

# Role of *smpB* in *E. coli* Antibiotic Sensitivity via CRISPR Interference

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**Abstract:** Multidrug-resistant bacteria are on the increase, which underscores the necessity of effective treatment. *E. coli smpB* gene encodes a system that helps in saving stalled ribosomes during translation stress, which could help the bacteria to survive in antibiotic stress by keeping the protein synthesis process going. The purpose of this research project was to determine whether CRISPR interference (CRISPRi) of *smpB* can make *E. coli* susceptible to antibiotics and modify cell growth under the experimental conditions. CRISPRi plasmids carrying two guide RNAs that target the *smpB* gene were cloned and transformed into *E. coli* strains LC-E75 and WM6026. Colony PCR and gel electrophoresis were used to check insertion and gave bands at the expected position of 416 bp. The sensitivity to antibiotics was then determined with disks of spectinomycin, chloramphenicol and a combination of both at low and medium concentration. The measurements of zones of inhibition were performed on 42 plates with various strains of *E. coli* and gRNA models. The results indicated that *smpB*-targeting CRISPRi was linked with higher antibiotic sensitivity in WM6026 strain compared to minimal impact in LC-E75 strain. A cell viability experiment in WM6026 indicated that CRISPRi activation did not have a significant impact on the growth without antibiotics. These results indicate that *smpB* can be involved in the antibiotic stress response and promote the continued research of the CRISPRi-based studies of bacterial genes.

**Keywords:** CRISPRi; *smpB*; Escherichia Coli; Antibiotic Sensitivity; Spectinomycin; Chloramphenicol.

## 1. Introduction

Antimicrobial resistance is a widespread problem that needs attention on an international scale, particularly in human and veterinary medicine, and multidrug resistance in *E. coli* is a major contributor [1, 2]. Horizontal gene transfer enables *E. coli* to accumulate resistance genes hence it has found application in identifying genetic targets associated with antimicrobial resistance [1].

*Escherichia coli* (*E. coli*), a rod-shaped bacterium that is common in the human gut, is often used as a model organism to investigate stress responses, such as exposure to antibiotics [3]. Multidrug resistant (MDR) *E. coli* strains have developed resistance to various groups of antibiotics, which present a considerable problem in treatment and burden of health care [2].

*smpB* gene of *E. coli* is positioned above the *ssrA* gene and is involved in clearing up stalled ribosomes on damaged or incomplete mRNA. This is called trans-translation and recycles ribosomes and assists in sustaining protein synthesis during stress [4, 5]. In the absence of *smpB*, ribosomes may pile up and translation quality control can be impaired. Furthermore, *smpB* was also noted among the genes overexpressed in MDR *E. coli* strains and this has indicated a potential association between the expression of *smpB* and increased drug resistance [6].

In case of a lower *smpB*-function under antibiotic stress, the translation maintenance and damage repair capability of the cell can also be decreased. *SmpB* is not directly an antibiotic-resistance gene but because of the bacteriostasis of its function, indicating ribosome rescue, it could aid bacterial survival in the presence of antibiotics [4, 5].

One previous report indicated that interference with trans-translation lowered tolerance to various antibiotics and stresses in *E. coli* and identified *smpB* as a possible genetic

goal to enhance antibiotic activity [7].

CRISPR interference (CRISPRi) is a CRISPR-Cas9 system modified to prevent transcription by a catalytically inactive dCas9 directed by a gRNA without cutting DNA [8]. To investigate the role of *smpB* in the antibiotic stress response, in this research the authors silenced *smpB* expression in *E. coli* with CRISPRi. The authors theorized that silencing *smpB* would weaken the recovery of ribosomes and make it more susceptible to translation-targeting antibiotics, particularly spectinomycin and chloramphenicol.

## 2. Materials and Methods

### 2.1. *psgRNA* DNA Minipreps for GGA Cloning

Purification of *psgRNA* plasmid DNA to be assembled into a Golden Gate Assembly was done by pelleting 800  $\mu$ L of each bacterial culture, suspending it in suspension buffer and lysing and neutralizing it. The authors centrifuged the supernatant and then transferred it to spin columns, added endo and wash buffers and eluted the DNA in two rounds using elution buffer. NanoDrop was used to measure final concentrations.

### 2.2. Golden Gate Assembly

The authors annealed *smpB* 28 and *smpB* 197 oligonucleotides, which were diluted to 10 fmol per  $\mu$ L. The authors diluted the GGA in tubes with each CRISPRi vector, water, gRNA sequence, 10x DNA ligase buffer, T4 DNA ligase and BsaI. The thermocycler program included a 1 h incubation temperature of 37°C, a final digestion temperature of 55°C and a final hold temperature of 4°C.

### 2.3. *E. coli* Transformation

The Golden Gate assembly was transformed into *E. coli*, which was done twice in the case of LC-E75, because the first

attempt of the assembly did not produce any colony. The second attempt was a competent cell preparation of a 1:100 culture which was washed with LB, 2xTSS and the GGA reaction were added and followed by a heat shock. In the case of WM6026, the authors took frozen competent cells and 2.5  $\mu$ L of the corresponding GGA reaction. The authors incubated the 100  $\mu$ L of each transformation mix on ice, followed by heat shock at 42°C and recovery in LB (with DAP in WM6026), then incubated overnight at 37°C.

## 2.4. PCR and Gel Electrophoresis of Colony

To confirm that colonies were successfully cloned, colony PCR was done on colonies of both *E. coli* strains of the CRISPRi plasmid. Colonies were resuspended in 100  $\mu$ L of LB; 20  $\mu$ L was transferred to PCR tubes containing extraction buffer to 15 mL culture tubes (LC-E75 LB + kan or WM6026 LB + amp + DAP). The authors used extraction thermocycler program (6 min at 65°C, 5 min at 95°C, hold at 4°C), then vortexed and placed the tubes on ice.

Primer mixes were prepared using combinations targeting either *smpB*<sub>28</sub> or *smpB*<sub>197</sub>. The authors added 23  $\mu$ L of primer mix and 2  $\mu$ L of colony extract to each PCR tube. PCR conditions were 3 min at 94°C; 32 cycles of 94°C (30 s), 60°C (30 s), and 72°C (45 s); followed by a final extension at 72°C for 2 min. The authors visualized PCR products by running 1.2% flash gels, loading 5  $\mu$ L of a mixture containing 4  $\mu$ L of PCR product and 6  $\mu$ L of loading dye into each lane, and running three lanes for each sample. Initial results for LC-E75 were inconsistent, so colony PCR and gel electrophoresis were repeated. Final gel images are provided in Supplementary Figures S1 and S2.

## 2.5. Disk Diffusion Assays

The authors prepared antibiotic disks by pipetting solutions directly onto blank paper disks and organizing them by treatment group: spectinomycin (50  $\mu$ g and 100  $\mu$ g), chloramphenicol (15  $\mu$ g and 30  $\mu$ g), and combined spectinomycin + chloramphenicol (25  $\mu$ g + 7.5  $\mu$ g and 50  $\mu$ g

+ 15  $\mu$ g). Each treatment included 18 disks, except for the no-antibiotic controls (3 disks). Disks were labeled, placed onto six sterile petri dishes by group, and stored at 4°C.

The authors then prepared 42 agar plates: 21 LB + Kan plates for LC-E75 (induced with 40  $\mu$ L tetracycline) and 21 LB + Amp + DAP plates for WM6026 (induced with 40  $\mu$ L IPTG). Using sterile cotton swabs and forceps, the authors seeded each plate with the appropriate *E. coli* strain and added three antibiotic disks per plate. After incubation, the authors measured the diameter of inhibition zones and scanned all 42 plates to compare antibiotic sensitivity across treatment conditions.

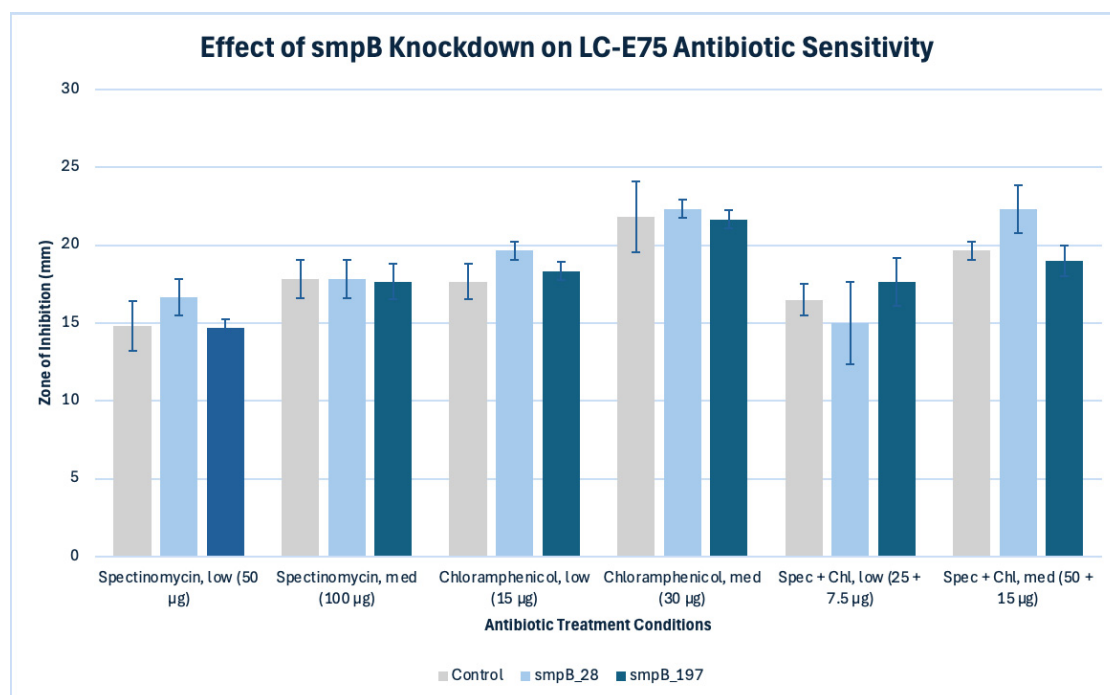
## 2.6. CRISPRi Cell Viability Test

The authors obtained LB + amp + DAP liquid cultures in four 15 mL tubes, with two for each gRNA. The authors picked a colony from each plate, inoculated it into its corresponding tube, and grew the cultures at 37°C for 3 h. The authors then made 50-fold dilutions by inoculating 100  $\mu$ L of each grown culture into four 5 mL LB broths, two with IPTG added (one with IPTG and one without for each gRNA culture). The authors incubated at 37°C for 1 h and measured cell viability using an absorbance reader every 30 min.

## 3. Results

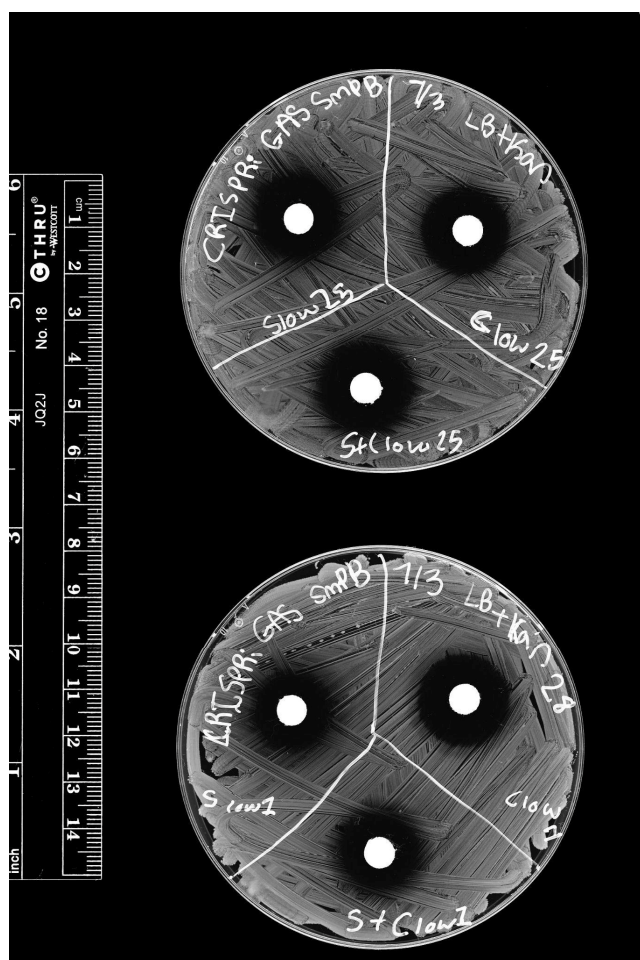
### 3.1. Antibiotic Sensitivity in LC-E75 *E. coli* Following *smpB* Knockdown

To assess the effect of *smpB* knockdown on antibiotic sensitivity, the authors treated transformed LC-E75 *E. coli* with spectinomycin, chloramphenicol, and combination therapies at varying concentrations, performing three trials of each. Across all tested conditions, *smpB* knockdown did not clearly affect the zone of inhibition compared with the control, as shown in Figure 1. Full zone-of-inhibition measurements for both strains are provided in Supplementary Table S1.



**Figure 1.** LC-E75 *E. coli* Antibiotic Sensitivity Effect of *smpB* Knockdown. Mean diameter of zone of inhibition (mm) of antibiotic disks (spec, chl and spec + chl) when comparing the *E. coli* LC-E75 *smpB*<sub>28</sub> and *smpB*<sub>197</sub> edited strain with a control of unedited *E. coli*. Standard deviation is represented by error bars

In order to provide a pictorial representation of the zone diameter measurements that are available in Figure 1, exemplary disk diffusion assay photographs are provided in Figures 2 and 3. These plates have the same inhibition trends between *smpB*-targeting and control strains.

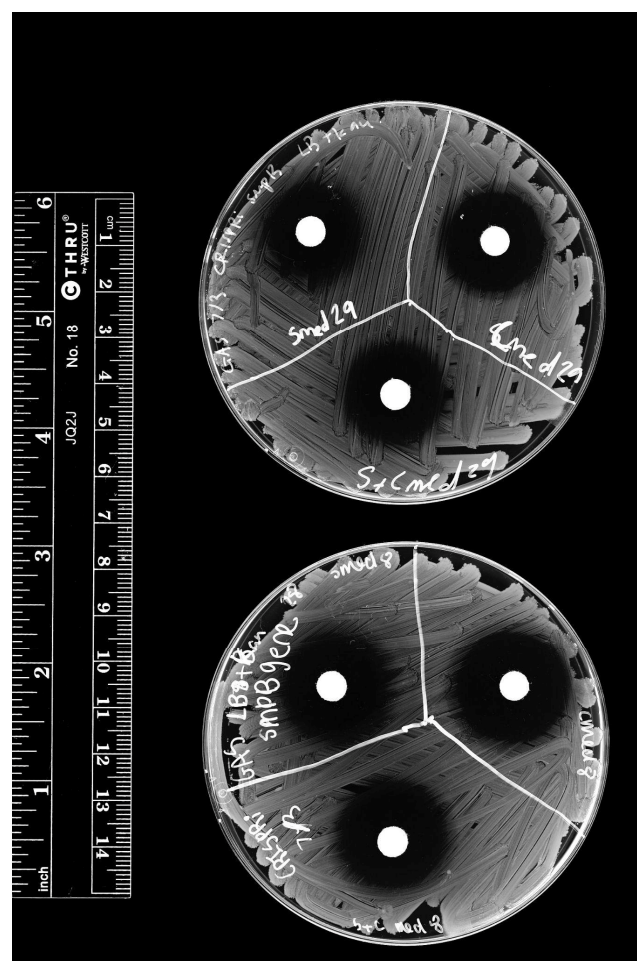


**Figure 2.** LC-E75 Representative Disk Diffusion Assay Results at Low Antibiotic Concentrations. Scan of antibiotic disk assay plates #1 (LC-E75 CRISPRi low concentration, *smpB*\_28; bottom) and #25 (LC-E75 low-concentration control; top), showing similar zones of inhibition. Both plates were treated with spectinomycin (50  $\mu$ g; top left), chloramphenicol (15  $\mu$ g; top right), and spectinomycin + chloramphenicol (25  $\mu$ g + 7.5  $\mu$ g; bottom).

### 3.2. Antibiotic Sensitivity in WM6026 *E. coli* Following *smpB* Knockdown

Likewise, the authors subjected transformed WM6026 *E. coli* to spectinomycin, chloramphenicol and combination therapies at different concentrations three times each. Knockdown of *smpB* in this strain was correlated with greater zones of inhibition among treatments as demonstrated in Figure 4 indicating greater antibiotic sensitivity in the conditions being tested. Full quantitative data is provided in Supplementary Table S1.

To visually support the zone diameter measurements presented in Figure 4, representative disk diffusion assay images are shown in Figures 5 and 6. In these representative plates, the CRISPRi-edited WM6026 strain shows larger zones of inhibition than the control.



**Figure 3.** LC-E75 Representative Disk Diffusion Assays at Medium Antibiotic Concentrations. Image of antibiotic disk assay plates, plate 8 (LC-E75 CRISPRi medium concentration, *smpB*\_28; bottom) and plate 29 (LC-E75 medium-concentration control; top), which have comparable zones of inhibition. The plates were treated with spectinomycin (100  $\mu$ g; top left), chloramphenicol (30  $\mu$ g; top right), and spectinomycin + chloramphenicol (50  $\mu$ g + 15  $\mu$ g; bottom).

### 3.3. Cell Viability of WM6026 *E. coli* with and without CRISPRi Induction

The cell viability assay in the WM6026 strain showed that CRISPRi induction did not noticeably affect overall viability or growth in the absence of antibiotics. The growth curves were similar across conditions, and the calculated doubling rates were also close, suggesting that gRNA production did not substantially affect growth under these conditions. Because IPTG induces gRNA expression, a control without IPTG was included.

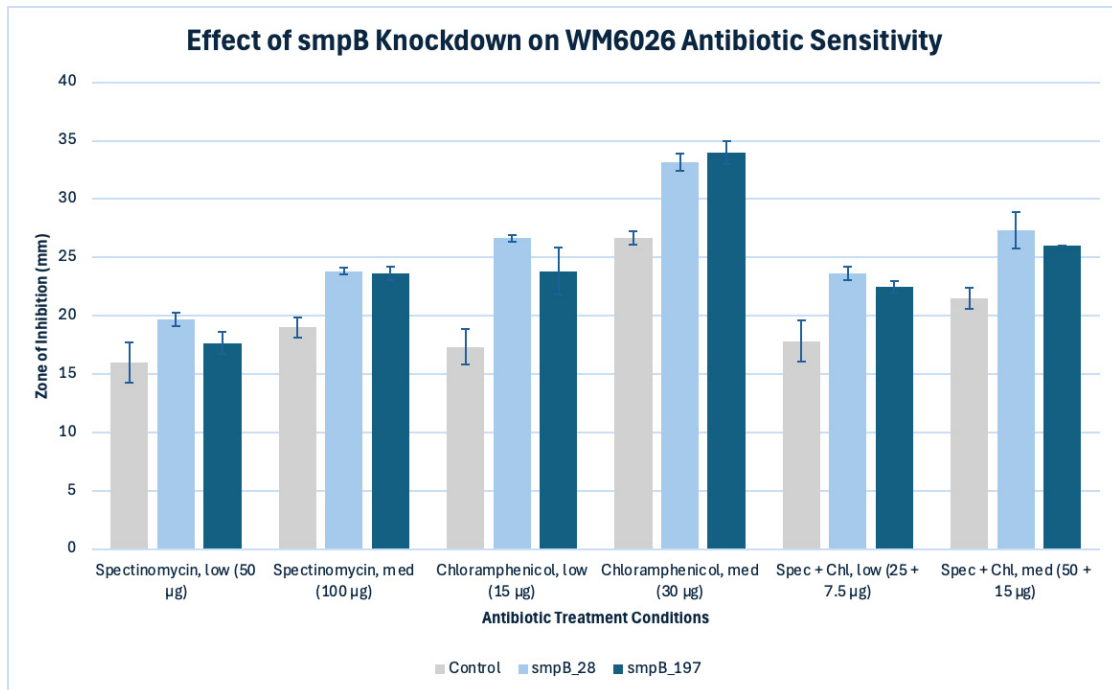
## 4. Discussion

The disk assays revealed measurable strain-specific patterns. The antibiotic sensitivity of the CRISPRi *E. coli* strains was compared with the corresponding control strain. For LC-E75, a substitute *psgRNA E. coli* strain was used as the control because a grown matching control culture was not available.

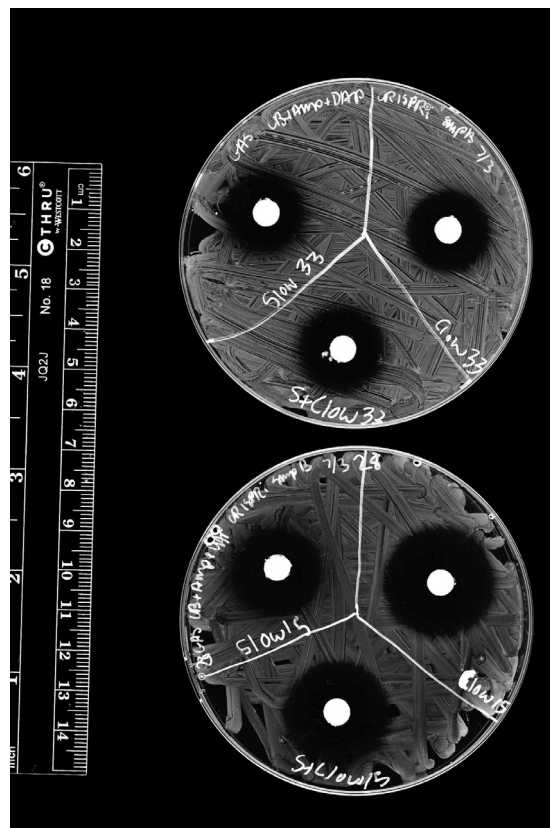
By knocking down *smpB* in two *E. coli* strains (LC-E75 and WM6026) with CRISPRi and conducting antibiotic disk diffusion assays, the authors observed that repression of *smpB* was associated with increased sensitivity to spectinomycin and chloramphenicol in the WM6026 strain, whereas LC-E75

showed minimal changes in susceptibility. The contrast was more noticeable among chloramphenicol disks overall, which may suggest a stronger effect under those tested conditions. The LC-E75 results were less clear, possibly because of guide compatibility, PCR quality, or induction conditions. In addition, sequencing results from the *psgRNA* DNA miniprep suggested that only one LC-E75 CRISPRi clone contained the

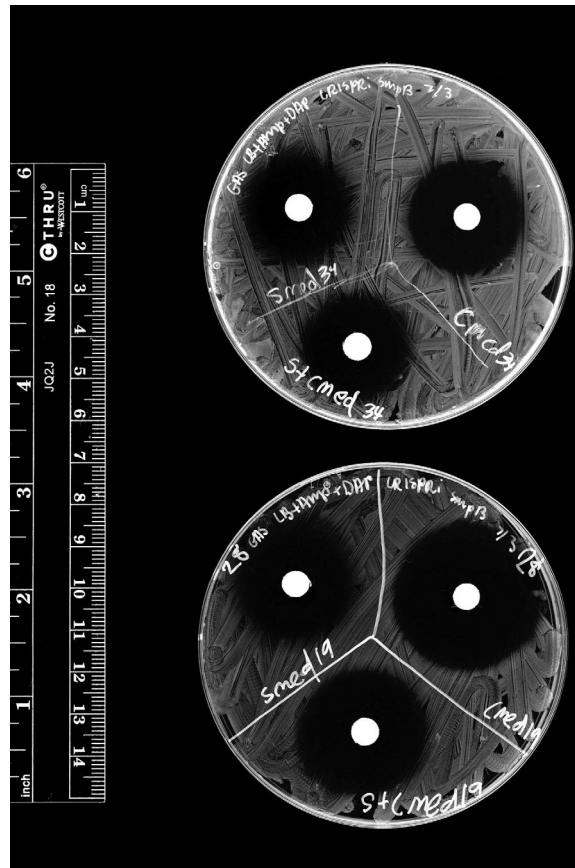
expected inserted guide RNA targeting *smkB*. This means that the unchanged sensitivity in LC-E75 may reflect incomplete or unsuccessful knockdown rather than a true lack of *smkB* involvement. Given its reported overexpression in MDR strains, *smkB* may support bacterial endurance during antibiotic treatment [6].



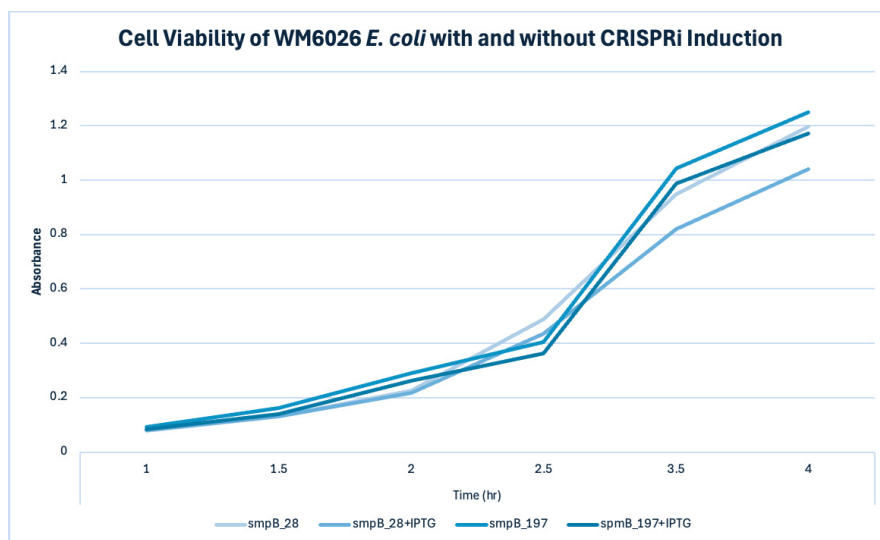
**Figure 4.** Effect of *smkB* Knockdown on WM6026 Antibiotic Sensitivity. Mean diameter of the zone of inhibition (mm) around antibiotic disks (spec, chl, and spec + chl), comparing the WM6026 strain edited at *smkB*\_28 and *smkB*\_197 with unedited WM6026 *E. coli* as a control. In most treatments, the edited strains showed larger mean zones than the control. Error bars show standard deviation



**Figure 5.** WM6026 Representative Disk Diffusion Assay Results at Low Antibiotic Concentrations. Scan of antibiotic disk assay plates #15 (WM6026 CRISPRi low concentration, *smkB*\_28; bottom) and #33 (WM6026 low-concentration control; top). Both plates were treated with spectinomycin (50 µg; top left), chloramphenicol (15 µg; top right), and spectinomycin + chloramphenicol (25 µg + 7.5 µg; bottom).



**Figure 6.** WM6026 Representative Disk Diffusion Assay Results at Medium Antibiotic Concentrations. Scan of antibiotic disk assay plates #19 (WM6026 CRISPRi medium concentration, *smpB*\_28; bottom) and #34 (WM6026 medium-concentration control; top). Both plates were treated with spectinomycin (100 µg; top left), chloramphenicol (30 µg; top right), and spectinomycin + chloramphenicol (50 µg + 15 µg; bottom)



**Figure 7.** Cell Viability of WM6026 *E. coli* with and without CRISPRi Induction. Absorbance readings of WM6026 *E. coli* over 4 h, comparing cultures induced with IPTG to controls without IPTG.

**Table 1.** Doubling Times and Growth Rates of WM6026 Under CRISPRi and Control Conditions

Condition	Slope	Doubling Rate (min)
<i>smpB</i> 28	0.4068	44.4
<i>smpB</i> 28 + IPTG	0.3787	47.7
<i>smpB</i> 197	0.3798	47.6
<i>smpB</i> 197 + IPTG	0.391	46.2

*Note.* Growth rates were calculated from the slopes of the exponential growth phase using log-transformed *OD*<sub>600</sub> values. Doubling rates indicate minimal differences between CRISPRi-induced and uninduced conditions, supporting that *smpB* knockdown did not substantially affect viability.

The cell viability test in WM6026 depicted that the growth curves and doubling rate were the same in the presence of IPTG induction and absence of it. This indicates that the variations that were recorded in the disk diffusion test did not merely arise as a result of a general growth defect in the absence of antibiotics. Rather, *smpB* knockdowns can be of greater importance in antibiotic stress than in normal growth. It is also demonstrated in the study that CRISPRi may be an effective method of studying the role of gene in bacteria under regulated circumstances.

To enhance future experiments, it would be beneficial to re-design guide RNAs, optimize culturing and induction environments and confirm the success of knockdown in advance of the assays, particularly with the LC-E75 strain. Since *smpB* is associated with translation-based rescue, the authors employed spectinomycin and chloramphenicol, which both address the ribosome. The antibiotics could also be tested outside of protein production in the future, like beta-lactams, to determine whether *smpB* is involved in the antibiotic stress response to a larger degree. In general, these results present a baseline to continue the investigation of the use of gene-based methods of antibiotic resistance.

Though it was only restricted to the strains and conditions tested, this study indicates that *smpB* can play a role in the responses of some *E. coli* strains to antibiotic stress. This renders *smpB* an attractive target of further research in future CRISPRi studies related to antimicrobial sensitivity.

## 5. Conclusion

Finally, this project established that CRISPRi targeting of *smpB* enhanced antibiotic sensitivity in WM6026 *E. coli* strain but had minimal effect on LC-E75. The WM6026 viability test also indicated that there was no significant change in growth in the absence of antibiotics when CRISPRi was induced. Collectively, these findings imply that *smpB* could be involved in the response of some *E. coli* strains to antibiotic stress and warrant further research on *smpB* in future CRISPRi investigations.

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## Appendix

### SUPPLEMENTARY MATERIAL

#### Supplementary Figure S1

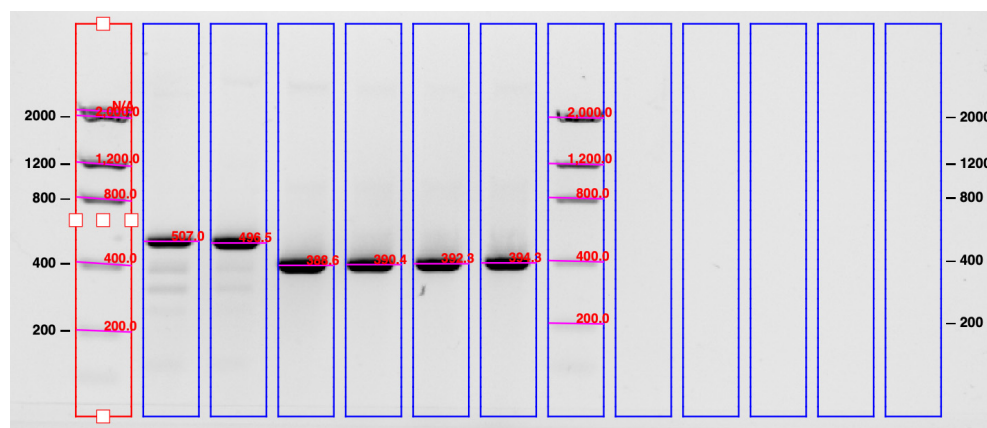


Figure S1. Gel Electrophoresis psgRNA/LC-E75 Results (Attempt 2)

Gel electrophoresis results confirming the presence of *smpB*-targeting gRNA sequence in plasmid DNA. Expected band size: ~416 bp. Lane 1: molecular ladder; lanes 2-4: *smpB\_28*; lanes 5-7: *smpB\_197*; lane 8: molecular ladder; lane 9: *smpB\_28* control; lane 10: *smpB\_197* control.

Molecular ladder band sizes from top to bottom: 2000, 1200, 800, 400, 200.

#### Supplementary Figure S2

Gel Electrophoresis pJMP/WM6026 Results



**Figure S2. Gel Electrophoresis pJMP/WM6026 Results**

Gel electrophoresis results confirming the presence of *smpB*-targeting gRNA sequence in plasmid DNA. Expected band size: ~416 bp. Lane 1: molecular ladder; lanes 2-4: *smpB*<sub>28</sub>; lanes 5-7: *smpB*<sub>197</sub>; lane 8: molecular ladder;

lane 9: *smpB*<sub>28</sub> control; lane 10: *smpB*<sub>197</sub> control. Molecular ladder band sizes from top to bottom: 2000, 1200, 800, 400, 200.

**Supplementary Table S1. Complete Raw Zone of Inhibition Measurements (mm) for all E. coli Samples**

Condition		Slope		Doubling Rate (min)						
smpB <sub>28</sub>		0.4068		44.4						
smpB <sub>28</sub> + IPTG		0.3787		47.7						
smpB <sub>197</sub>		0.3798		47.6						
smpB <sub>197</sub> + IPTG		0.391		46.2						
Spectinomycin, low (50 µg)										
	LC-E75					WM6026				
	Control	smpB 28	% diff	smpB 197	% diff	Control	smpB 28	% diff	smpB 197	% diff
1	15.5	16		14		18	19		17	
2	16	18		15		15	20		17	
3	13	16		15		15	20		19	
$\bar{x}$	14.83	16.67	12.36	14.67	-1.12	16	19.67	22.92	17.67	10.42
SD	1.61	1.15		0.58		1.73	0.58		0.94	
Spectinomycin, med (100 µg)										
	LC-E75					WM6026				
	Control	smpB 28	% diff	smpB 197	% diff	Control	smpB 28	% diff	smpB 197	% diff
1	19	18		17		19.5	24		24	
2	16.5	19		17		19.5	24		24	
3	18	16.5		19		18	23.5		23	
$\bar{x}$	17.83	17.83	0	17.67	-0.93	19	23.83	25.44	23.67	24.56
SD	1.26	1.26		1.15		0.87	0.29		0.58	
Chloramphenicol, low (15 µg)										
	LC-E75					WM6026				
	Control	smpB 28	% diff	smpB 197	% diff	Control	smpB 28	% diff	smpB 197	% diff
1	17	19		19		19	26.5		21.5	
2	19	20		18		17	27		25	
3	17	20		18		16	26.5		25	
$\bar{x}$	17.67	19.67	11.32	18.33	3.77	17.33	26.67	53.85	23.83	37.5
SD	1.15	0.58		0.58		1.53	0.29		2.02	
Chloramphenicol, med (30 µg)										
	LC-E75					WM6026				
	Control	smpB 28	% diff	smpB 197	% diff	Control	smpB 28	% diff	smpB 197	% diff
1	24	22		22		26	34		34	
2	19.5	23		21		27	33		33	
3	22	22		22		27	32.5		35	
$\bar{x}$	21.83	22.33	2.29	21.67	-0.76	26.67	33.17	24.38	34	27.5
SD	2.25	0.58		0.58		0.58	0.76		1	
Spectinomycin + Chloramphenicol, low (25 + 7.5 µg)										
	LC-E75					WM6026				
	Control	smpB 28	% diff	smpB 197	% diff	Control	smpB 28	% diff	smpB 197	% diff
1	16.5	14		19		18	23		23	
2	17.5	13		18		19.5	24		22.5	
3	15.5	18		16		16	24		22	
$\bar{x}$	16.5	15	-9.09	17.67	7.07	17.83	23.67	32.71	22.5	26.17
SD	1	2.65		1.53		1.76	0.58		0.5	

Spectinomycin + Chloramphenicol, med (50 + 15 µg)										
	LC-E75					WM6026				
	Control	smpB 28	% diff	smpB 197	% diff	Control	smpB 28	% diff	smpB 197	% diff
1	20	22		20		21	29		26	
2	19	21		19		21	26		26	
3	20	24		18		22.5	27		26	
$\bar{x}$	19.67	22.33	13.56	19	-3.39	21.5	27.33	27.13	26	20.93
SD	0.58	1.53		1		0.87	1.53		0	