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Developing an Antisense RNA Vector for Skp Chaperone Knockdown Analysis in *Escherichia coli* BW25113

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SUMMARY Seventeen-Kilodalton Protein (Skp) is a highly conserved periplasmic chaperone protein among Gram-negative bacteria. It plays a crucial role in the folding and assembly of outer membrane proteins (OMPs) in the periplasms. Studies utilizing *skp* knockout (Δ*skp*) mutant cells found that Skp also likely prevents periplasmic protein aggregation. This study aimed to develop a method to silence *skp* expression and characterize *skp* knockdown *Escherichia coli* cells. We employed anti-sense RNA (asRNA) silencing by constructing a *skp* asRNA insert and ligating it into a pHN678 vector, which contains an isopropyl-beta-thiogalactopyranoside (IPTG)-inducible promotor. The asRNA insert was designed to hybridize to a 40 base-pair (bp) region of the *skp* messenger RNA (mRNA) containing the Shine-Dalgarno (SD) ribosome binding site, as well as the first four codons of the coding sequence. Sequencing result of the asRNA vector, pJAHS, confirmed the successful and correct ligation of the insert. To characterize the knockdown cells, pJAHS was transformed into *E. coli* BW25113 cells for comparisons with Δ*skp E. coli* from the Keio collection. A zone of inhibition functional assay was used to assess the degree of *skp* knockdown in transformants. The results indicated that the Skp protein levels in pJAHS transformants were somewhere between wild-type (WT) and knockout levels. Growth of cultures at different temperatures suggested that the asRNA hybridization efficiency is greater at cooler temperatures, but this may have been an artifact of poor growth conditions. Lastly, growth-curve analysis of pJAHS transformants at varying concentrations of IPTG resulted in no significant phenotypical differences when compared to WT cells. This model allows for fine control of Skp expression and can guide and be utilized in future research of bacterial chaperone systems.

INTRODUCTION

Seventeen kilodalton protein (Skp) is one of the most well-researched outer membrane protein (OMP) chaperones [1]. The appearance of Skp resembles the eukaryotic cytoplasmic chaperone prefoldin, which is responsible for protecting partially folded proteins from aggregation and degradation [2, 3]. Skp has also been found to have functions in the maintenance of unfolded protein stability as well as chaperone mediated protein folding [4, 5]. Skp general chaperone has been found to maintain the polypeptide in an unfolded state while it is being ferried across the periplasm to prevent degradation. Skp, like many other chaperones has been proposed to be required for pathogenesis of certain Gram-negative bacteria by assisting with Type V Secretion System assembly [6, 7]. Specific genes encoding for autotransporters have been linked to pathogenesis in common bacterial pathogens such as *Bordetella pertussis*, *Salmonella enterica*, and *Pseudomonas aeruginosa* [6, 8, 9]. Research has suggested the involvement of protein chaperones in autotransporter biogenesis. Phan et. al. have suggested the roles of multiple different proteins, such as DsbA, Skp, SurA, and DegP, in maintaining the intermediate structure of the autotransporter in the periplasm [6, 9].

In this study, we aim to develop a method to knock down *skp* in a controllable manner and investigate the growth characteristics of *skp* knockdown *Escherichia coli* cells. Hence, we created an antisense RNA *skp* knockdown containing plasmid pJAHS to silence *skp*. We envisioned a plasmid that contains an antisense RNA (asRNA) component that binds complementarity to the *skp* mRNA sequence. This asRNA will be under the control of an inducible promoter, which when expressed, silences the *skp* gene by selective binding [10]. The asRNA transcribed is designed to bind in a complementary manner to the Shine Dalgarno

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(SD) sequence in *skp* mRNA to prevent ribosome binding hence any translation of the Skp protein. We incorporate our asRNA sequence into plasmid pHN678 to since features of this plasmid allowed optimal antisense function and manipulation of asRNA expression level through the modulation of an inducible promoter. pHN678 contains a lac operator region that can be manipulated to control expression levels of genes downstream. By using isopropyl-beta-thiogalactopyranoside (IPTG), the levels of asRNA can be potentially selectively induced to be expressed at various levels [11]. In addition, the pHN678 transcripts can stabilize the asRNA molecule and increase its resistance to degradation by host enzymes such as RNAses by forming a paired termini secondary structure flanking the asRNA sequence [11]. This increased stability of the transcript allows for massive accumulation of the *skp* asRNA when pHN678 is induced with IPTG, drastically improving the efficacy of the system [11]. To study the effect of *skp* knockdown on *E. coli* strain BW25113, we compared the cellular growth rate level of the WT strain to the *skp* knockdown strains containing the asRNA plasmid introduced. The growth levels were also compared to WT strains introduced with an empty vector of pHN678, as well as a *skp* single KO staring of BW25113 ordered from the Keio collection which acted as controls. Like previous research done on Skp mutants, the strain with pJAHS plasmid transformed were then compared to WT strain with either no plasmid or empty vector (pHN678) and against the *skp* single knockout *E. coli* cells from the Keio collection for characteristics such as growth rate, colony and single cell morphology, and antibiotic resistance [5]. We hypothesize that by selectively knocking down the Skp general chaperone through expression of an antisense RNA molecule we would expect to see a decreased growth rate, distinct colony morphology, and increased susceptibility to antibiotic resistance.

We hypothesize that both the *skp* genomic deletion and *skp* asRNA knockdown strains would result in a similar growth curve pattern that significantly differs from the WT and empty vector strains. However, our data has shown to be undetermined as the data across different assays seem to display contradictory results. Both the *E. coli* strains containing genomic deletion and temporary asRNA knockdown of *skp* do not resemble the WT BW25113 strain in any of the characteristics measured. However, there was also a significant difference observed between the *skp* knockout and *skp* knockdown, which calls for future investigations.

Overall, our findings include the correct identification of the Shine Dalgarno (SD) sequence for the Skp protein and designing a 60bp gene block using RNA primers in accordance that binds to a region overlapping the *skp* SD sequence. The RNA gene block was inserted downstream of a lac-inducible operon, and the engineered anti-sense RNA vector was transformed into WT *E. coli* DH5α strain. Transformants were selected on different antibiotic plates in addition with IPTG, and it was found that knocking down expression of *skp* general chaperons indeed obtains the same mutant phenotype as the *skp* knockout cells.

METHODS AND MATERIALS

Bacteria strains and plasmids used. *E. coli* K-12 substrains BW25113, and DH5α cells transformed pHN678 and pZ plasmids were obtained from the Dr. Dave Oliver (Department of Microbiology and Immunology, University of British Columbia). DH5α was used to propagate pJAHS and BW25113 was used in experimental conditions. *E. coli* K-12 substrain JW0173-1 was obtained from the Keio Collection of the Yale Coli Genetic Stock Center (CGSC). As a Δ*skp* strain, JW0173-1 was used to as a control to compare with pJAHS transformants. Both plasmids pHN678 and pZ were gifted by Dr. Liam Good (Royal Veterinary College, University of London). Lysogeny broth (LB, 1% w/v tryptone, 0.5% w/v yeast extract, 0.5% w/v NaCl; plates included 1.5% w/v agarose) was used for general growth and culturing. Media was spiked with 25 μg/mL chloramphenicol to select for pJAHS, pHN678, or pZ transformants. All overnight growth was carried out at 37˚C on a shaker at 200 rpm. Table 1 describes the strains and plasmids used in this study.

TABLE. 1 Summary of bacteria strains and plasmids used.

E. coli K-12 substrain	Description	Genotype
DH5α	Host strain for pZ and pHN678; Propagation strain for pJAHS	F-, *λ-, rph-1*
BW25113	Wild type control	F-, *Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ-, rph-1, ΔrffC741::kan, Δ(rhaD-rhaB)568, hsdR514*
JW0173-1	Harbours a *skp* deletion; Positive control for pJAHS	F-, *Δ(araD-araB)567, ΔhlpA781::kan, ΔlacZ4787(::rrnB-3), λ-, rph-1, ΔrffC741::kan, Δ(rhaD-rhaB)568, hsdR514*
Cloning Vector	**Description**	**Features**
pHN678	Cloning vector used to construct pZ and pJAHS; Optimized for asRNA expression	IPTG-inducible promoter [*Ptrc*], paired-termini flanking MCS, Cam^R
pZ	Derivative of pHN678; Antisense knockdown of *ftsZ* upon IPTG induction, leading to observable cellular elongation	IPTG-inducible promoter [*Ptrc*], paired-termini flanking MCS, Cam^R, *ftsZ* antisense insert
pJAHS	Derivative of pHN678; Antisense knockdown of *skp* upon IPTG induction	IPTG-inducible promoter [*Ptrc*], paired-termini flanking MCS, Cam^R, *skp* antisense insert

Generation of BW25113 competent cells. The American Society of Microbiology (ASM) protocol for the generation and transformation of chemically competent cells was modified and used to make *E. coli* BW25113 competent cells [12]. An overnight culture of BW25113 cells. An overnight culture of BW25113 was diluted 1:100 with fresh LB and grown on a shaker at 37°C until an OD_{600} of 0.4 was reached. OD_{600} measurements were taking using an Ultraspec 3000 Spectrophotometer (Pharmacia Biotech, 80-2106-20). The culture was chilled on ice for 20 minutes and then pelleted using a Beckman Coulter JA-20 Fixed Angle Rotor set at 4°C and 4,000 rpm for 10 minutes. The pellet was resuspended in 20ml of ice-cold 0.1M $CaCl_2$, incubated on ice for 30 minutes, and then centrifuged again. The pellet was resuspended in 5ml ice-cold 0.1M $CaCl_2$ with 15% glycerol and was then stored at -70°C until needed for transformations.

Isolation of pHN678 from DH5α. *E. coli* DH5α transformed with pHN678 was obtained from Dr. Oliver and Lalani et al. [14]. Cells were grown overnight in 5ml of LB and 25 µg/ml chloramphenicol on a shaker at 37°C. The plasmid was extracted from the culture using the PureLink Quick Plasmid DNA Miniprep Kits (Invitrogen, K2100) in accordance with the manufacture's protocol. Isolated plasmid concentration was measure using a NanoDrop 2000 spectrophotometer (Thermo Scientific, ND2000CLAPTOP) and then stored at -20°C.

Oligonucleotide generation and ligation into pHN678. Digestion of pHN678 was done following the Restriction Enzyme Double Digestion protocol (NEBcloner), optimized for NcoI (NEB, R0193S) and XhoI (NEB, R0146S) in NEBuffer r3.1 (B6003S). Following 15 minutes of incubation at 37°C, the enzymes were heat inactivated via incubation at 80°C for 20 minutes. The digested plasmid sample was used immediately in ligation to prevent DNA damage to the sticky ends. The annealing of oligonucleotides *skp*-as40F and *skp*-as40R (Table 2) was done using a modified version the Protocol for Annealing Oligonucleotides (Sigma-Aldrich). Lyophilized oligonucleotides were resuspended in sterile deionized water to a concentration of 100mM. Equal parts of each oligonucleotide were added to a tube were annealed using a Bio-Rad T100 Thermal Cycler (Bio-Rad, 1861096). Annealed inserts were also used immediately in ligation to prevent sticky ends damage. Ligation of the annealed *skp* antisense insert into the digested pHN678 plasmid was done based on the Ligation Protocol with T4 DNA Ligase (NEB, M0202). Following ligation, a 5µl aliquot of the constructed

pJAHS plasmid was kept on ice prior to transformation while the rest stored at -20°C for future use.

TABLE. 2 DNA sequences used to construct the *skp* antisense insert.

Identifier	Oligonucleotide Sequence (5' - 3')	T_m (°C)
***skp*-as40F (forward asRNA)**	catggCCACTTTTTCACAATAAACTCCTT ACCATCCCATTTGCACc	68
***skp*-as40R (reverse asRNA)**	tcgagGTGCAAATGGGATGGTAAGGAGT TTATTGTGAAAAAGTGGc	68

Transformation of pJAHS in DH5α and BW25113. Transformation of chemically competent DH5α cells (Thermo Scientific, EC0112) was done following a modified version of the ASM protocol for the generation and transformation of chemically competent cells [12]. This was done to propagate the constructed pJAHS plasmid. The frozen cells were thawed, heat-shocked and then transformed with 5µl of pJAHS. Following 1 hour of growth in 1ml of LB, the cells were spread on LB agar plates containing 25µg/ml chloramphenicol and incubated overnight at 37°C to select for pJAHS transformants. The following day, random isolated colonies from the plates were selected and cultured in liquid LB media containing 25µg/ml chloramphenicol and were then grown overnight on a shaker at 37°C. Plasmids were isolated from the overnight cultures using the PureLink Quick Plasmid DNA Miniprep Kits (Invitrogen, K2100) in accordance with the manufacture's protocol. An aliquot of pJAHS was kept in -20°C for future use, while another aliquot was sent for sequencing, and the last aliquot was transformed into BW25113, following the same ASM protocol and selection used previously. A separate batch of BW25113 cells were also transformed with plasmid pHN678.

Sequencing of pJAHS for confirmation of antisense insert. A 50µl aliquot of pJAHS, concentrated at 33ng/µl, was sent to Plasmidsaurus for whole plasmid sequencing using Oxford Nanopore Technologies (ONT). Confirmation of the antisense insert within pJAHS was determined by analyzing the sequencing results using SnapGene.

Zone of inhibition functional assay of IPTG-induced *E coli.* JW0173-1, BW25113, and BW25113 transformants harbouring pHN678 and pJAHS. A zone of inhibition assay was used to assess the efficacy of the antisense knockdown. This was done by following a modified version of the ASM Kirby-Bauer Disk Diffusion Susceptibility Test Protocol and comparing the phenotypes of *skp* knockdown and *skp* knockout cells. Stock solution of 100mM IPTG was diluted to 10mM using deionized water. Mueller-Hinton agar plates were streaked with 50µl of 10mM IPTG prior to culturing. Overnight cultures of *E. coli* JW0173-1, wild-type (WT) BW25113, and BW25113 harbouring pHN678 or pJAHS were diluted to an OD_{600} reading of 0.18 and were then spread on the Mueller-Hinton IPTG agar plates using a sterile cotton swab, following the techniques and pattern outlined in the ASM protocol. Using forceps, 5 discs of 30ng/µl vancomycin were placed on the plate in a box-shaped pattern with a disc in the middle. The plates were incubated for 48 hours at 37°C. Resulting inhibition zones were measured using a ruler.

Growth rates of uninduced and IPTG-induced *E coli.* JW0173-1, BW25113, and BW25113 transformants harbouring pHN678 and pJAHS. Growth rate analysis was done to assess Skp silencing effects on growth and whether growth-related phenotypic similarities existed between *skp* knockdown and *skp* knockout cells. Overnight cultures of BW25113, BW25113 harbouring pJAHS or pHN678 with 25µg/ml chloramphenicol, and JW0173-1 with 50µg/ml kanamycin were diluted to an OD_{600} of 0.1 using fresh LB media. Cultures would be grown in triplicates at 4 different concentrations of IPTG. Using a 96-well plate, 160µl of each sample was added to 12 wells (48 wells total; 4 samples across 12 wells). For the 12 wells containing each sample, 40µl of 0mM, 1mM, 20mM, and 50mM IPTG were added to 3 of the wells. This resulted in triplicates of each condition for each sample. The plate was incubated for 24 hours at 37°C in a BioTek Epoch 2 Microplate Spectrophotometer (Agilent, EPOCH2NS) with OD_{600} readings taken every 30 minutes.

Growth of uninduced and IPTG-induced *E coli.* DH5α harbouring pZ, and BW25113 transformants harbouring pHN678 and pJAHS. To test whether the *skp* antisense knockdown efficacy was temperature dependent, cultures were grown at varying temperatures on plates with varying concentrations of IPTG. LB agar plates with 25ng/µl chloramphenicol were streaked with 50µl of either 0mM, 1mM, 10mM, or 20mM IPTG (12 plates, 3 for each IPTG concentration). Plates were then spotted with 50µl of diluted (1:10) DH5α harbouring pZ, and BW25113 transformants harbouring pHN678 and pJAHS overnight cultures. The spotted plates were incubated overnight at either 25°C, 30°C, or 37°C. Growth was assessed and compared by the unaided eye.

Microscopic observation of uninduced and IPTG-induced DH5α harboring pZ, and BW25113 harbouring pHN678, and pJAHS. To visualize the morphology, colonies grown on LB agar plates contains 25µg/ml chloramphenicol and 50µl of 0mM, 1mM, 10mM, or 20mM IPTG were observed using a stereo microscope at 40x magnification. Wet mounts of samples were also prepared and observed at 1000x magnification with oil immersion.

RESULTS

Insertion of *skp* asRNA between the XhoI and NcoI restriction sites on the pHN678 plasmid. To determine if the gene block that was created was inserted between the NcoI and XhoI restriction enzyme sites on the pJAHS plasmid, Oxford Nanopore Sequencing was done on the whole plasmid and the sequence between the cut sites was observed in SnapGene. Custom complementary antiparallel primers were ordered and ligated together to create our *skp* asRNA gene block to be inserted into the pHN678 plasmid. XhoI and NcoI restriction enzymes were used to cut and create sticky ends on both the gene block and pHN678 plasmid before the gene block was inserted with ligase. Sequencing results showed that our engineered asRNA gene block was identical to our desired construct and inserted into the target site in the pHN678 plasmid to create the novel pJAHS plasmid as shown in Figure 1.

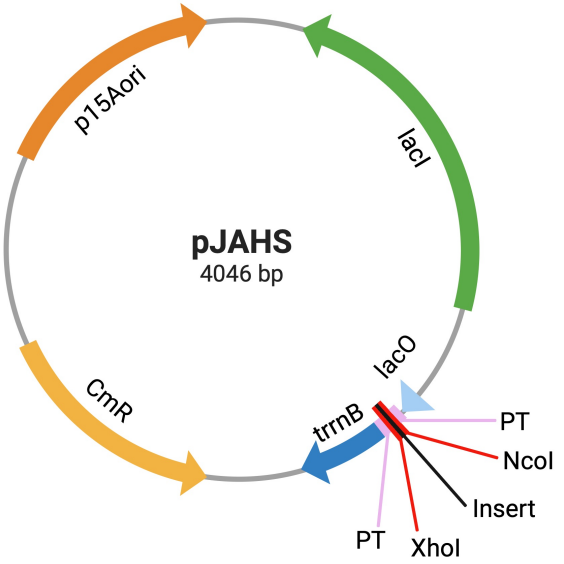

FIG. 1 *skp* asRNA construct was inserted between XhoI and NcoI cut sites on pHN678 plasmid to create the novel pJAHS plasmid. SnapGene images of the pJAHS plasmid and its components including constitutively expressed LacI, a chloramphenicol resistance marker, and the XhoI and NcoI restriction enzyme recognition sequences. (A) View of the entire plasmid construct with important genes and markers labelled. Red box highlights the XhoI and NcoI cut sites where asRNA gene block was inserted into the plasmid. (B) Sequence reads of the region between the NcoI and XhoI cut sites containing the engineered *skp* asRNA gene block.

Addition of IPTG induced expression of *skp* asRNA by pJAHS plasmid and knocked down *skp* expression in the cell. To find out if our asRNA construct would knockdown Skp expression in BW25113 *E. coli* cells, a zone of inhibition assay using vancomycin was conducted. Wild type cells, a complete *skp* knockout obtained from the Keio strain collection, as well as BW25113 *E. coli* transformed with pJAHS or pHN678 were spread plated with a sterile cotton swab onto media containing IPTG before placing 30ug/ml vancomycin antibiotic disks on top. The empty vector (pHN678) had the smallest zone of inhibition at >1 mm diameter, and the wild type cells had the second smallest at 0.5 cm diameter. Our knockdown construct, whose asRNA was expressed via the addition of IPTG, had a larger zone of inhibited growth with a diameter of 1 cm which resembled the phenotype of the *skp* knockout which had a diameter of inhibition of 1.4 cm as shown in Figure 2. An increased zone of inhibition caused by the antibiotic vancomycin in our asRNA construct matching the

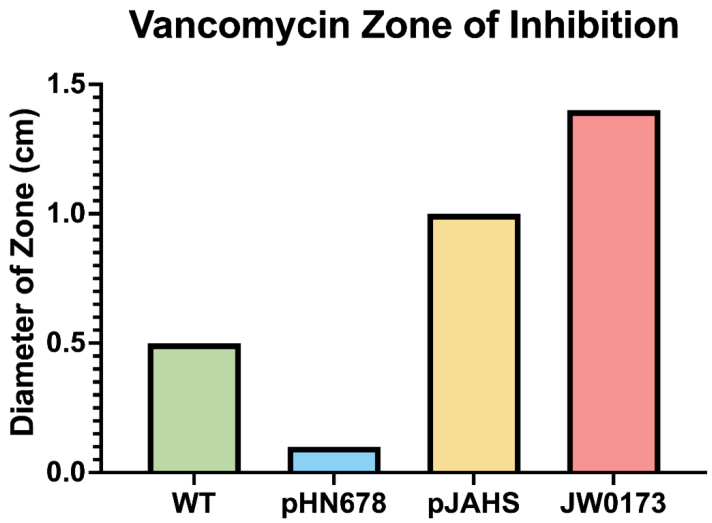

FIG. 2 Diameter of inhibited growth by vancomycin on wild type, empty vector, *skp* knockdown, and *skp* knockout BW25113 *E. coli* strains. Measurements of the diameter of no growth surrounding vancomycin antibiotic disks on media plated with WT BW25113, BW25113 + pHN678, BW25113 + pJAHS, or BW25113Δ*skp*. X-axis shows which bacterial sample is being represented. Y-axis shows the diameter in cm of no growth surrounding the antibiotic disc. Values above each bar represent exact values for the zone of inhibition assay which was run once.

phenotype of a *skp* knockout indicates that inducing expression of *skp* asRNA in pJAHS by adding IPTG knocks down the expression of *skp* in BW25113 *E. coli* cells.

Growth of BW25113 *E. coli* cells was not inhibited by a knockdown or knockout of *skp* nor the addition of IPTG. To determine if knocking down *skp* using the pJAHS asRNA affected the growth of BW25113 *E. coli* cells, samples were loaded into a 96 well plate containing different concentrations of IPTG and their growth was read over 24 hours at 37° C. Wild type BW25113, BW25113 with a *skp* knockout, and BW25113 transformed with either pJAHS or pHN678 were plated in triplicate in a 96 well plate containing 0 mM, 10 mM, 20 mM or 50 mM of IPTG (as shown in Figure 3A, B, C, D). The samples were diluted to an OD between 0.08 and 0.13 to start the growth curve. In all samples, the OD increased over the first 13 hours after which it plateaued and remained constant at an OD between 0.99 and 1.1. The addition of varying concentrations of IPTG also did not cause significant changes to the maximum OD or growth rates observed for any of the samples. The rate of growth for all samples under all conditions as well as the maximum OD that was reached were consistent and did not significantly vary indicating that the addition of the plasmids themselves did not affect cell growth nor did the decrease in *skp* expression in the cells.

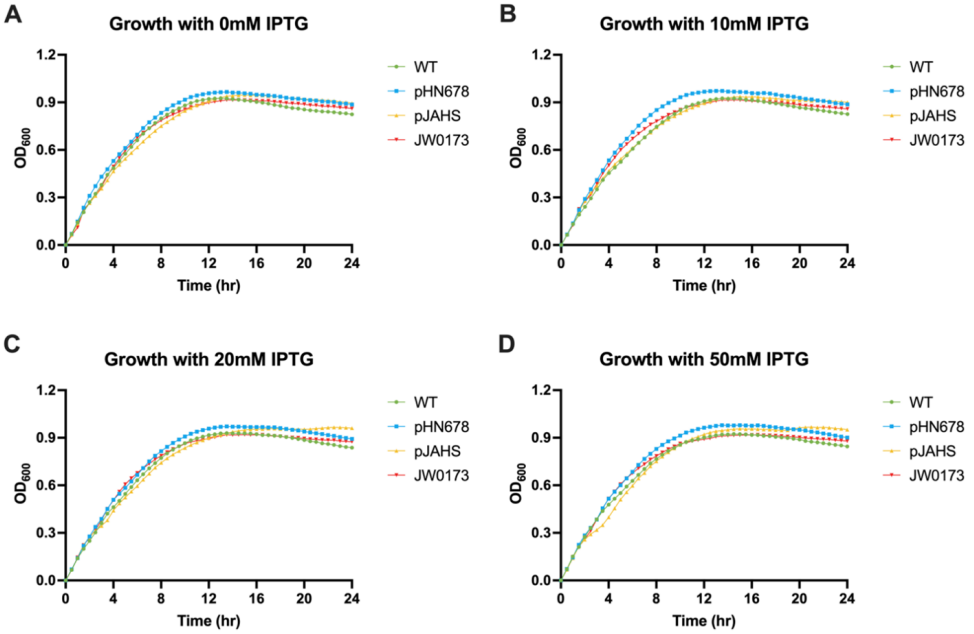

FIG. 3 Growth of BW25113 *E. coli* cells is not significantly altered by knocking down or knocking out *skp*, the addition of IPTG or the addition of plasmids pHN678 and pJAHS. Growth curves of Keio strain collection *skp* knockout, wild type BW25113 *E. coli,* and BW25113 transformed with either pHN678 or pJAHS over 24 hours. IPTG was added in concentrations of (A) 0 mM, (B) 10 mM, (C) 20 mM, or (D) 50 mM. Growth rates and final OD values are very similar for each sample under all IPTG concentrations. Each sample was diluted to an OD between 0.08 and 0.13 and samples were run in triplicate. Values shown are normalized to the minimum read value.

Lower temperatures cause less growth in transformed BW25113 *E. coli* cells. To determine if different temperatures would lead to better annealing of the asRNA to the mRNA and lower expression levels of *skp*, BW25113 cells transformed with pJAHS, pZ, or pHN678 were spot plated onto media containing 0 mM, 1 mM, 10 mM, or 20 mM IPTG and then

incubated at 37°C, 30°C, or 25°C overnight. The growth of cells containing plasmid pZ was lowest at 25C, intermediate at 30°C, and highest at 37° C regardless of IPTG concentration. At 37°C in the pZ condition, there was slightly less growth at 20 mM IPTG than at lower concentrations. The pHN678 and pJAHS colonies appeared to be slightly more transparent in the 25°C condition (indicating slightly less growth) than in the 30°C and 37°C conditions which appeared the same for all IPTG concentrations as shown in Figure 4. Because the colony morphologies appeared consistent across each of the IPTG concentrations and between the empty vector and pJAHS construct, this suggests that any changes in growth were not due to the *skp* knockdown, but rather due to the lower temperatures themselves being non-optimal for growth of BW25113 cells.

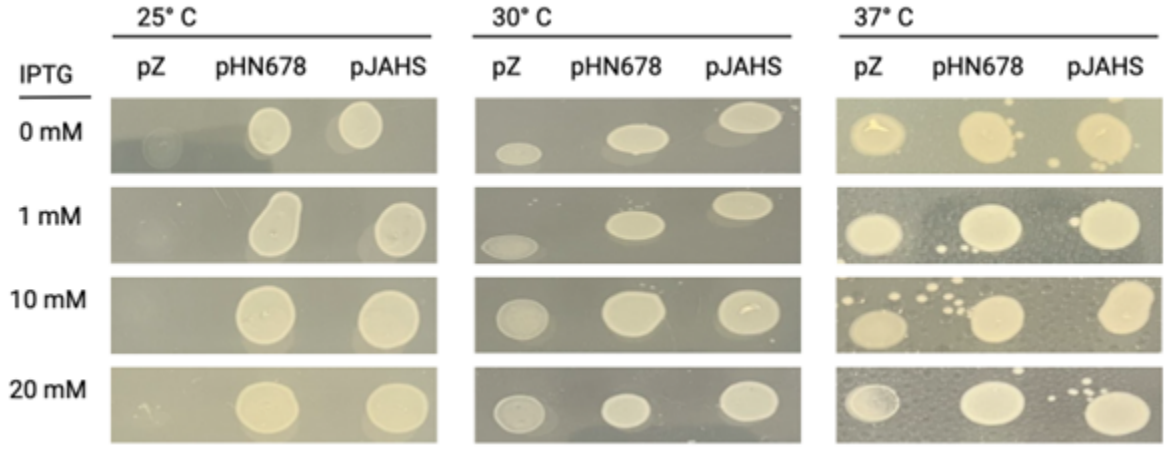

FIG. 4 Lower temperatures greatly inhibit growth of BW25113 *E. coli* cells transformed with pZ and slightly decrease growth of cells transformed with pHN678 and pJAHS. Growth of BW25113 *E. coli* cells containing either pZ, pHN678, or pJAHS plasmid at different temperatures and different IPTG concentrations. Values on the left represent IPTG concentration for that row of growth. Temperature and sample are indicated above each column of growth. Samples were plated and allowed to grow at designated temperatures overnight. IPTG concentration does not alter growth except for pZ at 37°C.

DISCUSSION

This study investigated the role of Skp on growth rate in *E. coli* by silencing *skp* with asRNA. Although Skp expression was not measured directly using techniques such as western blot with direct binding anti-Skp antibodies, the expression of the *skp* asRNA sequence contained in pJAHS was indirectly verified using a zone of inhibition test with vancomycin instead. Previous studies highlighted the increased sensitivity to hydrophobic antibiotics in Δskp mutant *E. coli* compared to the wildtype, resulting in a significantly larger zone of inhibition in the Δskp mutant [5]. An intermediate phenotype for antibiotic sensitivity against Vancomycin (moderate sensitivity) observed in the *skp* KD strain created using pJAHS when compared to WT (low sensitivity) and *skp* KO (High sensitivity) confirmed the expression of our asRNA expression (Fig. 3). The expression of the asRNA sequence contained in pJAHS was indirectly verified using a phenotype comparison with pZ as well. Since the original plasmid for both pZ and pJAHS was pHN678, if pZ was able to silence the expression of *ftsZ* and result in visible elongation of *E. coli*, we expected that the asRNA sequence to Skp would also be expressed.

The zone of inhibition assay revealed significant difference in between the *skp* knock down mutants compared to the WT BW25113, but also with respect to the Keio *skp* single knockouts (Figure 2, Figure 5). This was surprising since we used varying levels of IPTG concentration to induce the expression of the asRNA but we were never able to get a similar phenotype to the Keio *skp* knockout. A potential explanation to this intermediate level of sensitivity to antibiotics observed in the *skp* KD is that the concentration of IPTG used in this study was too low, and it has not yet reached saturation point of the asRNA. The result of this is that there is still some level of Skp protein being translated which results in slightly elevated antibiotic resistance compared to the complete *skp* KO, but decreases resistance compared to

the WT. Due the integral role of Skp in outer membrane protein folding, we expected that the phenotype of knocking down *skp* in *E. coli* would be drastic in terms of growth rate [15]. However, the 24 hour growth curve analysis revealed no significant difference in growth rate between the *skp* KD and WT. Contrary to our original hypothesis of seeing a major difference in growth curve, this result could be rationalized as it was found later during the study that *skp* KO do not show a significant decrease in growth rate on its own due to its hypothesized redundant role with other general chaperone protein DegP [16]. Previous studies have conducted growth rate analysis on double knock mutant *E. coli* strain for *skp* and *degP* and found that it was lethal [15, 16]. One of the biggest flaws in our experiment is that there were no direct assays done to measure the levels of Skp protein levels in the *E. coli* strain containing the pJAHS plasmid under different levels of IPTG influence. In terms of experimental design, another potential limitation was the length of the gene block used to create the asRNA. The length of the asRNA insert was arbitrarily chosen to be 40 base pairs (bp). This was due to previous studies highlighting that gene blocks should not exceed 60 bp.

Future experimentations should look to prove the efficacy of the system we have created that utilize IPTG concentrations to control for expression of *skp* asRNA. This can involve using more direct methods of detection for *skp* asRNA concentration, and whether varying levels of IPTG are causing any significant difference. One feasible solution is to directly use anti-Skp antibodies to measure the level of *skp* expression BW25113 strains containing pJAHS using a western blot. Measuring the *skp* expression directly while the strains are subject to different levels of IPTG would give a clear interpretation of the system's efficacy. It would also help find what is the optimal concentration of IPTG to elicit the strongest level of *skp* knockdown activity. Once the *skp* knockdown activity of the system has been determined, we can observe morphological and phenological differences more clearly if we introduce the pJAHS plasmid into a *degP* single knockout mutant of BW25113. Since the double knockout mutant of *degP* and *skp* of *E. coli* has been highlighted by previous research to present a much more distinct morphology, we can compare the observed phonology and morphology of BW25113 strains with pJAHS and single *degP* knockout under the influence of IPTG to BW25113 double knockout mutants [15, 16]. However, the efficacy of the asRNA contraption may vary depending on the length of the gene block, and future research should test the efficacy of gene block systems length on asRNA efficiency for selectively knocking down general chaperone protein Skp. For future experiments, one of the primary aims should be to answer the question of whether Skp expression levels affect the levels of BrkA secreted.

Conclusions In this study we set out to create a plasmid containing a *skp* antisense RNA to selectively and controllably knockdown general chaperon *skp* expression in *E. col*i. In our experiment, we were able to successfully create an 40bp antisense RNA sequence for *skp* gene that inserted downstream of lac operon on the commercial plasmid pHN678 (Figure 1). The successful integration of the insert was identified through full plasmid sequencing, and the plasmid with the specific insert was renamed pJAHS. Subsequently, we were also successful in transforming competent BW25113 to uptake pJAHS, confirmed through colony growth on antibiotic plates containing 25ng/ml chloramphenicol. Colony and single cell morphology of pJAHS containing strain grown overnight at various temperatures (25°C, 30°C, 37°C) was characterized using stereo microscopes, and no significant effect on morphology was observed to be correlated with temperature. We conducted growth rate analysis on the pJAHS containing *E. coli* strains by measuring OD using 96 well plate compared to WT and *skp* KO strains and found no significant changes at various levels of IPTG. Interestingly, there was also no difference observed between the WT BW25113 and *skp* KO, suggesting that Skp's role as general chaperon is non-essential or may poses redundant functions with other chaperon proteins.

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