THE ROLE OF THE HOK/SOK LOCUS IN BACTERIAL RESPONSE TO STRESSFULL GROWTH CONDITIONS

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ABSTRACT

The *hok*/*sok* locus is renowned for its plasmid stabilization effect via post-segregational killing of plasmid-free daughter cells. However, the function(s) of the chromosome-encoded loci, which are more abundant in pathogenic strains of a broad range of enteric bacteria, are yet to be understood. Also, the frequent occurrence of this toxin/antitoxin addiction system in multi-drug resistance plasmids suggests additional roles. In this study, the effects of the *hok*/*sok* locus on the growth of bacteria in stressful growth-limiting conditions such as high temperature and antibiotic burden were investigated using hok/*sok* plasmids. The results showed that the *hok*/*sok* locus prolonged the lag phase of host cell cultures, thereby enabling the cells to adapt, respond to the stress and eventually thrive in these growth-limiting conditions by increasing the growth rate at exponential phase. The *hok*/*sok* locus also enhanced the survival and growth of cells in low cell density cultures irrespective of unfavourable growth conditions, and may complement existing or defective SOS mechanism. In addition to the plasmid stabilization function, these effects would enhance the ability of pathogenic bacteria to establish infections and propagate the antibiotic

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resistance elements carried on these plasmids, thereby contributing to the virulence of such bacteria.

Keywords: *Hok*/*sok*, plasmid, antibiotic resistance, stress response, bacterial growth.

1. INTRODUCTION

The *hok*/*sok* locus is a well established type 2 toxin/antitoxin system which was originally discovered due to its stabilizing activity on the inheritance of plasmid R1 [1]. It encodes a highly potent host killing (Hok) toxin, and an antisense RNA antitoxin, suppression of killing (Sok). The Sok antisense RNA regulates the expression of the *hok* mRNA by binding to the *hok* mRNA and initiating RNase III decay of the duplex, thereby inhibiting the translation of the *hok* transcript [2, 3]. However, in daughter cells that have lost the plasmid, the acquired Sok RNA are more rapidly degraded than the *hok* mRNA, thereby releasing the more stable *hok* mRNA for translation and subsequent cell death by the toxin produced. In this way, the *hok*/*sok* locus efficiently ensures that all surviving daughter cells inherit the plasmid by killing plasmid-free daughter cells, thereby playing a major role in plasmid maintenance and stability [4]. This plasmid stabilizing activity was subsequently characterized and found to localize on the *parB* region of the R1 plasmid [5]. The R1 plasmid carries several genes that encode resistance traits and in this way is able to impart multi-drug/antibiotic (ampicillin, chloramphenicol, kanamycin, streptomycin and sulphonamides) resistance to its host bacteria [6-8]. In addition to being found in plasmids, *hok*/*sok* loci have also been identified in the chromosomes of enterobacteria [9]. It has been established that the chromosomally-encoded loci do not mediate plasmid stabilization. However, their function(s) are not yet understood. Interestingly, chromosomally-encoded *hok*/*sok* loci are more abundant in pathogenic strains of *E. coli* [10].

The *hok*/*sok* toxin/antitoxin locus is one of the most frequently reported plasmid addiction systems occurring in plasmids that encode extended spectrum beta-lactamases, especially CTX-M ESBLs [11]. Several other plasmid stability systems have been described in *E. coli*, and have been shown to inhibit cell division until plasmid replication is achieved [12]. Furthermore, other toxin/antitoxin systems have been described, and are associated with stress response elements that help bacteria survive unfavourable growth conditions[13]. Given that the pathogenic strains of bacteria in which the *hok*/*sok* loci are more abundant most often encounter and overcome stressful growth condition (such as high temperature in fevers), it is possible that the *hok*/*sok* locus may also be involved in helping the bacteria survive these stressful growth conditions. Being found in close association with antibiotic resistance genes, the *hok*/*sok* locus may also contribute to antibiotic resistance by helping bacterial cells survive periods of exposure to antibiotics. These roles in stress response would be in addition to their established roles in plasmid maintenance, which would be very helpful to the host bacteria in the propagation of resistance mechanisms; thereby contributing to the pathogenicity and virulence of such bacteria. Hence, this study investigated the effects of the *hok*/*sok* locus on the growth of bacteria in stressful growth conditions such as high temperature and antibiotic stress.

2. Materials and methods

2.1. Plasmid construction

E. coli strains and plasmids used in this study are listed in Table 1. Plasmids pCCB1, pCCB2 and pCCB3 were constructed by inserting the *parB* locus of *E coli* plasmid R1 into plasmid vectors pUC19, pLAU80 and pHNZ respectively, to create new *hok*/*sok* study tools with better controls. The primers were designed with restriction sites for XbaI and HindIII (for

cloning into pUC19 and pLAU80 vectors), and NsiI and SbfI sites (for cloning into pHNZ vector). PCR amplification of the insert was done using Phusion® High Fidelity PCR Master Mix, with the *hok*/*sok*$^{+}$ pPR95 plasmid DNA as template. Vectors and inserts were subsequently digested with the appropriate restriction enzymes and ligated at a ratio of 1:3 with T4 DNA ligase. 5µl sample of the ligation mixtures were fractionated on 1% agarose gel to ensure successful ligation of the vectors and insert by comparing the bands on gel electrophoresis with those of the vectors and insert respectively. Competent Top10 and SS996 cells were then transformed with 2µl of the ligation sample, and plasmid extracted from 3 single colonies of each plasmid using QIAgene Miniprep kit. The plasmid preps were digested with appropriate restriction enzymes and compared with the vectors and insert bands on 1% agarose gel electrophoresis to check whether plasmids contained both the vectors and inserts. Transformants containing intact desired plasmids were then further analysed to ensure their integrity by PCR amplification, phenotype checks and sequencing. PCR amplification of the *parB* locus was done using the plasmid prep as template to ensure that the new plasmid contained the desired insert (*parB*). The yellow fluorescence and growth inhibition phenotype of pLAU80 and pHNZ vectors were checked by inducing expression with arabinose and IPTG respectively, on a lawn of the transformants on LB agar plates. The plates were then examined using G:Box imaging machine for fluorescence or growth inhibition. Sequencing was done by Eurofins MWG Operon Custom DNA Sequencing (www.eurofinsdna.com).

Table 1: Plasmids and bacterial strains

Plasmid/strain	Relevant features/genotype	Reference/source
pUC19	*hok/sok*$^{-}$, Amp^{R}	Invitrogen
pCCB1	*hok/sok*$^{+}$, Amp^{R}	This work

plAU80	*hok*/*sok*$^{-}$, *ftsZ-yfp*, Amp^{R}, Arabinose inducible	[14]
pCCB2	*hok*/*sok*$^{+}$, *ftsZ-yfp*, Amp^{R}, Arabinose inducible	This work
pHNZ	*hok*/*sok*$^{-}$, *ftsZ*-antisense, Chlr^{R}, IPTG inducible	[15]
pCCB3	*hok*/*sok*$^{+}$, *ftsZ*-antisense, Chlr^{R}, IPTG inducible	This work
Top 10	*F– mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara leu) 7697 galU galK rpsL (StrR) endA1 nupG*	Invitrogen
SS996	*Ωgfp {Δ(attλ)::sulApWgfp-mut2} sulA*$^{+}$ *sulB103 recA*$^{+}$	[24]

2.2. Temperature shift growth assay

Selective antibiotic media, LBamp (LB broth/agar + 100µg/ml ampicillin) were used for all growth cultures (unless otherwise stated) in order to eliminate cells that have lost the *hok*/*sok*$^{+}$ plasmid without allowing the expression of Hok toxin, thereby maintaining only cells containing the plasmid in the cultures. Overnight cultures were prepared by inoculating a single colony from the plate into 2ml of LB broth (Sigma) and incubating at 37^{o}C in a Stuart orbital incubator S1500 for 16hrs with shaking (180 rpm). The optical density of cultures was measured using SpectraMax spectrometer at 600nm. Overnight cultures were diluted to the required cell density in LBamp broth. 200µl of the diluted culture were then transferred into 96 well plates and incubated for 18-22hrs using Biotek Powerwave XS universal spectrometer and Gen 5 software to monitor culture growth (OD measured at 550nm). The lag time was scored as the average time taken to achieve an increase of 0.03 units in culture OD of replicate samples. The growth curve of the bacterial cells was first monitored to determine the early logarithmic phase (about 4hrs). After the growth profiles of these strains were determined under standard conditions, temperature shift experiments were

conducted by increasing the incubation temperature from 37^{o}C to 42^{o}C (after initial 4hrs of growth at 37^{o}C).

2.3. Antibiotic susceptibility assays

The antibiotic susceptibility of bacterial strains containing *hok*/*sok* plasmids were tested by both disk diffusion method and MIC determination by broth dilution method. For disk diffusion tests, overnight broth cultures of *hok*/*sok*$^{+}$ and *hok*/*sok*$^{-}$ strains diluted to about 10^{6} CFU/ml, were plated on agar plates containing the specified antibiotics using sterile cotton buds to make a "lawn" of cells. The antibiotic disks (Oxoid) were then placed on the plate and incubated at 37^{o}C overnight. The sizes of the zones of inhibition around disks were compared with that of the control strains. MIC determination by broth dilution method was done using 96 well plates. For this test, overnight cultures were diluted to about 10^{6} CFU/ml with LB broth containing increasing concentrations of the desired antibiotics and 200µl added to each well. Plates were incubated at 37^{o}C in Biotek Powerwave XS universal spectrometer, and growth data obtained and analysed with Gen 5 software. MIC was scored as the lowest concentration of the drug at which no observable growth of the bacterial culture occurred after 18hrs of incubation.

2.4. Data analysis

All graphs and growth curves were produced with Microsoft Office Excel 2007. Statistical analyses were done using IBM SPSS Statistics version 19. Analysis of the effect of the *hok*/*sok* locus on the lag time of bacterial cell cultures was performed by comparing the mean lag times of *hok*/*sok*$^{+}$ and *hok*/*sok*$^{-}$ cells using independent samples exact test (Mann–Whitney U test) at 0.05 significance level.

3. Results

3.1. New hok/sok plasmids (pCCB1, pCCB2 and pCCB3)

Plasmid pCCB1 was constructed by inserting the *hok*/*sok* locus (*parB*) into pUC19 vector (Figure 1), making pUC19 a better control plasmid which differs only in the absence of *hok*/*sok* locus. Plasmids pCCB2 and pCCB3 were also constructed by inserting the *hok*/*sok* locus (*parB*) into pLAU80 (which contains *ftsZ-yfp)* and pHNZ (which contain *ftsZ* antisense) respectively. The integrity of the newly constructed plasmids were verified by restriction enzyme digestion, PCR amplification, phenotype check and sequencing. Sequencing results were compared to the expected sequences of the constructed plasmids, and matched the predicted sequences of the new plasmids.

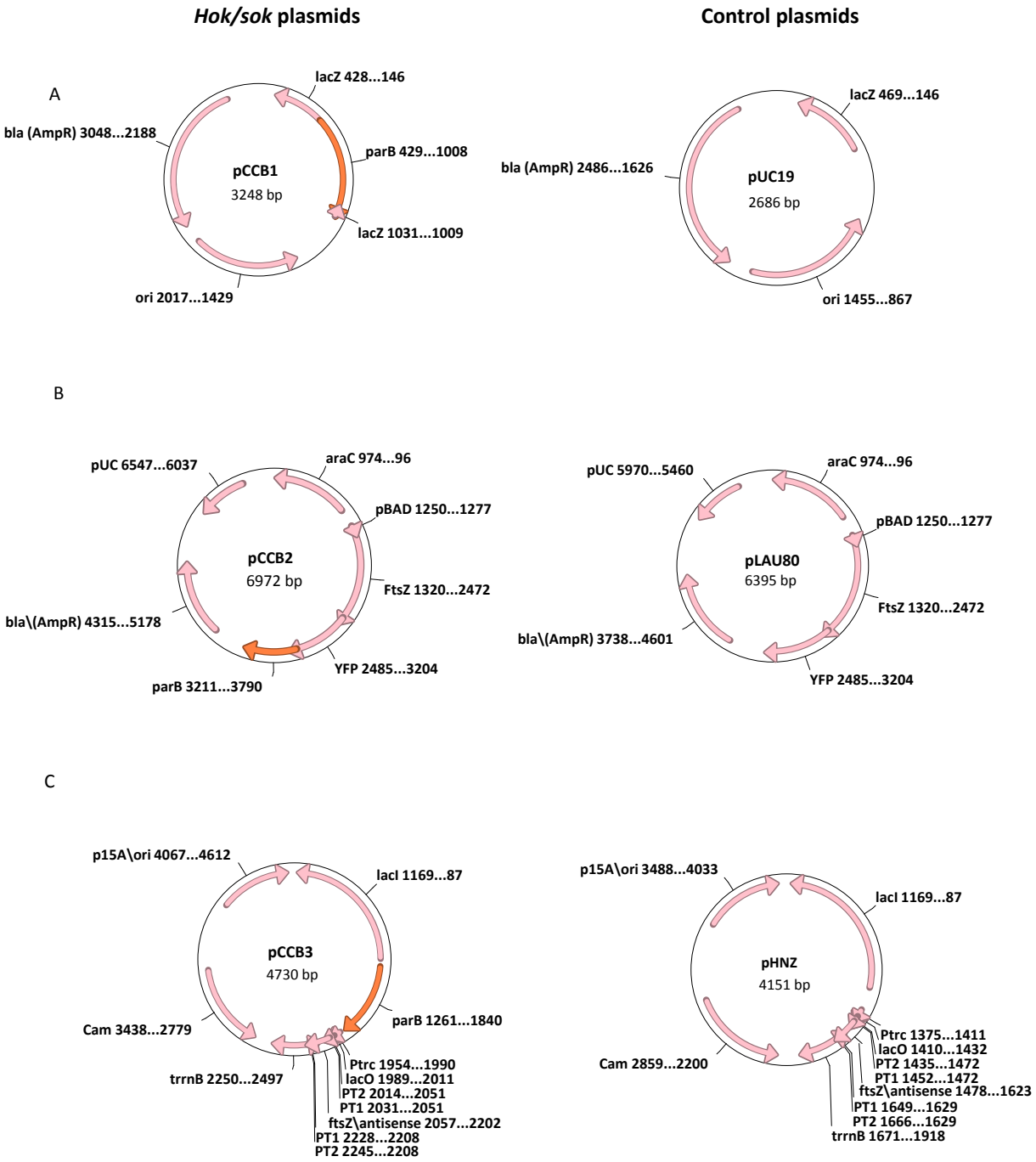


Figure 1: Graphic maps of the newly constructed *hok*/sok plasmids and their control plasmids. Plasmids pCCB1 (A), pCCB2 (B) and pCCB3 (C) were constructed by inserting the *parB* (*hok*/*sok*) locus into pUC19, pLAU80 and pHNZ plasmid vectors respectively, thereby creating study tools with more appropriate controls.

3.2. Effect of the hok/sok locus on the growth of E. coli under temperature stress

When the growth of *E. coli* Top 10 cells containing the *hok*/*sok*$^{+}$ (pCCB1) and *hok*/*sok*$^{-}$ (pUC19) plasmids was monitored at normal growth temperature (37°C), the *hok*/*sok*$^{+}$ cells exhibited a prolonged lag phase (Figure 2A). The presence of the *hok*/*sok* locus significantly increased the lag time of Top 10 host bacteria cell cultures by approximately one and half hours (p =0.002), from an average of 2hrs 45mins in the *hok*/*sok*$^{-}$ cells to 4hrs 10mins in *hok*/*sok*$^{+}$ cells (Figure 2A inset). When the growth temperature was shifted to 42°C, the lag phase of the *hok*/*sok*$^{-}$ cells became prolonged, whereas that of the *hok*/*sok*$^{+}$ cells appeared to be shortened (Figure 2B). The *hok*/*sok*$^{+}$ also showed a higher rate of exponential growth (indicated by the steeper slope of the exponential growth phase) at both incubation temperatures (37^{o}C and 42^{o}C) when compared to the *hok*/*sok*$^{-}$ cells grown under similar conditions. In addition, the inhibitory effect of high incubation temperatures on the growth of the bacterial cells was hindered in the *hok*/*sok*$^{+}$ cells at 42^{o}C, allowing the culture to reach a higher optical density than the *hok*/*sok*$^{-}$ cells. In general, these results show that the *hok*/*sok* locus delays the onset of growth of bacteria cell cultures, and enables the cells to grow rapidly at a later stage, despite the prevailing/unfavourable growth temperature. The results also show that temperature stress stimulates rapid growth of *hok*/*sok*$^{+}$ cells, whereas it inhibits the growth of *hok*/*sok*$^{-}$ cells.

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

In an attempt to investigate the mechanism of induction of growth arrest observed as delayed onset of growth of the *hok*/*sok*$^{+}$ cells at normal growth temperature, a strain of *E. coli* which is insensitive to the SOS-induced cell division inhibition protein SulA (SS996) was

used as the host strain for the *hok*/*sok* plasmids. When the growth patterns of these cells were monitored at normal growth temperature (37^{O}C) there was also a significant increase (p =0.002) in the lag time of the *hok*/*sok*$^{+}$ cells at 37^{O}C when compared to the *hok*/*sok*$^{-}$ cells (Figure 2C). In this case, the lag time was increased from an average of 1hr 50mins in *hok*/*sok*$^{-}$ cells to an average of 4hrs 30mis in *hok*/*sok*$^{+}$ cells (Figure 2C inset). The lag phase of the *hok*/*sok*$^{+}$ cells was more prolonged in the SS996 strain (by approximately 3hrs) than in Top 10 strain (which was increased by approximately 1.5hrs). At 42^{O}C, the *hok*/*sok*$^{-}$ cells showed a more prolonged lag phase and inhibited growth than was observed with Top 10 strain (Figure 2D) whereas the *hok*/*sok*$^{+}$ cells showed very rapid increase in culture OD at both incubation temperatures. In addition, the growth rate of *hok*/*sok*$^{-}$ SS996 cells at 37^{O}C was quite comparably higher than that of Top 10 strain, and competes well with the growth rate of *hok*/*sok*$^{+}$ cells of both strains.

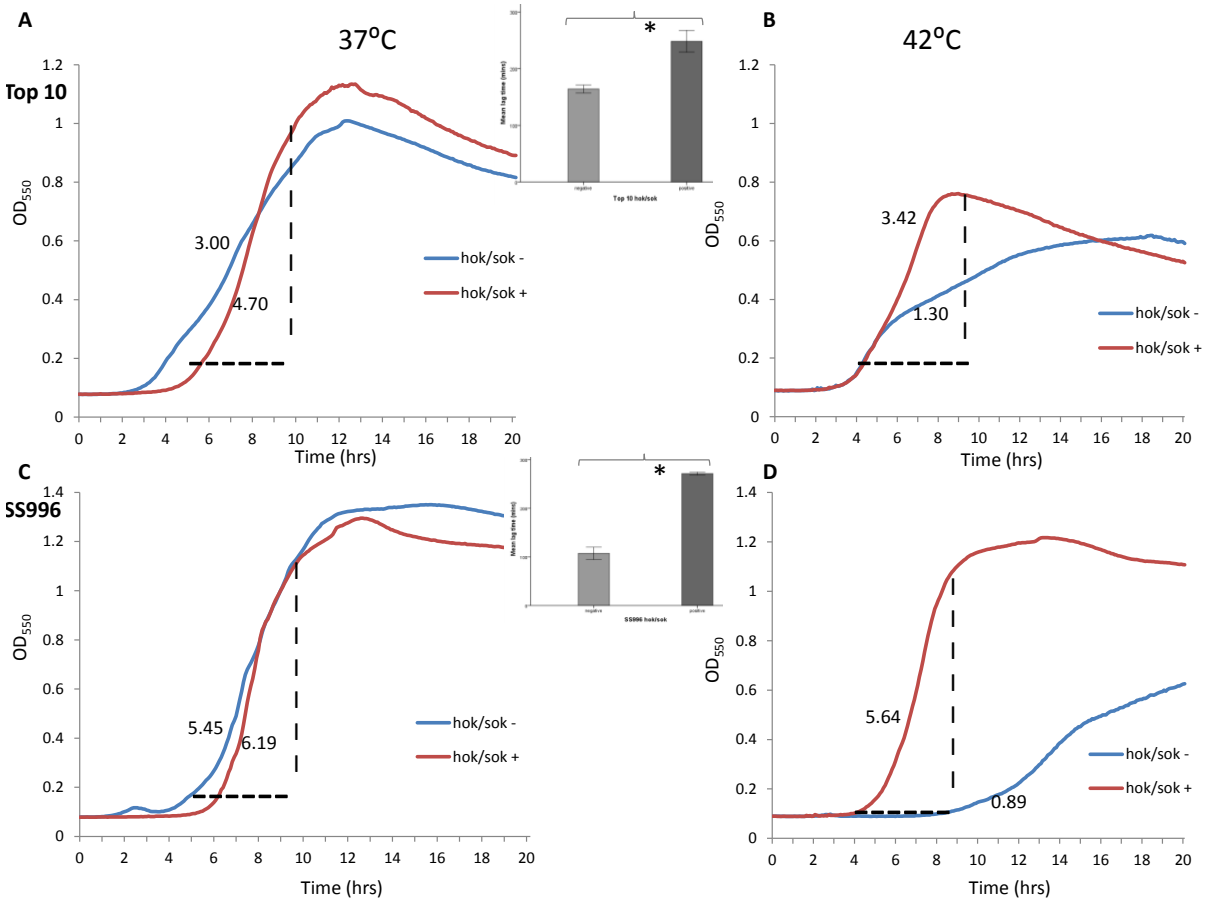


Figure 2: Effect of *hok/sok* locus on the growth of bacteria under temperature stress

Graphs show the growth curves of *E. coli* Top 10 (A, B) and SS996 (C, D) strains containing the *hok*/*sok*$^+$ (pCCB1) or *hok*/*sok*$^-$ (pUC19) plasmids in LBamp. Insets show bar charts of the mean lag time for *hok*/*sok*$^+$ and *hok*/*sok*$^-$ cells of each strain. Data represents the means of 6 independent experiments. Error bars: +/- SD, p=0.002 at 0.05 significance level.

3.4. Effect of the hok/sok locus on the growth of bacterial cells at low starting culture cell density

In ten-fold serial dilution cultures, the growth of the *hok*/*sok*$^+$ cells was generally rapid at the exponential phase, and reached higher OD than the *hok*/*sok*$^-$ cells in all cell densities and incubation temperatures (Figure 3). In other words, the growth of the *hok*/*sok*$^+$ cell cultures was not inhibited at lower cell densities as was observed for the control cells. In the SS996

host strain, there was a striking difference between the growth of the *hok*/*sok*$^{+}$ and *hok*/*sok*$^{-}$ cells at very low cell densities, with the *hok*/*sok*$^{+}$ cells growing normally and reaching a very high OD (≈1.3) even at lower cell densities while the growth of *hok*/*sok*$^{-}$ cells was severely impaired (Figure 3A). For cells growing under temperature stress at 42^{O}C, the growth of SS996 *hok*/*sok*$^{-}$ cells was completely inhibited at low cell densities whereas the *hok*/*sok*$^{+}$ cells grew to a very high OD at all cell densities (Figure 3B).

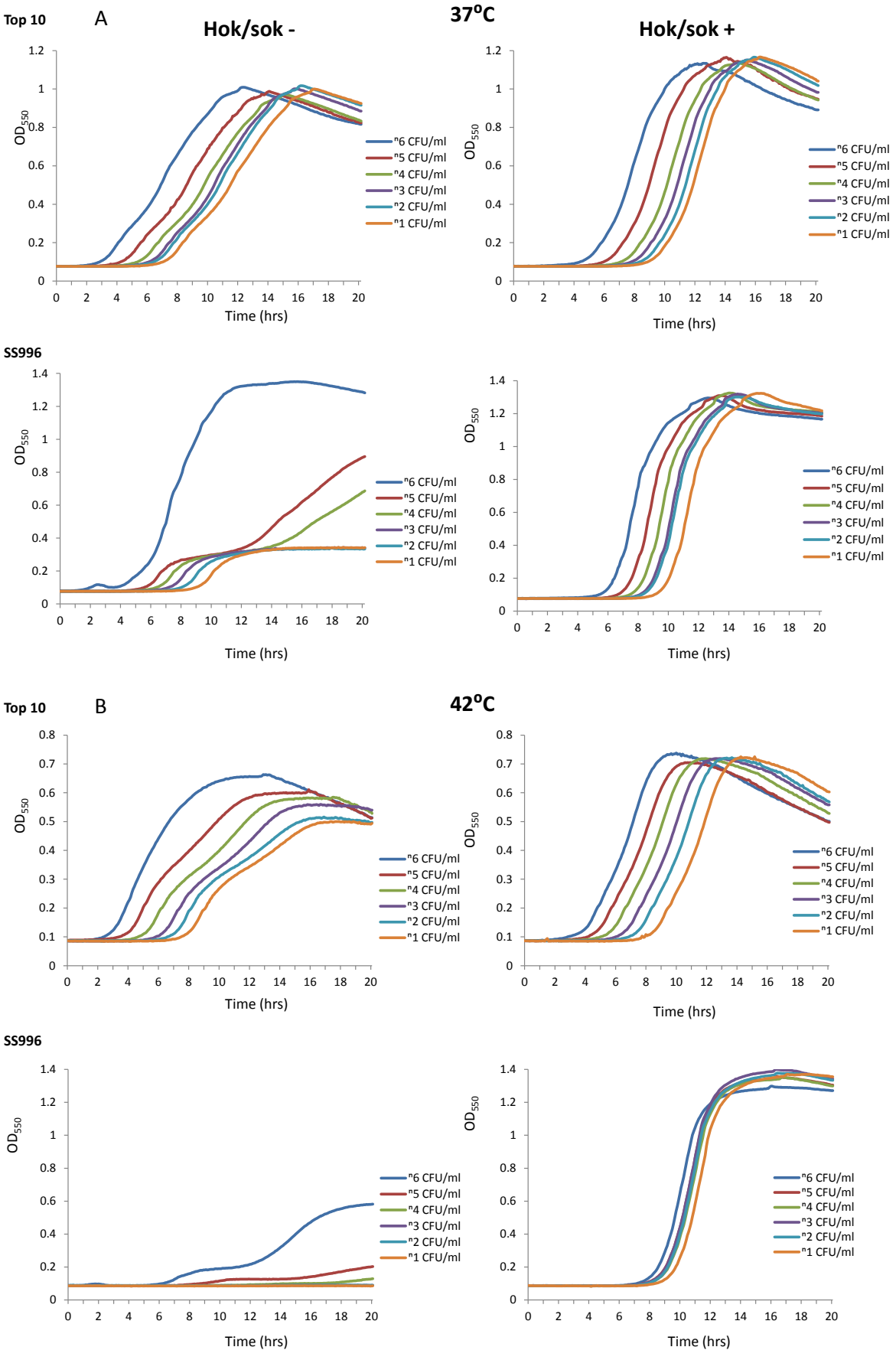

Top 10
A
37°C
Hok/sok -
Hok/sok +
SS996
B
42°C
OD550
Time (hrs)
~6 CFU/ml
~5 CFU/ml
~4 CFU/ml
~3 CFU/ml
~2 CFU/ml
~1 CFU/ml

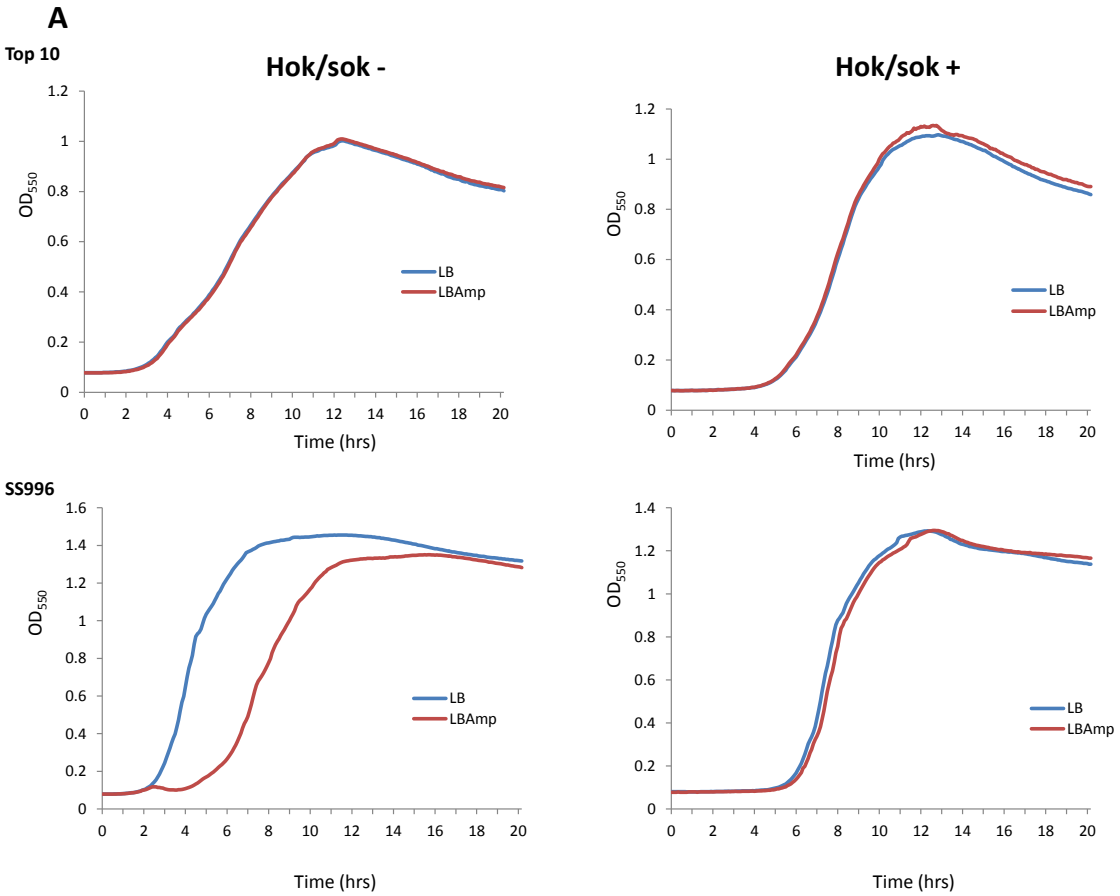
Figure 3: Effect of the *hok*/*sok* locus on the growth of bacterial cells in low starting culture cell densities. Graphs show the growth curves of *hok*/*sok*$^{+}$ and *hok*/*sok*$^{-}$ *E. coli* (Top 10 and SS996) in a ten-fold serial dilution in LBamp at 37^{o}C (A) and 42^{o}C (B), with the *hok*/*sok*$^{+}$ cells showing improved growth especially at lower cell densities (^{n}=1x10^{n}).

3.5. Effect of the hok/sok locus on the growth of bacteria in antibiotic selective media and plasmid stability

The *hok*/*sok*$^{+}$ and control plasmids (pCCB1 & pUC19) both contain ampicillin resistance gene, thus enabling selection of cells containing the plasmid in ampicillin media. When the growth of the bacterial cells in antibiotic selective medium was compared with the growth in non-selective medium, the Top 10 cells showed similar growth pattern in both selective and non-selective media for both the *hok*/*sok*$^{+}$ and *hok*/*sok*$^{-}$ cells but the growth of SS996 cells were found to vary in the two media. SS996 *hok*/*sok*$^{+}$ cells grew similarly well in both selective and non-selective media, whereas the growth of the *hok*/*sok*$^{-}$ cells was inhibited in ampicillin-containing media, LBamp (Figure 4A). This disparity in the growth of SS996 *hok*/*sok*$^{-}$ cells in antibiotic selective and non-selective media was found to be more apparent at lower starting culture cell densities. This observation was verified with cultures grown on solid media, which also showed that all the cells grew well in both LB and LBamp agar plates, except SS996 *hok*/*sok*$^{-}$ cells which did not grow well in LBamp plates. Similar to what was observed in the liquid cultures, the SS996 *hok*/*sok*$^{-}$ cells grew well in LB plates, whereas growth was only observed in areas with high inoculating colony density in LBamp plates (Figure 4B).

However, when the cells were grown on agar plates containing ampicillin, X-gal and IPTG, the plates showed blue colonies for both SS996 and Top 10 *hok*/*sok*$^{-}$ cells, but with a few

white colonies for SS996 strain (Figure 5A). Since the control plasmid, pUC19 contains a functional *lacZ* gene (which was disrupted by the insertion of *parB* locus to yield pCCB1- refer to Figure 1), the presence of blue colonies indicates the presence of the plasmid. Therefore, the result showed that few of the SS996 cells have lost the control plasmid, although most of the cells still contained the plasmid. Gel electrophoresis analysis of the plasmids extracted from *hok*/*sok*$^{+}$ and *hok*/*sok*$^{-}$ cell cultures grown under similar conditions showed a higher plasmid yield of the *hok*/*sok*$^{+}$ plasmids than the control plasmid in both strains (Figure 5B). In addition, the SS996 *hok*/*sok*$^{-}$ cells appeared to lose the plasmid over time, as the band intensity of plasmid extract from an old stock was very low compared to that of newly transformed stock. Nevertheless, the blue colonies from older stock still showed a good plasmid yield.


A
Top 10
Hok/sok -
Hok/sok +
SS996
OD550
Time (hrs)
LB
LBAmp

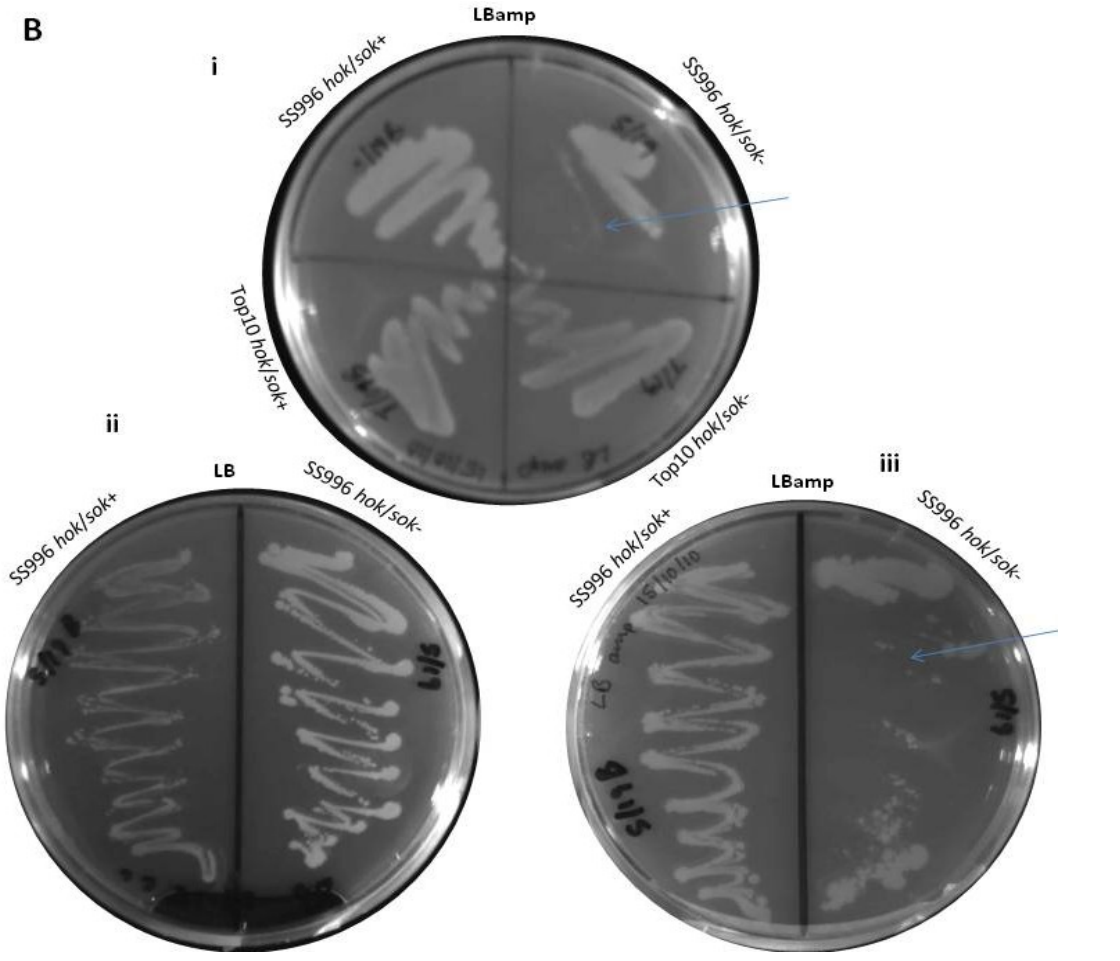

B
i
LBamp
SS996 hok/sok+
SS996 hok/sok-
Top10 hok/sok+
Top10 hok/sok-
ii
LB
iii
LBamp

Figure 4: **Effect of the *hok*/*sok* locus on the growth of bacteria cells in antibiotic-selective media.**

Graphs show growth curves of *hok*/*sok*$^+$ and *hok*/*sok*$^-$ Top 10 and SS996 cells in 100µg/ml ampicillin selective media (LBamp) and non-selective media (LB) at 37^{o}C. Agar plates show (i) growth of all 4 strains on LBamp agar, (ii) growth of SS996 *hok*/*sok*$^+$ and *hok*/*sok*$^-$ strains on LB agar, (iii) differential growth of SS996 cells on LBamp agar. Arrows indicate regions of inhibited growth of SS996 *hok*/*sok*$^-$ cells.

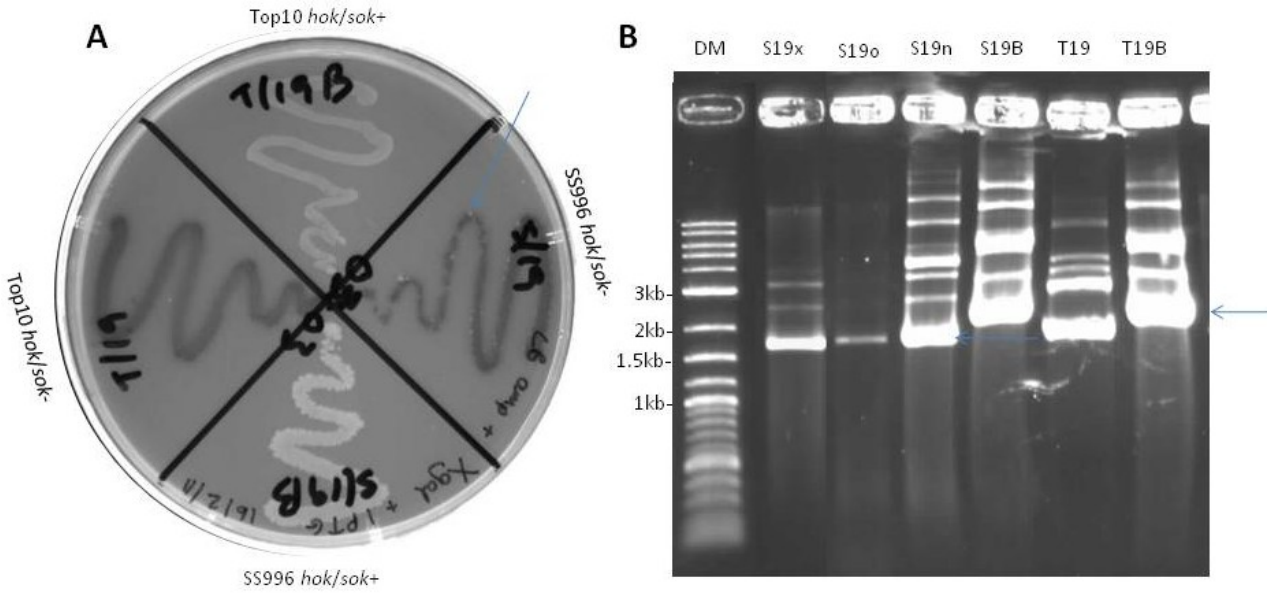


Figure 5: **Effect of the *hok*/*sok* locus on plasmid maintenance**

Agar plate shows partial loss of plasmid pUC19 from SS996 cells (white and blue colonies on LBamp + X-gal + IPTG agar indicated by arrow); Gel shows higher plasmid yield from Top 10 and SS996 *hok*/*sok*$^+$ cells (T19B and S19B) than the control *hok*/*sok*$^-$ strains (T19, S19-x=blue colonies from x-gal plate, o=cells from an old stock, n=newly transformed cells). Arrows indicate plasmid bands. DM=NEB 2-log DNA ladder.

3.6. Effect of the hok/sok locus on β-lactam antibiotic susceptibility

Antibiotic susceptibility tests by disk diffusion showed zones of growth inhibition for the β-lactam antibiotics amoxicillin and ampicillin in *hok/sok*$^{-}$ cells, but not in *hok/sok*$^{+}$ cells (Figure 6A). These inhibition zones increased with increasing drug concentration. Similar effects on growth were also observed in cultures grown in LB broth with increasing concentrations of ampicillin (Figure 6B). However, Nitrocefin showed colour change in both *hok/sok*$^{+}$ and control cells, which indicates that β-lactamases are produced in both strains. This shows that the SS996 *hok/sok*$^{-}$ cells still retained the plasmids despite being susceptible to ampicillin and amoxicillin. In other words, the susceptibility of the SS996 control cells to ampicillin is not entirely due to plasmid loss. Growth inhibition was not observed with penicillin and cloxacillin B. Rifampicin D, which is an RNA polymerase inhibitor, showed zone of inhibition in both *hok/sok*$^{-}$ and *hok/sok*$^{+}$ cells. For MIC determination, complete inhibition of growth for the SS996 *hok*/*sok*$^{-}$ cells was only observed at 25mg/ml ampicillin concentration (Table 2), which is about 1000X the MIC of ampicillin in *E coli*. This also shows that the cells are still resistant to ampicillin; therefore the observed susceptibility may not be wholly attributed to plasmid loss. In general, these results show that the *hok*/*sok*$^{+}$ cells were able to survive and grow well in conditions of high ampicillin concentrations, in contrast to the *hok*/*sok*$^{-}$ cells, indicating that the *hok/sok* locus helps cells overcome/tolerate the growth inhibitory effects of ampicillin or reduce their susceptibility to the β-lactam antibiotics.

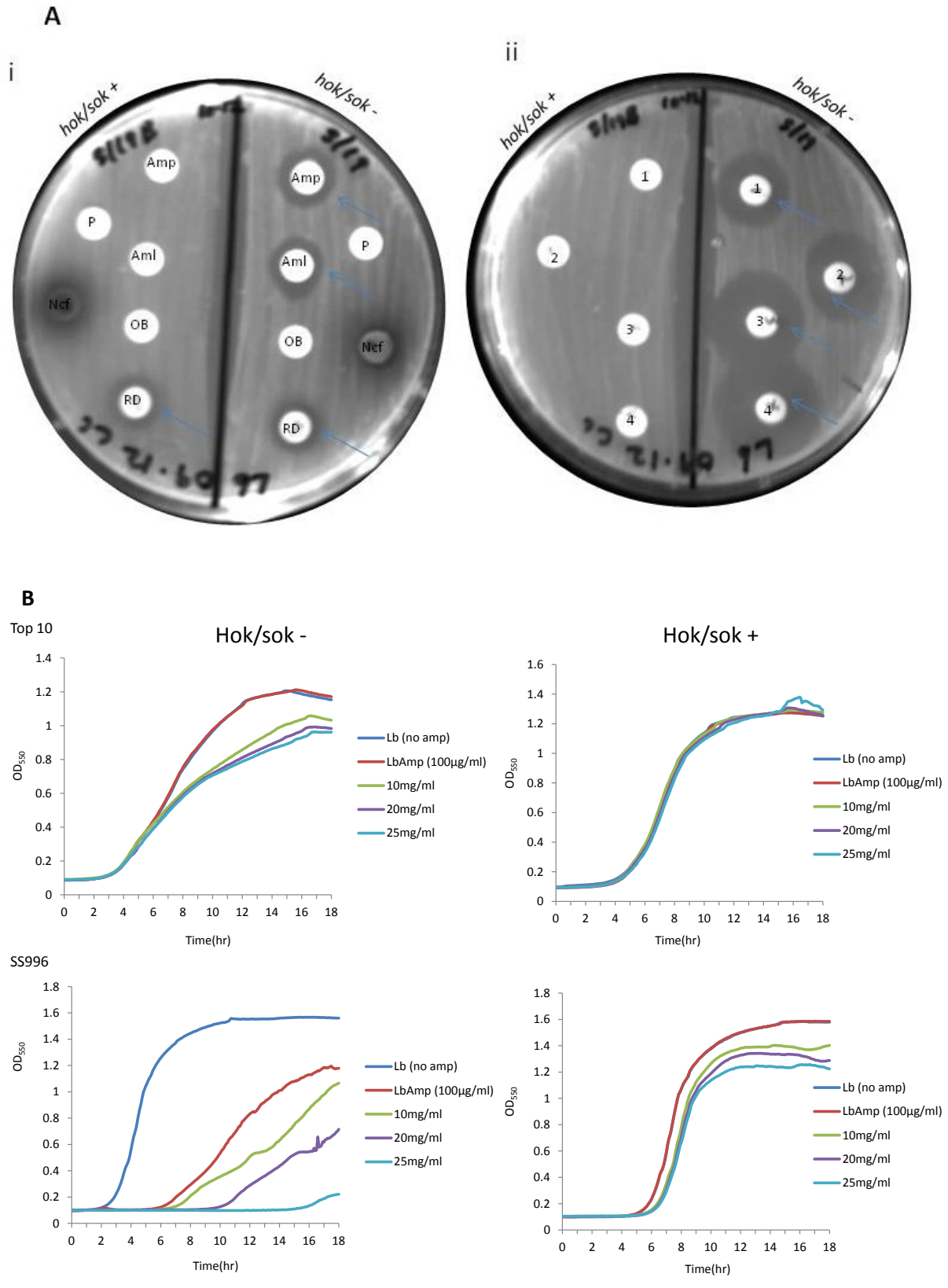


Figure 6: **Effect of the *hok*/*sok* locus on β-lactam antibiotic susceptibility.**

A: LB agar plates show susceptibility tests with (i) several β-lactam antibiotics (amp=2µg ampicillin, aml=2µg amoxicillin, OB=5µg cloxacillin B, P=5 units penicillin G, Ncf= nitrocefin)

and rifampicin D (RD, 5µg); (ii) increasing amounts of ampicillin (1, 2, 3, 4=100, 200, 300, 400µg ampicillin) applied to disks. Arrows indicate zones of inhibition. B: Graphs show growth curves of cells containing the *hok*/*sok* plasmid, pCCB1 or the control plasmid, pUC19 in increasing concentrations of ampicillin.

Table 2: MIC (µg/ml) of ampicillin in *E. coli* cells containing plasmid-borne *hok*/*sok* locus

Host strain	***Hok*/*sok*$^-$**	***Hok*/*sok*$^+$**
Top 10	>30000	>30000
SS996	25000	>30000

4. Discussion

Toxin/antitoxin systems such as *hok/sok* are present on the majority of large plasmids isolated from clinical strains of enteric pathogens [16-20]. The plasmid stabilization function of the *hok*/*sok* locus was found to be affected by factors such as temperature, growth rate and dilution rates in a study in which a temperature sensitive plasmid was used without antibiotic selection [21]. Conversely, this study investigated the effect of the *hok*/*sok* locus on the growth of bacterial cells using plasmids that contain the *hok*/*sok* locus and antibiotic selection to eliminate plasmid-free cells without inducing cell killing by Hok toxin expression. A close look at the plasmids previously used for studies of the *hok*/*sok* locus [140,10] showed that there are differences between the plasmid backbones of the *hok*/*sok*$^+$ plasmid (pPR95) and the *hok*/*sok*$^-$ plasmid often used as control (pOU82). This could compound experimental results since the two plasmids differ in more ways than just the *hok*/*sok* locus.

Hence, a more appropriate set of *hok*/*sok*$^{+}$ plasmids that have better control plasmids were constructed.

The results of this study show that the *hok*/*sok* locus generally increased the lag phase and the rate of exponential growth in all strains used. This may imply that *hok*/*sok* locus inhibits the onset of growth of bacteria during the lag phase, and in some way this enables the cells to adapt to the growth conditions and prepare them for survival in stressful conditions. This view is supported by the observation that the *hok*/*sok*$^{+}$ cells survive better under temperature stress, indicating that the *hok*/*sok* locus confers a selective advantage on host cells when grown in adverse conditions. However, the situation is quite complex, as the outstanding phenotype observed is influenced by the genetic makeup of the host strain and the type of stress encountered. For example, the effect of temperature stress was quite obvious in both strains, whereas the effect of antibiotic stress was more obvious in SS996 strain. In all strains however, the *hok*/*sok* locus enhanced the growth of cells in low cell density cultures, especially at high incubation temperature.

Remarkably, we observed that the *hok*/*sok* locus confers a selective advantage to the cells in ampicillin-selective media, and this was especially apparent in SS996 strain (which is defective in SOS response). The SS996 has a mutation in *ftsZ* that makes the protein insensitive to the SOS-induced cell division inhibitor SulA, thus allowing continued growth of the cells in conditions that would otherwise induce the SOS response and inhibit cell growth [22, 23]. It has been reported that β-lactams and fluoroquinolones induce SOS response that temporarily inhibit cell division and help bacteria survive their lethal effects [24, 25]. For the β-lactic antibiotics, the mechanism of induction of this SOS response has been attributed to defective cell wall synthesis instead of DNA damage. Irrespective of the mechanism of

induction, survival of such bacteria exposed to antibiotic treatment depends on a functional SOS response and subsequent inhibition of cell division, often leading to filamentation of the cells. The combined effects of this SOS response and *bla* gene expression from the plasmid vector used in this study allow bacterial growth in ampicillin-selective media. However, in SS996 host strains, these growing SS996 cells become more susceptible to antibiotic killing in ampicillin-selective media due to the fact that ampicillin and other β-lactam antibiotics kill only dividing cells [26]. This increased susceptibility to ampicillin was more apparent in conditions where *bla* gene expression from the plasmid is insufficient to destroy the drug (low cell densities and/or high drug concentrations). Interestingly, this sensitivity to ampicillin is reversed in the *hok*/*sok*$^{+}$ cells, suggesting that the *hok*/*sok* locus may induce inhibition of cell division (in the prolonged lag phase) via a mechanism that is at least partly related to the SOS response. This effect is similar to the normal function of FtsZ in the SOS response. Since the SS996 strain has an allele of FtsZ that allows continued growth of the cells despite SOS response induction, it is possible that the effects of the *hok*/sok locus on bacterial growth observed in this study may be mediated via an effect on FtsZ. This would temporarily inhibit cell growth and give the cells time to produce enough β-lactamase to destroy the drug before growth resumes. These results also indicate that the *hok/sok* locus can functionally complement the SOS response (especially in SOS defective strains such as the SS996) by helping the cells adapt to and thrive in growth-limiting conditions such as temperature and antibiotic stress. Bearing in mind that the R1 plasmid (and many others) in which the *hok*/*sok* locus occurs naturally is associated with multi-drug resistance, these results strongly indicate that the *hok*/*sok* locus contributes to antibiotic resistance. The observed tolerance to higher concentrations of antibiotic, in combination with plasmid stabilization via post-segregational killing of plasmid-free cell [4], would

enhance selection of highly resistant strains in natural conditions. In addition, the ability of the *hok*/*sok*$^{+}$ cells to thrive in conditions of low cell densities and high temperature could help cells survive at low density during times of stress, for example during early stages of infection or low grade exposure to the bacteria. In normal living conditions, human and animal hosts are often exposed to low amounts of pathogenic bacteria that fail to establish an infection because the organisms are too few to successfully colonize the host. If the presence of the *hok*/*sok* locus in bacteria cells enhances the growth of low numbers of the organism, it stands to reason that it will help pathogenic strains to successfully infect their hosts even in conditions of low pathogen exposure, thereby contributing to their virulence. By enhancing the survival of the host cells in these unfavourable circumstances/growth conditions, the *hok*/*sok* locus would subsequently enhance the propagation of the resistance elements carried on the plasmid. Therefore, a better understanding of the *hok*/*sok* system could be beneficial in the global war against antibiotic resistance.

5. Conclusions

This study showed that the *hok*/*sok* locus enables bacteria to respond favourably to stressful or restrictive growth conditions. Specifically, the *hok*/*sok* locus enhances the growth of bacterial cells in conditions that would otherwise inhibit bacterial growth such as high temperature and low starting culture cell densities. It also enables the cells to continue to grow in or tolerate higher concentrations of ampicillin, thereby contributing to beta-lactic antibiotic resistance. In this way, the *hok*/*sok* locus enables the host bacteria to survive and even thrive in otherwise detrimental conditions, which would expectedly lead to the propagation of the associated resistance elements often contained in the plasmid. It may

therefore be possible to exploit the *hok*/*sok* system as a drug target to induce self-killing in such cells and enhance antibiotic efficacy.

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