar to what was observed in both Top 10 and CSH50 hok*/sok*$^{+}$ cells during the lag phase at 37^{o}C, and Top 10 *hok/sok*$^{+}$ cells growing under temperature stress at 42^{o}C. In other words, SS996 *hok/sok*$^{-}$ cells had a similar morphology to the *hok*/*sok*-induced elongation of cells in the lag phase of growth, and in cells growing under temperature stress. This indicates that the *hok*/*sok*-induced growth changes may occur naturally in the SS996 strain (in the absence of the *hok*/*sok* locus). On the other hand, the presence of the *hok*/*sok* locus in the SS996 strain could not elicit the growth changes observed in other strains (Top 10 and CSH50). This indicates that the mechanism by which the *hok*/*sok* locus inhibits cell division during the lag phase is impaired or defective in the SS996 strain. However, at 42^{o}C the SS996 *hok/sok*$^{+}$ cells showed a sub-population of very long cells (filaments), whereas the control cells were not elongated like the Top 10 and CSH50 cells. Bearing in mind that the SS996 strain is insensitive to the temperature-induced inhibition of cell division (as also observed here) due to the presence of a mutant form of FtsZ, these results suggest that the *hok*/*sok* locus may exert its effect on bacterial cell

192 growth via effects on FtsZ activity during cell division. In addition, the elongation of SS996
193 *hok*/*sok*$^{+}$ cells at 42^{o}C when compared to the *hok*/*sok*$^{-}$ cells further indicates that the
194 *hok*/*sok* locus may complement the defective response mechanism to temperature stress.



195

196 **Figure 3**: **Effect of the *hok*/*sok* locus on the morphology of SOS-negative *E. coli* cells**
197 **(SS996) at normal and high growth temperatures.**
198 Fluorescence microscopy images of *hok/sok*$^{+}$ and *hok/sok*$^{-}$ cells before and after
199 temperature shift, showing normal-sized *hok/sok*$^{+}$ cells (no cell elongation) at 37^{o}C, and a
200 sub-population of filamented *hok/sok*$^{+}$ cells at 42^{o}C. Temperature stress did not induce cell
201 elongation in *hok/sok*$^{-}$ (control) cells. Images were acquired at x630 magnification. Scale
202 bar= 2µm. Data is representative of four repeat experiments.

3.4. Effect of ftsZ silencing on the growth of hok/sok host bacteria cells using antisense PNA

To investigate whether the elongation of cells and cell division changes observed in *hok/sok*$^+$ cells are due to an effect on FtsZ activity, *ftsZ* was selectively silenced in both *hok/sok*$^+$ and *hok/sok*$^-$ bacteria cells containing the low copy *hok*/*sok* plasmids (pPR95 and pOU82) using antisense peptide nucleic acid (PNA). The susceptibility of the *hok/sok*$^+$ cells to the growth inhibition induced by the PNAs was compared to that of the *hok/sok*$^-$ cells. In CSH50 strain, the anti- *ftsZ* PNA induced a prolonged lag phase/growth arrest (up to about 12hrs) in the *hok/sok*$^-$ (control) cells, but not in the *hok/sok*$^+$ cells (Figure 4). This is similar to (although more severe than) the *hok*/*sok*-induced prolonged lag phase and growth arrest previously reported [5].The control PNA (Sp4) did not produce this effect. In the SS996 strains, the anti-*ftsZ* PNA induced a more prolonged lag phase/growth arrest in the *hok*/*sok*$^-$ (control) bacteria cell cultures than the control PNA, but not in the *hok/sok*$^+$ cell cultures. Since this prolonged lag phase effect was replicated only in the *hok*/*sok*$^-$ (control) cells by anti-*ftsZ* PNA, this indicates that the *hok*/*sok* effect may be mediated via inhibition of FtsZ activity. On the other hand, diminished anti-*ftsZ* PNA effect in the *hok/sok*$^+$ cells indicates that the *hok*/*sok* locus may rescue host bacteria cells from the inhibitory effects of the PNA. In other words, the presence of *hok*/*sok* locus enabled the host bacteria cells to defy the growth inhibitory effects of anti-*ftsZ* PNA, thus enabling the *hok*/*sok*$^+$ cells to grow in the presence of the PNA with less inhibition than the *hok*/*sok*$^-$ cells. In the CSH50 strain, the presence of the *hok*/*sok* locus particularly rescued the bacteria cells from the prolonged growth arrest induced by the anti-*ftsZ* PNA, as well as a small protective effect from the control PNA. This also indicates that in addition to a specific effect on FtsZ action, the *hok*/*sok* locus generally improves bacterial survival in stressful/adverse growth conditions. In the SS996 strain, both

the growth inhibitory effect induced by the anti-*ftsZ* PNA and the protective effect of the *hok*/*sok* locus on the prolonged lag phase was not as obvious as in the CSH50 strain. The protection against the control PNA was also obscured. This again indicates that there are additional factors affecting the growth effects of the *hok*/*sok* locus in SS996 strain. Particularly, the reduced effect of *ftsZ* inhibition on culture growth in SS996 cells indicates that the mechanism of *hok*/*sok*-induced growth effects is impaired in SS996 strain, suggesting an effect on FtsZ activity.

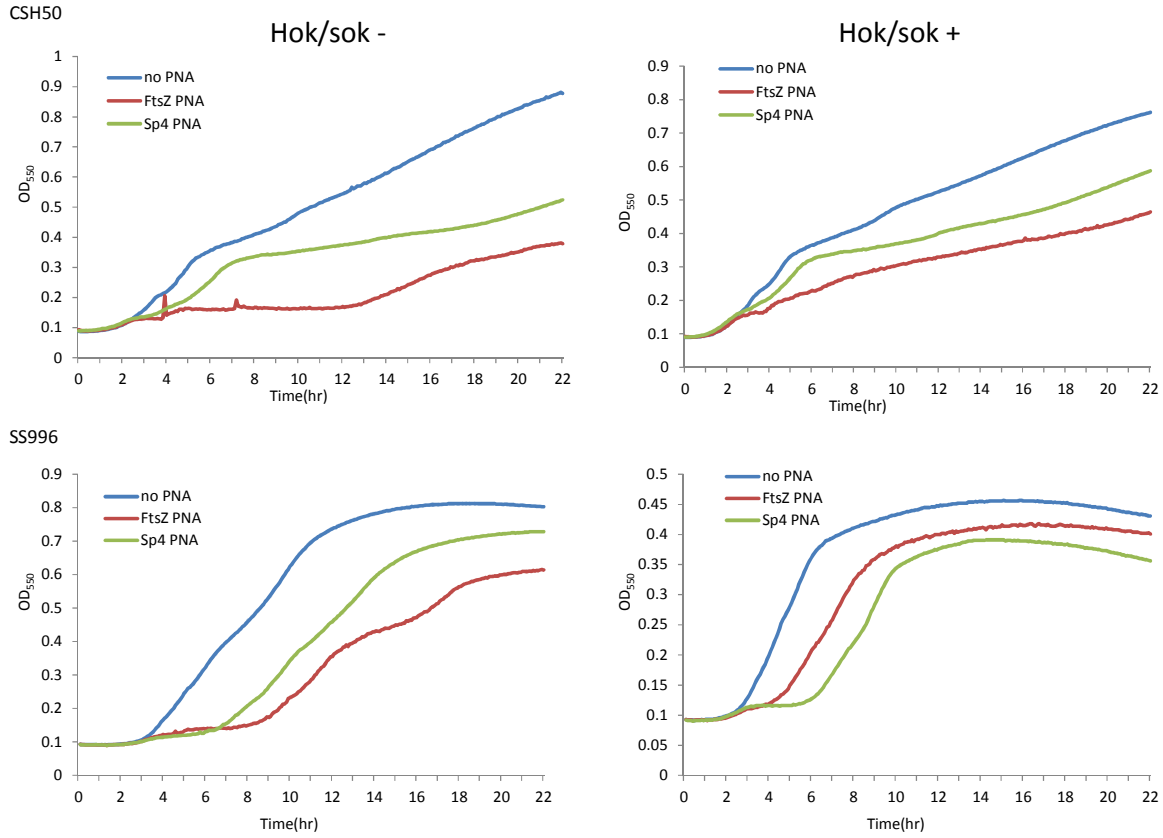


Figure 4: **Effect of *ftsZ* silencing using antisense PNA on the growth of *hok/sok*$^+$ and *hok*/sok$^-$cells.** Graphs show growth curves of *E. coli* CSH50 and SS996 cells containing the *hok*/*sok*$^+$ plasmid (pPR95) or the *hok*/*sok*$^-$ plasmid *(*pOU82). The cells were either untreated (no PNA) or treated with 1µM *ftsZ* antisense PNA or a positive control PNA (SP4, anti-*acpP*). Data is representative of four repeat experiments.

3.5. Effect of ftsZ silencing on the growth of hok/sok cells using expressed antisense plasmid

To avoid the possible complications that could arise from PNA-induced growth inhibition in the previous experiments, we also used plasmids that express *ftsZ* antisense RNA from an inducible promoter (pHNZ) to further investigate the role of FtsZ in the *hok*/*sok*-induced growth effects. Again, if the *hok*/*sok* locus inhibits cell division via an effect on FtsZ, then *ftsZ* inhibition would be expected to produce different effects on cell growth in *hok*/*sok*$^+$ and *hok*/*sok*$^-$ cells. Specifically, the prolonged lag phase normally seen in *hok*/*sok*$^+$ cells would be replicated in the *hok*/*sok*$^-$ (control) cells, whereas the *hok*/*sok*$^+$ cells would be rescued from this effect. When *ftsZ* was inhibited by inducing expression of the antisense plasmid with IPTG, the *hok*/*sok*$^-$ cells showed a prolonged lag phase or transient growth arrest at the early log phase in both CSH50 and SS996 strains (Figure 5); similar to what was observed in the antisense PNA experiments. Again, *ftsZ* inhibition by expressed antisense produced less growth inhibition in SS996 strain than in CSH50 strain, as was also observed in the PNA experiments. This inhibition of growth at the early phase of growth was greatly minimized or absent in the *hok*/*sok*$^+$ cells when the expression of *ftsZ* antisense RNA was induced with IPTG. Since *ftsZ* is an essential gene for bacterial growth, its inhibition would ordinarily inhibit growth in any normal bacteria cell. Therefore, the inability of *ftsZ* inhibition to inhibit growth in *hok*/*sok*$^+$ cells indicates that the *hok*/*sok* locus may also possess the ability to compensate for *ftsZ* inhibition and improve culture growth. This further indicates that the *hok*/*sok* locus may achieve its effects on bacterial growth via a mechanism involving alterations in FtsZ activity.

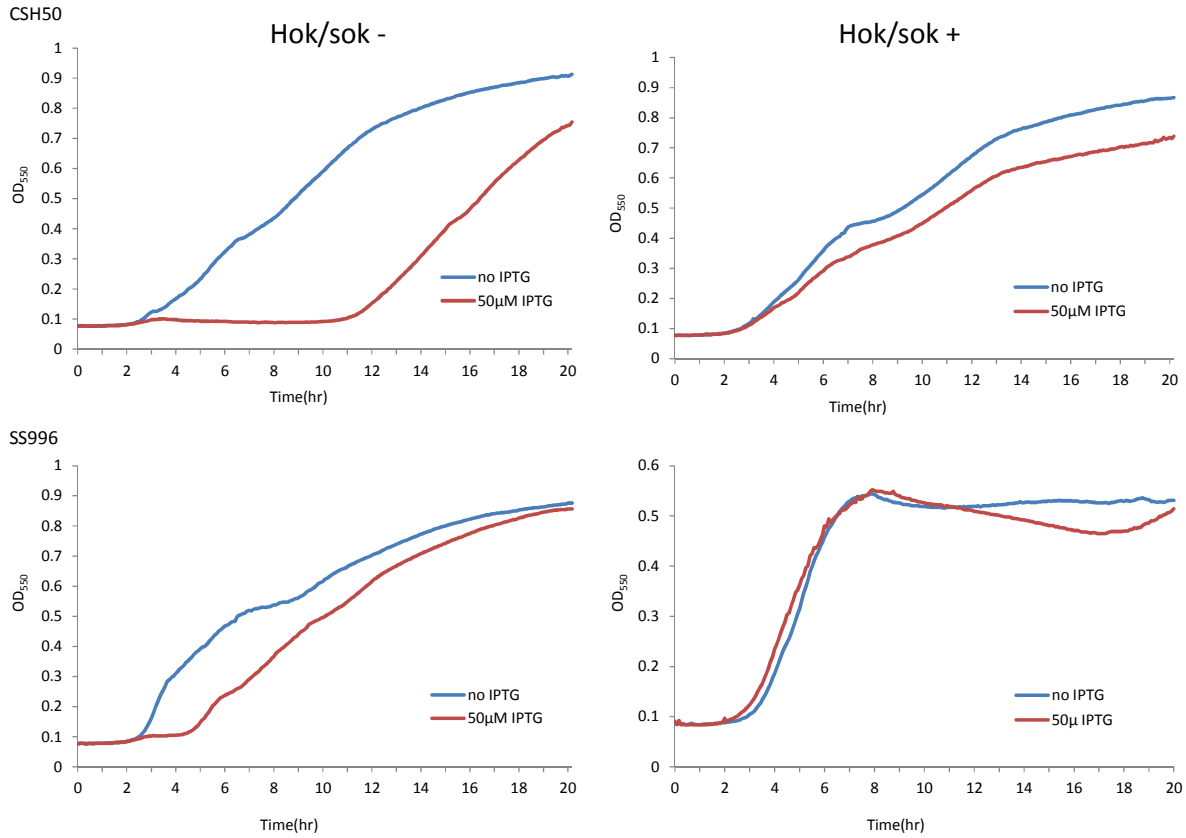


Figure 5: **Effect of *ftsZ* silencing on the growth of bacterial cells using plasmid that express *ftsZ* antisense RNA.**

Graphs of the growth curves of *E. coli* CSH50 and SS996 cells containing the *hok*/*sok*$^+$ plasmid (pPR95) or the *hok*/*sok*$^-$ plasmid *(*pOU82) with *ftsZ* antisense plasmid (pHNZ), showing early log phase growth arrest in *hok/sok*$^-$ cells and little or no growth inhibitory effect in *hok/sok*$^+$ cells with IPTG induction of *ftsZ* antisense RNA expression. Data is representative of four repeat experiments.

To further elucidate the role of FtsZ in these culture growth changes, we also examined the morphology of Top 10 and SS996 host cells when *ftsZ* inhibition was induced with IPTG via expressed antisense plasmids (pHNZ and pCCB3). The *hok*/*sok*$^+$ (pCCB3) and *hok*/*sok*$^-$ (pHNZ) cells in both strains appeared elongated/filamented (indicating inhibition of cell division, as

would be expected of cells in which FtsZ activity is inhibited). But whereas the elongated Top 10 *hok*/*sok*$^-$ cells were smooth in appearance, the *hok*/*sok*$^+$ cells showed much nuclear segmentation/constriction (Figure 6). This is indicative of enhanced cell growth/division activity when compared to the control cells. In the SS996 strain, the control cells appeared as filaments with multiple nuclear segmentations, indicating some level of cell division activity (due to the presence of a FtsZ allele that is resistant to cell division inhibition). On the other hand, the SS996 *hok*/*sok*$^+$ cells showed smaller cells, with a combination of smooth and segmented appearance. These morphologic observations are consistent with the results of the growth experiments (which showed normal growth in *hok*/*sok*$^+$ cells induced for *ftsZ* antisense expression, and shorter period of growth arrest/inhibition in SS996 *hok*/*sok*$^-$ cells). Since the SS996 has an impaired SOS response due to a mutant form of FtsZ, the differences in the effect of FtsZ inhibition between the two strains strongly suggests that the *hok*/*sok* effects could be mediated via alterations in FtsZ activity. These results also suggest that the *hok*/*sok* locus may enhance cell division in division-impaired cells.

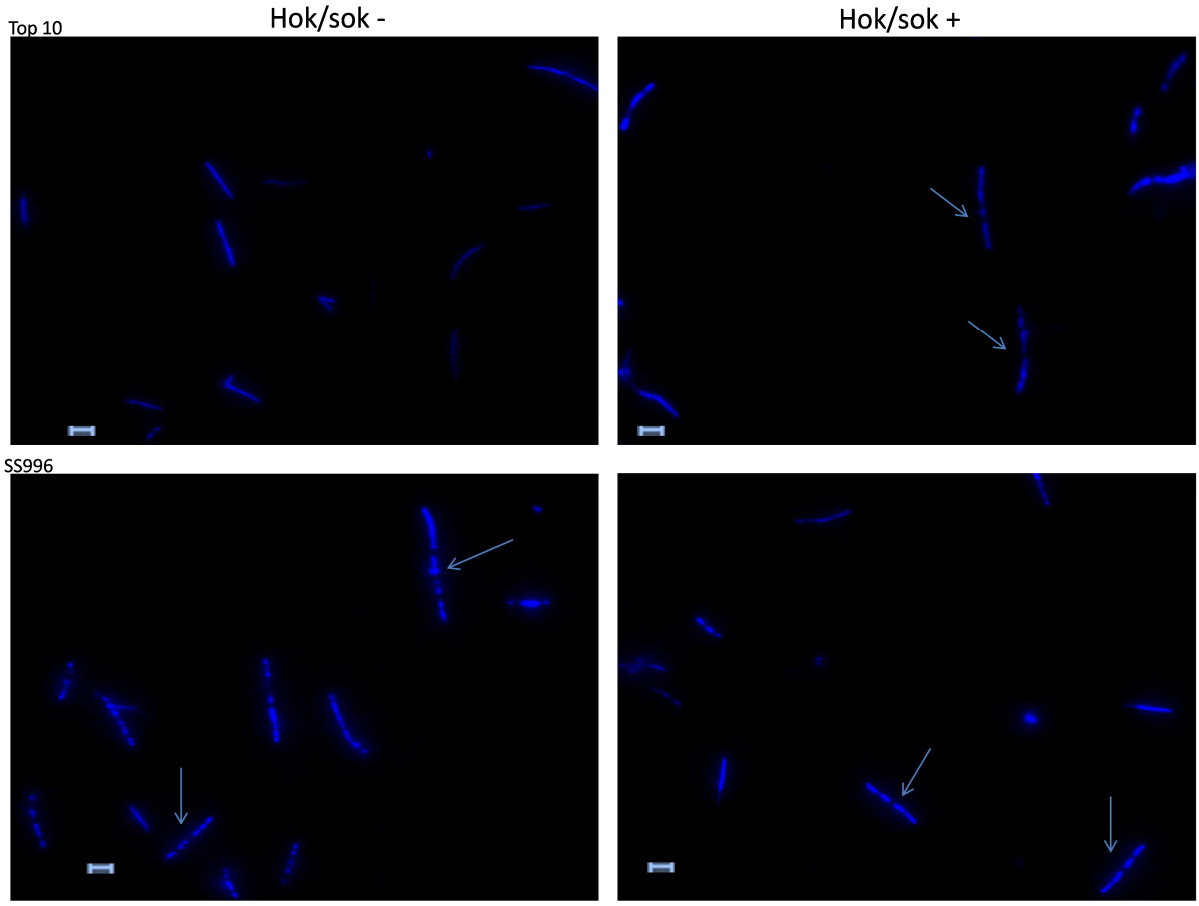


Figure 6: **Effect of *ftsZ* silencing via expressed antisense RNA on the morphology of host bacteria cells**

Fluorescence microscopy images of cells containing the *hok*/sok$^+$ (pCCB3) and *hok*/sok$^-$ (pHNZ) *ftsZ* antisense plasmids show filamented cells in all 4 strains, with multiple nuclear segmentations in *hok*/sok$^+$ cells and SS996 *hok*/sok$^-$ cells. Arrows indicate nuclear segmentation in cells. Images were acquired at x400 magnification. Scale bar= 2µm. Data is representative of four repeat experiments.

3.6. Effect of ftsZ over-expression on the growth of hok/sok cells

If the *hok*/*sok* locus inhibits cell division via inhibition of FtsZ, then *ftsZ* over-expression could rescue the cells from *hok*/*sok*-induced growth arrest/prolonged lag phase. We therefore investigated the effect of *ftsZ* over-expression using the plasmid ASKA- on the growth of *hok*/*sok*$^+$ (pPR95) and *hok*/sok$^-$ (pOU82) cells. In both CSH50 and SS996 strains,

the lag phase of both *hok*/*sok*$^+$ and control cultures was not affected when *ftsZ* over-expression was induced with IPTG (Figure 7). This indicates that *ftsZ* over-expression rescued the cells from the *hok*/*sok*-induced growth arrest and prolonged lag phase. However, growth inhibition was observed in the CSH50 *hok*/*sok*$^+$ cells when *ftsZ* over-expression was induced with IPTG, especially at the exponential growth phase. Since *ftsZ* over-expression is toxic to bacterial cells and also inhibits cell division [19-21], this result indicates that there are more toxic/ higher amounts of FtsZ in the CSH50 *hok*/*sok*$^+$ cells at the log/exponential growth phase. This is consistent with the rapid exponential growth reported previously in *hok*/*sok*$^+$ cells [5], and indicates that the *hok*/*sok* locus may induce *ftsZ* expression or improve FtsZ activity at the exponential growth phase. Again, this effect is masked in the SS996 strain which has a mutant form of FtsZ, indicating that the mechanism by which FtsZ activity is enhanced by the *hok*/*sok* locus is impaired in this strain.

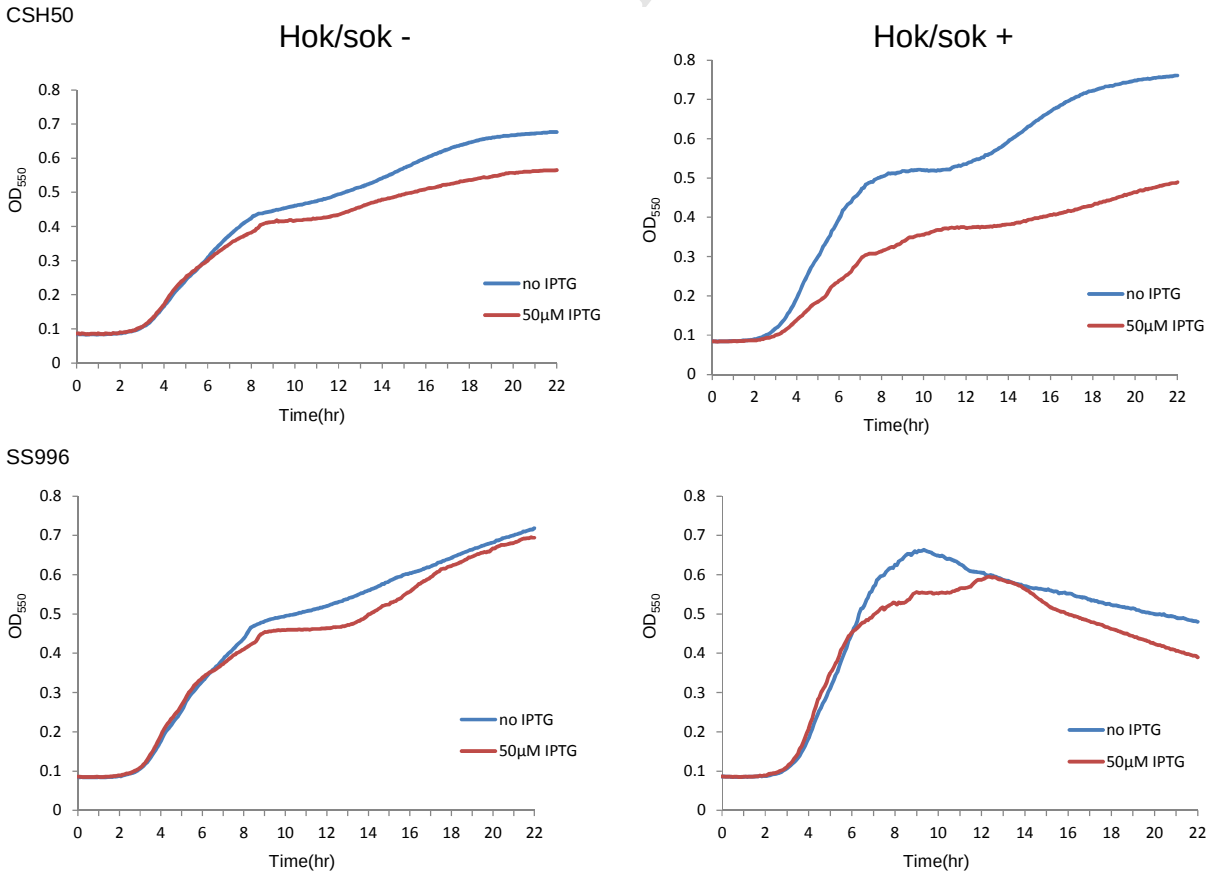
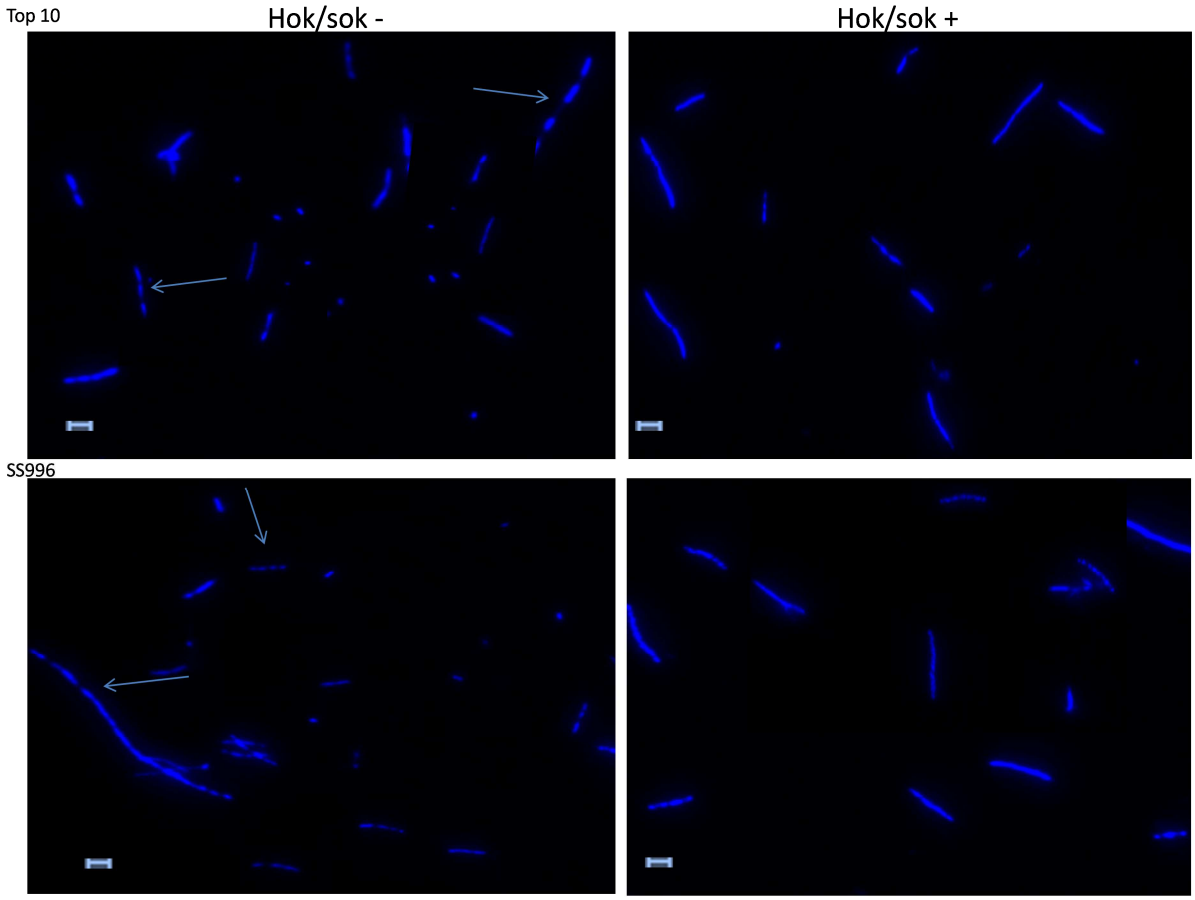


Figure 7: **Effect of *ftsZ* over-expression on the growth of *hok/sok*$^{+}$ and $^{-}$ cells.**
Graphs show cultures with no early growth arrest/prolonged lag phase, and greater inhibition of growth (FtsZ toxicity) in CSH50 *hok*/*sok*$^{+}$ cells at the exponential phase when *ftsZ* expression is induced with IPTG cells. Data is representative of four repeat experiments.

To further investigate the effect of *ftsZ* over-expression in the *hok*/*sok*-induced growth effects, we examined the morphology of cells containing plasmids that over-express *ftsZ* (pCCB2 for *hok*/*sok*$^{+}$ or pLAU80 for *hok*/*sok*$^{-}$). Whereas the *hok*/*sok*$^{-}$ (control) cells of both strains showed elongated/filamented cells with multiple nuclear segmentations and many small-sized cells/minicells (Figure 8), the *hok*/*sok*$^{+}$ cells showed smooth elongated/filamented cells (without nuclear segmentations). The filamented cell morphology with multiple nuclear segmentations indicate low level *ftsZ* over-expression/FtsZ activity, whereas the smooth filamented cell morphology is indicative of FtsZ toxicity due to higher levels of *ftsZ* over-expression/FtsZ activity. Hence, these results suggest that *ftsZ* over-expression/FtsZ activity is enhanced in the *hok*/*sok*$^{+}$ cells, indicating that the *hok*/*sok* locus may alter FtsZ activity in host bacteria cells.



335

336 **Figure 8**: **Effect of *ftsZ* over-expression on the morphology of *hok*/*sok*$^{+}$ (pCCB3) and**
337 ***hok*/sok^{-} (pLAU80) cells at early growth phase.** Cells are elongated (filamented) in all
338 strains, but *hok*/*sok*$^{-}$ cells show multiple nuclear segmentations (indicated by arrows),
339 whereas *hok*/*sok*$^{+}$ cells have smooth filamentation. Images were acquired at x400
340 magnification. Scale bar= 2µm. Data is representative of four repeat experiments.
341

342 ## 4. Discussion

343 Inhibition of cell division is typically associated with cell elongation or filamentation, as the
344 cells increase in size but fail to divide. It is widely associated with bacterial SOS response to
345 DNA damage; but there have been other reports of filamentation not associated with DNA
346 damage in bacteria [20, 22, 23]. These reports have indicated high temperature and
347 pressure as factors that induce cell elongation (filamentation) in *E. coli*, and suggest the

involvement of the cell division protein, FtsZ, which polymerizes to form the Z-ring at the mid-cell during cell division.

The observation of cell elongation during the prolonged lag phase induced by the *hok*/*sok* locus, and improved cell division in *hok*/*sok*$^+$ cells growing under temperature stress both suggest alterations in cell division and/or FtsZ activity. Also, the differences between the *hok*/*sok* mediated effects on culture growth, cell division and morphology observed in CSH50/Top 10 and SS996 host strains seem to suggest a mechanism involving FtsZ activity. The observation of a prolonged lag phase, early log phase growth arrest and cell elongation in the control cells with anti-*ftsZ* PNA or expressed antisense indicates that these effects (which were also observed in the *hok*/*sok*$^+$ cells at 37°C in culture growth and microscopy experiments) may be mediated via inhibition of FtsZ [5]. The smooth appearance of elongated Top 10 *hok*/*sok*$^+$ cells in the lag phase are typical of *ftsZ* deficiency/inhibition [21], and the degree of inhibition seem to be affected by the plasmid copy number (compare the lag phase morphology of Top 10 hok/*sok*$^+$ cells containing high copy number plasmid with the CSH50 strain containing low copy number). On the other hand, the observation that the toxic effects of *ftsZ* over-expression are more apparent in *hok*/*sok*$^+$ cells at the exponential growth phase and the observation of dividing cells in *hok*/*sok*$^+$ cells at 42^{o}C suggest that the *hok*/*sok* locus may also enhance cell division/FtsZ activity at the exponential growth phase or in cells growing under stress. This is also consistent with the rapid exponential growth of *hok*/*sok*$^+$ cells earlier reported in both normal and stressful growth conditions [5], as well as the observation of cells with multiple nuclear segmentations in *hok*/*sok*$^+$ cells when *ftsZ* was silenced with expressed antisense. Increasing the level of FtsZ is known to induce minicell formation at low levels and produce smooth filaments at high levels [21]. This is consistent

with our findings when *ftsZ* over-expression was induced, leading to filaments with multiple nuclear segmentations and small-sized cells in *hok*/*sok*$^-$ cells, and smooth filaments in *hok*/*sok*$^+$ cells (which is indicative of high level *ftsZ* over-expression). It is very likely that the *hok*/*sok* locus inhibits cell division via inhibition of FtsZ activity during the lag phase of growth of bacterial cultures to enable the cells adapt to their environment, as has been previously suggested [5]. We suspect the *hok*/*sok*-induced inhibition of FtsZ activity at the early growth phase may subsequently induce *ftsZ* over-expression, leading to enhanced cell division and high exponential growth rate.

Although inhibition of cell division is widely associated with bacterial SOS response to DNA damage, the observation of elongated *hok*/*sok*$^+$ cells in a strain of *E. coli* that lacks the SOS response (SS996) suggests that the *hok*/*sok*-induced elongation of cells and inhibition of cell division in cells growing under temperature stress is not mediated via SOS induction. This is in agreement with the report that *hokE* is not up-regulated like other SOS genes following UV-irradiation [24]. Nevertheless, the *hok*/*sok* locus seems to involve a mechanism downstream of the SOS pathway, probably at the point of FtsZ action. The mutant form of FtsZ expressed in the SS996 strain is unable to bind SulA in the SOS response pathway [17, 18], and possibly other proteins [22, 25, 26], which could lead to the differences in the observed growth effects of the *hok*/*sok* locus in SS99 strain.

5. Conclusion

This study has shown that the bacterial growth changes associated with the *hok*/*sok* locus may be mediated via alterations in FtsZ activity during cell division. FtsZ is a potent drug target, and its roles in these protective mechanism(s) provided by the *hok/sok* locus could

possibly be exploited to induce self-killing in the host bacteria cells. However, the involvement of FtsZ in the inhibition of cell division may also involve a complex interaction with the products of other *fts* genes (e.g. FtsA and FtsQ) necessary for the localization of FtsZ and assembly of the Z-ring [19, 21, 22]. Therefore, a better understanding of the exact mechanisms of the involvement of FtsZ in the *hok*/*sok*-induced growth inhibition and how it could be exploited as a drug target would require additional genetic and biochemical investigations.

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- The role of FtsZ in *hok*/*sok* induced bacterial growth changes was investigated
- *Hok*/*sok*$^{+}$ cells appeared bigger and elongated at lag phase of normal growth
- Cells growing under temperature stress showed evidence of increased division
- The *hok*/*sok* induced growth changes are mediated via altered FtsZ activity
- FtsZ could be exploited as a drug target to combat *hok*/*sok* protective effects on host cells