A. Title: "Unique Role of the *E. coli* Min System in Motility and Flagellation Beyond Cell Division"

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B. Introduction:

The struggle of humans against infectious diseases is a major concern, as these diseases are the foremost cause of death globally (1). Various pathogens such as bacteria, viruses, fungi, and parasites cause infectious diseases, which mostly challenge public health. Despite the advancements made in medical science, infectious disease still contributes significantly. Early detection, prevention, and treatment are critical to managing the disease burden. Recently it was found that some bacterial disease shows coinfection with viral diseases. According to a recent report, 4-8% of COVID-19 patients exhibit bacterial coinfections and show more severe health issues, while 61.77% of patients with COVID-19 received antibiotic treatment (2,3)

Bacteria are widespread tiny microscopic organisms and now-a-days bacterial infection is highly prevalent. Only a small percentage cause severe diseases that significantly affect public health. According to a global burden of disease study in 2019, it was found that 13.7 million infectious-related deaths, out of which 7.7 million deaths were associated with 33 bacterial pathogens (4). 33 bacterial pathogens are responsible for these deaths but only, five bacteria *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* were responsible for 54.9% of total deaths (4). However, all these bacteria use a common strategy to cause infection and disease. The infection cycle of most of bacteria including *E. coli* has four phases: attachment to host cells, invasion, multiplication, and dispersion. Cell surface appendages like flagella and pili play an important role in attachment, while invasion allows bacteria to enter and multiply inside host cells. Through multiplication or cell division bacteria establish infection inside host cells and via motility, they move to the new target sites (5). Pathogenic *E. coli*, such as EIEC, and ETEC, can secrete toxins, invade and grow within epithelial cells, and damage host cells, eventually dispersing to infect new cells (6). Understanding both cell division and motility is crucial for bacterial pathogenesis. Since both processes are essential for bacterial survival and infection, targeting common factors that are vital for both pathways could be an effective strategy for controlling bacterial infections. Identifying and targeting these shared factors may offer a promising approach for developing new treatments.

Bacteria are divided by binary fission forming a Z-ring or division ring at mid-cells (7). More than a dozen proteins are involved in the formation of the Z-ring, and this process is highly regulated by two independent systems: the Min system and the nucleoid occlusion system. Min system composed of MinC, MinD, and MinE which oscillates from pole to pole and inhibits Z-ring assembly at pole. Whereas the nucleoid occlusion system inhibits the formation of Z-ring over nucleoid, with any disruption in these processes potentially impacting bacterial survival and serving as a target for antibiotic development (8).

It was found that most of the clinically isolated bacteria are motile and motility plays an important role in pathogenesis, chemotaxis, and escape from macrophage (9-12). Recently it was found that motility plays an important role in the development of antibiotic resistance and some efflux pump genes are also upregulated in motile bacteria (13,14). Flagella is the motility organelle of bacteria and it is necessary for adhesion, establishing infection, colonization inside host cells, and biofilm formation. From the study, it was found that the bacteria that lack flagella show defects in colonization and invasion (15). So, targeting bacterial motility machinery presents a promising approach for controlling infections.

Inside a bacteria system, many cellular processes are interlinked. For example, carbohydrate metabolism is directly linked to bacteria cell division, and DNA replication is also interconnected with cell division. Similarly, some bacteria stop they are motility before undergoing division and after division, they regain their motility. However, how these two processes are linked was not clearly understood in the model organism like *E. coli*. Our study focuses on the relationship between cell division, motility, and flagella biogenesis. Our finding shows the cell division regulatory machinery that is Min system regulates motility in *E. coli* as well as flagella biosynthesis also via interacting with an AtoSC two-component system.

C. Objectives:

1. To find out the role of cell division protein MinCDE on bacteria motility.
2. To find out the regulatory mechanism of Min system on flagella biogenesis

D. Materials:

HEPES, KCl, Tris, sodium bicarbonate, sodium phosphate, SD media, 3-amino-1,2,4-triazole, and Isopropyl β- d-1-thiogalactopyranoside from MP were purchased, and all types of nutrient media like LB broth and LB agar were purchased from HiMedia. Mostly the primary antibodies anti-flagellin, and anti-GroEL, antibodies were purchased from Abcam and the secondary antibodies were purchased from Sigma. However, the primary anti-GFP antibodies was purchased from Invitrogen. The remaining chemicals used were of molecular biology grade and were obtained from Sigma–Aldrich.

E. Methods:

1. Strains, Plasmids:

E coli Δ*min* (JS964) was a kind gift from Dr. Joe Luthkenhaus from the University of Kansas Medical Center, and wild-type lab strain MG1655 (CGSC6300) was used in all experiments (16). The Δ*atoSC* strain was a kind gift from Hirofumi Aiba (Nagoya University) (17).

2. Protein overexpression and purification:

For the purification of His-MinD, His-Cyto-AtoS were purified using the previously described methods. The expression plasmid containing MinD and Cyto-AtoS pET28a was grown at 37°C in Luria–Bertani medium containing 50 µg/ml kanamycin. At OD600 ~0.5, 1 mM IPTG was added to the cells to induce recombinant protein production (18,19). Cells were harvested using centrifugation after 4 hrs of post-induction. The pellets were resuspended with ice-cold lysis buffer and lyse using Stansted pressure homogenizer (SPH). After lysis the lysate were subjected to centrifugation and supernatant was subjected to affinity chromatography using Cobalt–NTA agarose column (20). The column was washed with 10 column volumes of washing buffer containing 50 mM HEPES pH 7.4, 300 mM KCl, and 20 mM imidazole. His6X-tagged proteins were then eluted with 50 mM HEPES (pH 7.4) buffer containing 300 mM KCl and 300 mM imidazole. The purification of GST-MinD was done using glutathione Sepharose column and the bind GST protein was eluted with elution buffer containing 50 mM HEPES pH 7.4, 300 mM KCl, and 40mM reduced glutathione. The eluted fractions were dialyzed with a buffer having 50 mM HEPES pH 7.4, 150 mM KCl, and 10% glycerol.

3. Swarming and Motility assay:

E. coli wildtype, Δ*min,* and min complementation strains were grown in M-media which is made of LB broth and 0.5% NaCl. From this, 3µl of culture was inoculated on plates having 0.3% agar in M-media and grown at 37 ºC up to 18 hours and motility was observed on the naked eye. For visualizing motility in live cells each strain was grown in a flat wide bottom flask at 37 ºC up to ~0.5 OD600 and 2µl of cells were placed on 0.3% agarose pad over a slide and observed under the live cell imaging microscope (Zeiss, cell discoverer).

4. Flagella staining and imaging:

Nowadays Alexa-488 fluorescent dye is widely used to stain flagella. We stain the flagella of *E. coli* wildtype, Δ*min,* and min complementation strains using methods as described previously (21). Each strain was grown at 37 ºC, 200 rpm overnight. Next day morning from the overnight culture 1% of the inoculum was sub-inoculated into the M-media and grown for 6-7 hrs at 60 rpm and 37 ºC in a flat wide bottom flask. After that culture was pelleted and re-suspended in buffer containing 0.01M potassium phosphate pH 7.4, 67 mM NaCl, 0.4 mM EDTA, 0.002% tween20. To 500 µl of the bacterial suspension, 10 µl of Alexa-488 (5 mg/ml) and 50 µM sodium bicarbonate were added. The mixture was incubated at room temperature for at least 2 hours. The suspension was then washed twice with the above-mentioned buffer. A few drops of bacterial sample were taken on a slide and flagella were observed under a 100x oil immersion confocal microscope (Leica STED-SP8 microscope). Few drops of sample from above growing culture were also taken on a formvar-carbon coated copper grid and negatively stained using 2% uranyl acetate. The samples were then visualized using a JEOL transmission electron microscope (TEM).

5. RT-PCR analysis of flagellar genes:

E. coli wildtype, Δ*min* cells were grown in LB broth media at 37 °C, 200 rpm. The total RNA from the exponentially growing cells was isolated using a Qiagen RNA isolation kit and 200ng of RNA were taken for c-DNA preparation using M-MuLV reverse transcriptase (Invitrogen). After cDNA synthesis the resulting DNA was quantified and subjected to qRT-PCR using the Applied biosystem SYBER Green Master mix and the desired primers. The relative expression of target genes was determined using the $2^{-\Delta\Delta CT}$ method, with GAPDH and 16sRNA serving as reference genes

6. Western blot for flagellin:

Equal number of *E. coli* wildtype, Δ*min,* and min complementation strains (MinC, MinD, MinE, MinDE, and MinCDE) were loaded onto the SDS-PAGE and separated by electrophoresis. Then the proteins were transferred to PVDF membrane by blotting. The membrane was immune-stained with the Rabbit Anti-flagellin polyclonal antibody and anti-Rabbit IgG as secondary antibody. The expression of flagellin was quantified densitometrical using ImageJ software.

7. Yeast-Two hybrid assay:

For the yeast two-hybrid analysis, HIS3 was used as a nutritional reporter system. Briefly, yeast strain HFY7c was grown at 30 °C in YPD broth until mid-log phase (OD_{600} = 0.5-0.8). Once it reached the mid-log phase competent cells were prepared and various combinations of GAL4-AD-MinD, AtoC, AtoS, and GAL4-DB-MinC, AtoC, AtoS, FlhD, FlhC were co-transformed into the competent cells. Positive transformants were screened on synthetic defined media (SD media) lacking of leucine and tryptophan. To verify protei-protein interaction, co-transformants were spotted on Leu–Trp–His– selection media plates without and with different concentrations of 3-AT and grown at 30 °C for 3 days. Yeast transformants that grow on plates lacking histidine indicated positive protein-protein interaction.

8. Pull down assay of His-cyto-AtoS and GST-MinD:

GST-MinD and His-Cyto-AtoS were incubated at an equal molar ratio of 5 μM each in buffer containing 50 mM HEPES pH 7.4, 150 mM KCl at room temperature for 30 minutes. After incubation, the reaction mixture was incubated with cobalt resin for one hour at 4 ºC. Control reactions involved only His-Cyto-AtoS (5 μM) and GST-MinD (5 μM). The entire mixture was transferred into the spin column and washed three times with buffer containing 50 mM HEPES pH 7.4, 150 mM KCl, and 20 mM imidazole. Finally, the samples were eluted with the above buffer containing 300 mM imidazole. The eluted fractions were analyzed on a 12% SDS-PAGE and western blot using anti-GST antibody and anti-His antibody (both at 1:10,000 dilution) as primary antibodies and anti-mouse HRP-conjugated secondary antibody (Invitrogen, 1:200000 dilution).

9. Promoter activity assay:

The promoter activity of *flhDC* and *fliA* was studied using a reporter assay. For this we cloned the *egfp* gene in the pET22b vector at NdeI and XhoI site then its T7 promoter was removed by digestion with BglII and NdeI restriction enzyme and in places of this the native *flhDC* and *fliA* of *E. coli* K-12 was cloned. The clone was confirmed by colony PCR and sequencing. To check the promoter activity, we introduced *fliA(p)*/ EGFP-pET22b plasmid along with minD-pACYCDuet-1 and AtoSC-pCDFDuet-1 in to *E. coli* C41 strains. The bacteria containing respective plasmids were grown at 37°C until the OD600 ~0.6, and were induced with 1 mM IPTG for 3 hours. Equal amounts of cells were loaded onto an SDS-PAGE and western blotting was performed using anti-GFP monoclonal antibody followed by anti-mouse IgG secondary antibody. Anti-GroEL antibody was used as a loading control. EGFP expression was quantified densitometrically using ImageJ software.

To check the effects of different Min components on *fliA* e promoter activity we cloned this promoter under a high copy pCDFDuet-1-eGFP plasmid after removing its both T7 promoters. This plasmid was then transferred into different *E. coli* wildtype, Δ*min,* and different min complementation strains. From the transformant plates single bacteria colonies were streaked on LB agar plates having desired antibiotics and 0.1 mM IPTG, then grown at 37°C for 12 to 16 hours. Then images were captured using a Bio-Rad Chemidoc imaging system. To measure eGFP intensity, cells were grown to OD600 ~0.5 at 37°C, then induced with 1 mM IPTG for 3 hours. After washing the cells with 0.85% NaCl, an equal number of cells were used in a fluorimeter to measure eGFP fluorescence intensity.

***10. atoDAEB* promoter activity**

To study *atoDAEB* promoter activity this promoter was cloned into the promoterless eGFP-pET22b vector at BglII and NdeI restriction site. The vector was then introduced into *E. coli* (WT) and into *E. coli* (Δ *min*), along with MinDE and AtoSC. The cells were cultured at 37 ºC with the appropriate antibiotics and the presence of 10 mM acetoacetate. The protein expression was triggered with 1 mM IPTG for 2 hours at OD600 ~0.5-0.6. After taht equal quantity of cells were loaded onto an SDS-PAGE, and a western blot was performed using anti-GFP monoclonal antibody and anti-GroEL antibody.

F. Results:

Lack of Min system increases the motility and flagellation:

While observing *E. coli* wildtype and Δ*min* cells under the microscope the Δ*min* cells random and faster movement. Whereas the *E. coli* wildtype cells were non-motile (Fig: 1A). We verify this random movement of Δ*min* cells was due to an increase in motility by doing a soft agar motility assay. Again, we found that the complementation of the entire Min system that is MinCDE and MinDE restored the wildtype phenotypes but the complementation of individual min proteins such as MinC, MinD, and MinE can't restore it (Fig: 1B and C).

Hypermotility could be due to increased flagellation or flagellar activity. So, we have stained the flagella using the fluorescent dye Alexa488 which binds to amine-rich protein flagellin and can be observed under a fluorescent microscope. Our results show that Δ*min* cells are hyper-flagellated characterized by dense flagella located all over the cells. Interestingly, flagella are in a curled shape, which is a characteristic feature of functional flagella. However, we have not observed any flagella in wild-type cells (Fig. 2A). We also did negative staining to visualize flagella through TEM and the result was the same (Fig; 2B). The Δ*min* cells are highly flagellated and the complementation MinCDE and MinDE reduce the number of flagellated cells significantly, while the wild-type cells contain no flagella (Fig: 2C).

Flagella genes were upregulated in *E. coli* Δ*min* cells:

To understand how the hyperflagellated morphology is happening in Δ*min* cells, we checked the expression of flagellar structural genes and their regulators in *E. coli* wildtype and Δ*min* cells. The flagellar expression is strictly controlled by transcription factor FlhDC, a master regulator, and FliA a flagella-specific sigma factor (22). We performed RT-PCR for transcription factors- FlhDC, FliA, and structural proteins FliC, FliE, and motor proteins MotA, MotB, and RcsB, a global transcription factor. Our result shows that most of the flagella genes such as *fliA*, *motA*, *motB*, and *fliC*, were significantly upregulated (Fig: 3A). These results may suggest that MinD regulates flagellar gene expression by increasing the expression of the FliA. Although the global regulator RcsB and transcription factor FlhDC both showed almost unchanged expression levels in the absence of the Min, our findings imply that the Min system may play a role in flagella biogenesis but not necessarily in the flagellar function.

The above observation was further supported by western blot analysis using anti-flagellin antibodies which detect the flagellar filament protein FliC (flagellin). We found that the *E. coli* Δ*min* cells express more flagellin than the wild-type cells and the complementation of MinCDE and MinDE in Δ*min* cells further decreased the flagellin production (Fig 3B and 3C). This result is in line with our RT-PCR findings.

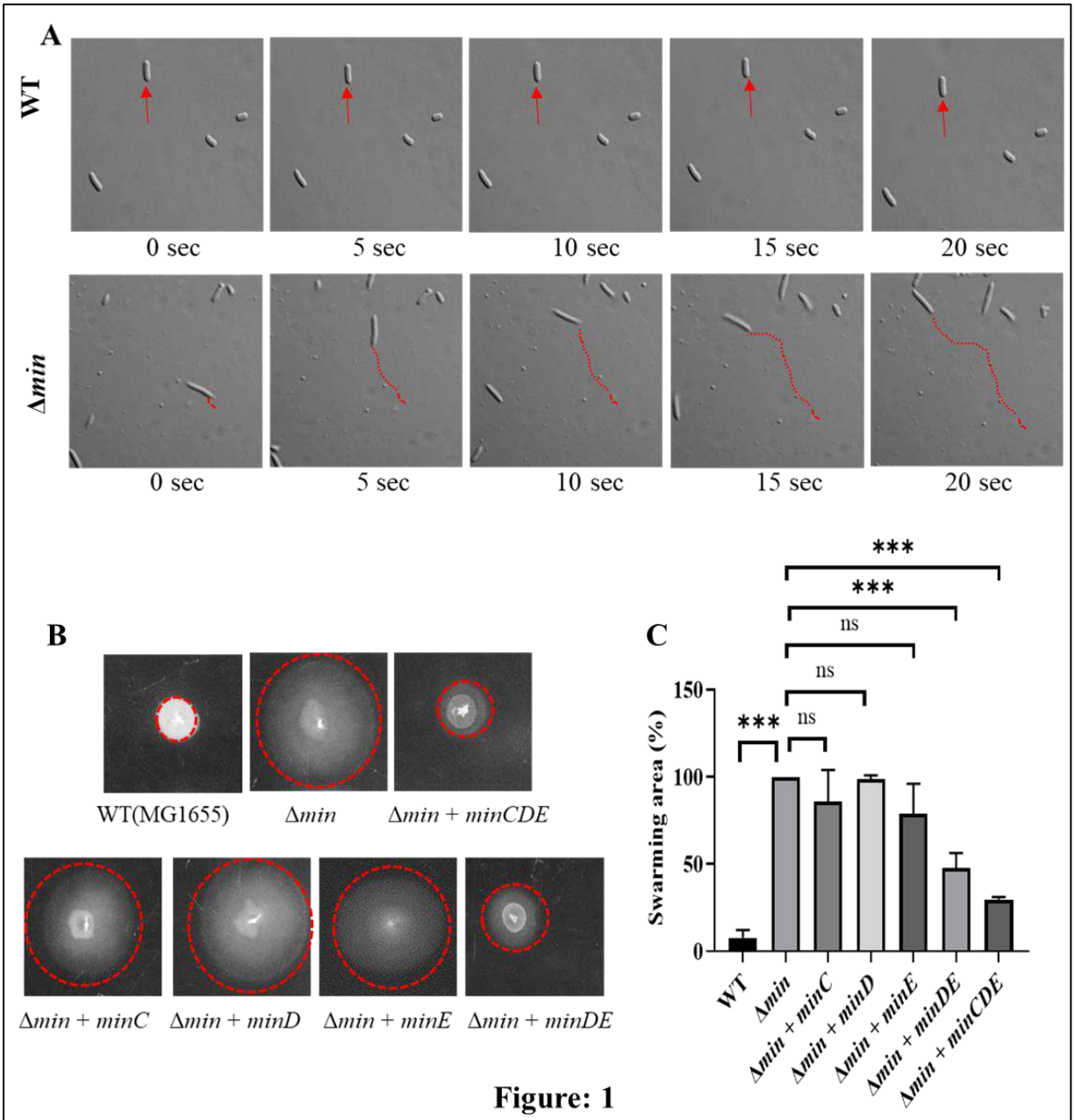

Figure: 1

Figure 1. Absent of min system increase bacterial movement: *E. coli* MG1655 (WT) wild type and *E. coli* JS964 (Δ*min*) were grown in motility media. A few drops were added on to agar over a glass slide and observed under the microscope (Olympus BX51). The figure shows bacterial movement and snapshots of different time points. The red lines indicate the path followed by the bacteria during its movement. ***Panel B*** shows the swarming motility of different *E. coli* strains. ***Panel C*** shows the swarming area percentage in each strain in the form of bar graph. Error bars shows the ±SEM determined and p values (<0.05) were determined using an unpaired parametric t-test (n = 3).

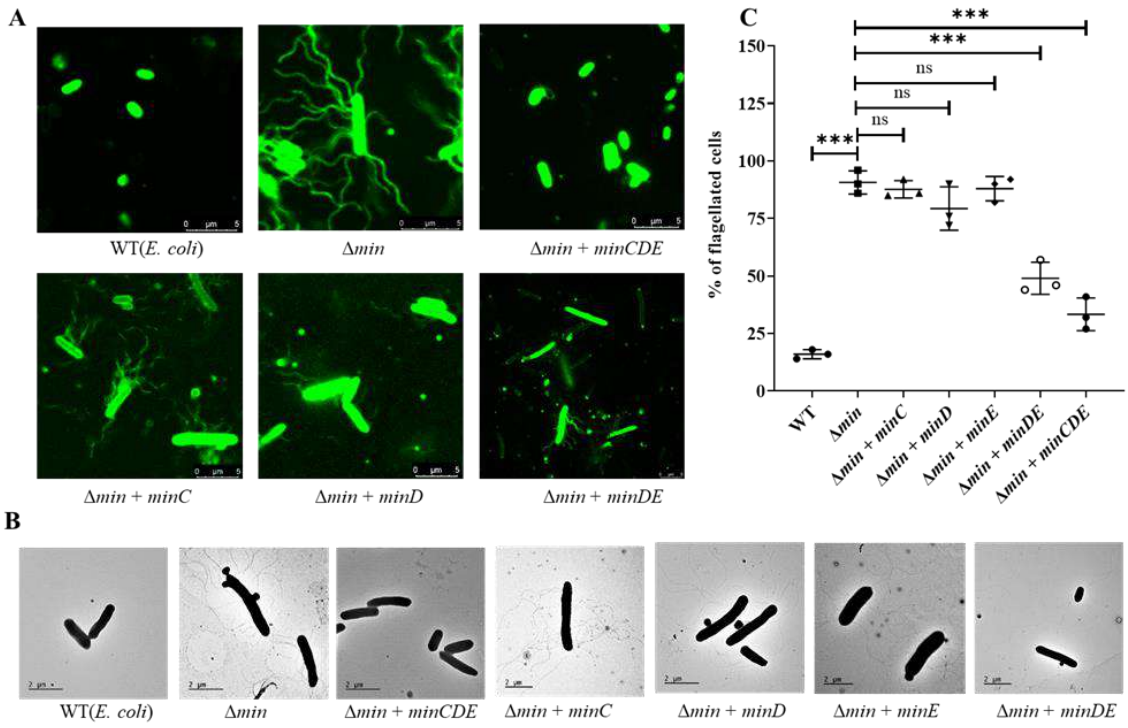

Figure: 2

Figure 2. ***The phenotype of Δmin Cells*****:** *E. coli* MG1655 (wild type), *E. coli* (Δ*min)*, and Δ*min* cells complemented with various Min components were grown, and their bacterial morphology was observed using different microscopes. ***Panel A:*** Fluorescence microscopy images of flagella stained with Alexa Fluor-488 (scale bar = 5 µm). ***Panel B:*** Transmission Electron Microscopy images showing flagella in different strains (scale bar = 2 µm). ***Panel C:*** Scatter plot showing the percentage of flagellated cells in each strain with error bars representing ±SEM of three replicates (n = 50), determined using an unpaired t-test.

Effects of Min System on *fliA* Promoter Activity in *E. coli*

FlhDC controls the expression of *fliA* in *E. coli* by binding to its promoter region.. To investigate the impact of the Min system on *flhDC* or *fliA* expression, we used an enhanced green fluorescent protein (eGFP) reporter experiment to measure the *flhDC* and *fliA* promoter activity in wild-type and Δ*min* cells (Fig. 4, A, B, and C). By utilizing an anti-GFP antibody on a Western blot to measure GFP expression, the promoter activity was investigated, and found that *fliA* promoter is only activated in Δ*min* cells. However, no *flhDC* promoter activity was detected in both wild-type and Δ*min* cells.

Furthermore, we found that eGFP expression was decreased when Δ*min* cells were complemented with MinDE or MinCDE, but the complementation of individual Min components did not decrease GFP intensity GFP intensity directly implied *fliA* promoter activity (Fig.4). Using a fluorimeter we also

evaluated the GFP intensity in the presence of MinDE decreased the eGFP expression in comparison to the *E. coli* Δ*min* cells (Fig.).

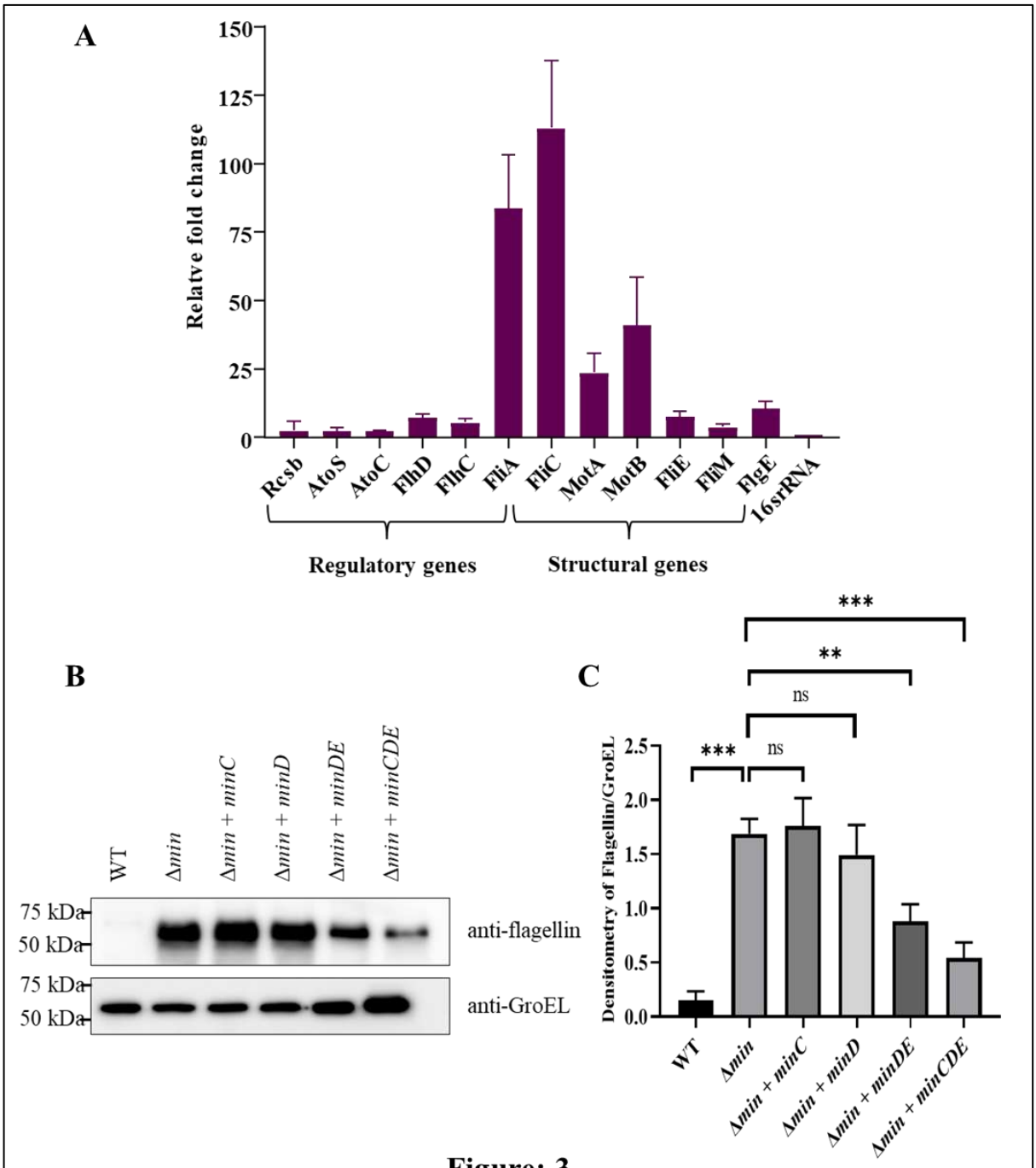

Figure: 3

Figure 3. ***Upregulation of flagellar gene in Δmin cells. Panel A*** shows the RT-PCR for flagellar gene expression *in E. coli* (Δ*min*) versus wild-type cells. ***Panel B*** shows the western blot of flagellin expression in wild type, Δ*min*, and complemented Δ*min* strains. ***Panel C*** display the relative flagellin intensity, normalized to GroEL, with error bars (±SEM) and p-values from a t-test. Experiments were repeated three times.

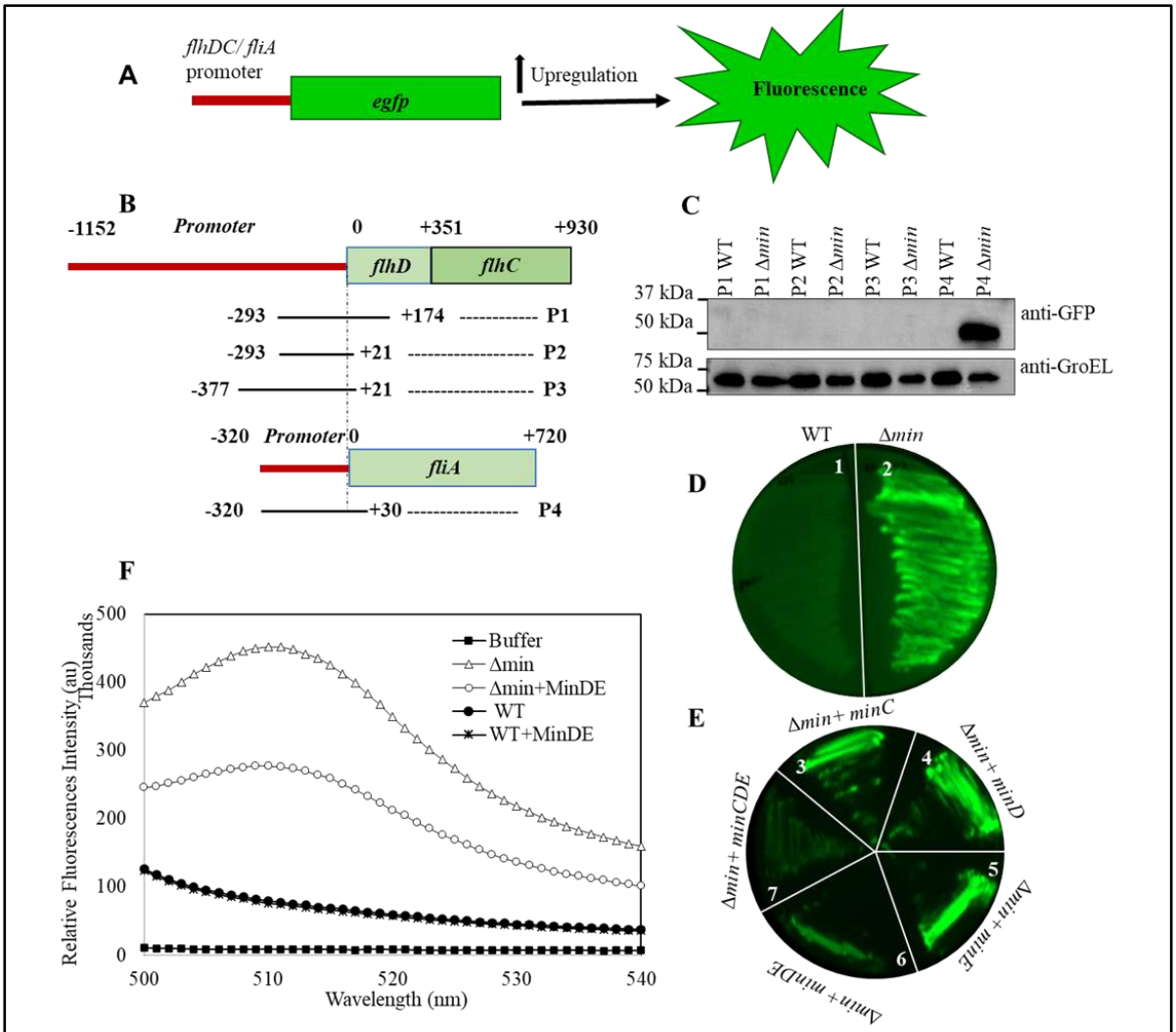

Figure: 4

Figure 4. *fliA* eGFP reporter assay. Native *flhDC* and *fliA* promoters were cloned with an eGFP reporter to study expression, detected using anti-GFP and anti-GroEL antibodies. ***Panel A.*** Pictorial representation of the eGFP reporter assay. ***Panel B.*** Schematic of truncated *flhDC* (P1, P2, P3) and *fliA* (P4) promoter constructs. : P1 = *flhDC* 467 bp, P2 = f*lhDC* 314 bp, P3 = *flhDC* 398 bp, P4 = *fliA* 350 bp. ***Panel C.*** Western blot showing eGFP expression with different promoter constructs in *E. coli* (Δ*min*) and wild type. ***Panel D and Panel E*** *fliA* promoter expression with various Min protein complementation in Δ*min* cells. ***Panel F.*** eGFP fluorescence intensity in WT, Δ*min*, and Δ*min* cells complemented with MinDE.

MinDE does not interact with FlhDC flagella master regulator:

The above qRT-PCR data and reporter data suggested that there is an upregulation of the *fliA* gene as well as *fliA* promoter activity in Δ*min* cells. Thus, there is a possibility that MinDE functions as a transcriptional repressor by binding directly to the promoter region of FliA and controls flagellation. However, our bioinformatics analysis suggested the absence of any DNA binding motif in MinDE (ref). So, MinDE probably is not binding to the promoter region of *fliA* but it may bind to FlhDC complex, a class I transcriptional factor that controls *fliA* expression. So, we checked if there is any direct interaction of MinDE with FlhDC protein using yeast two-hybrid assay. The yeast two-hybrid assay showed that MinDE does not interact with FlhDC (Fig; 5).

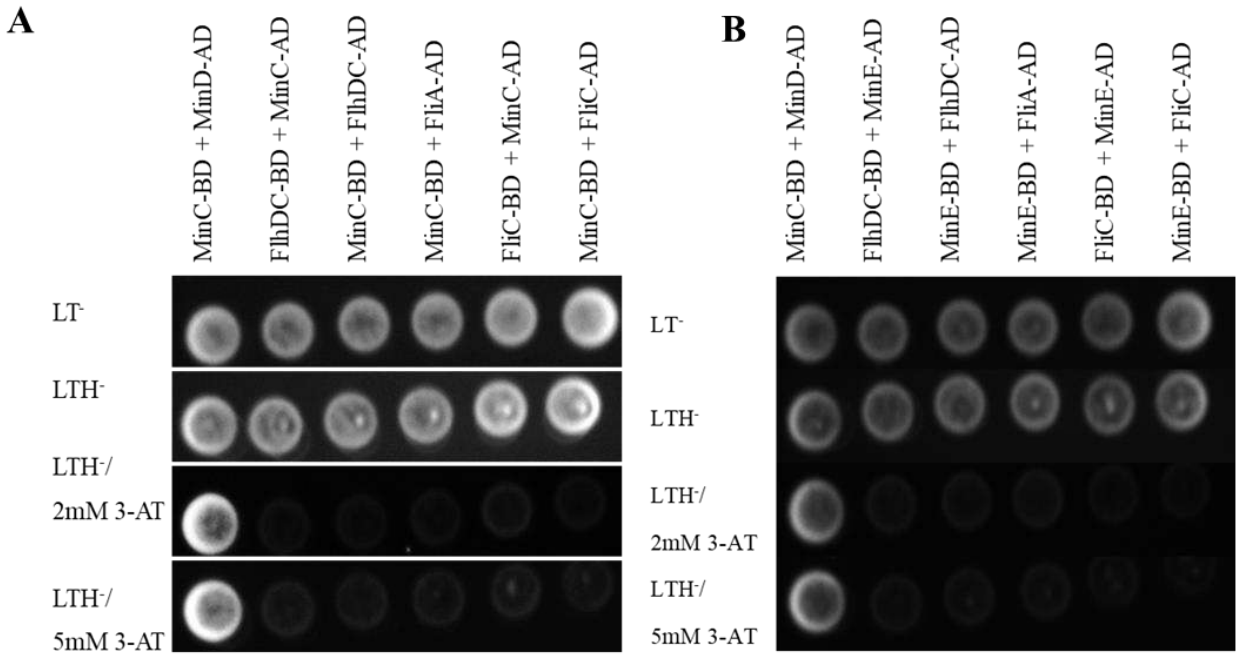

Figure: 5

Figure 5. Interaction of Min protein with *fliA* promoter and FlhDC: ***Panel A:*** Yeast two-hybrid assay demonstrating the interaction of MinD with FlhDC, FliA, and FliC proteins. ***Panel B:*** Yeast two-hybrid results showing the interaction of MinE with FlhDC, FliA, and FliC. Positive interactions are indicated by colony growth on selective plates.

Flagella regulator FlhG and FleN are MinD homologue:

Since MinD and FlhDC do not interact directly, this made it easier and more motivating for us to find MinD partner who regulates flagellar motility. We used BLAST search to find the MinD homologs in other bacteria. Remarkably, our findings indicated that MinD homologues, FlhG/FleN, are flagella regulatory proteins. Highly conserved areas were found between these proteins in the multiple sequence alignment of MinD homologues (Fig. 6A). In *Vibrio alginolyticus,* FlhG is a MinD-like ATPase that attaches to the cell membrane and regulates the number of flagella (23,24). Similarly, FleN regulates *Pseudomonas aeruginosas* flagella count (25). We also carried out structural alignment between FlhG/FleN and MinD (Fig: 6C). The findings demonstrated MinD show high structure similarities with FleN aand FlhG.

FlrA/ FleQ are the AtoC homologue and MinD interacts with AtoSC:

Similar to MinD, its homologs FlhG/FleN do not have DNA binding domains, but they interact with the sigma 54 factors FlrA/FleQ to control the expression of flagellar genes. To trigger the expression of the flagellar gene, FlrA and FleQ bind to the *fliA* promoter (26). But FlhG/FleN and FlrA/FleQ absent in *E. coli*. We postulated that MinD may interact with FlrA/FleQ homolog. Over 40% of AtoC's similarity to FlrA/FleQ was found using BLAST search against the *E. coli* K12 genome, and structural alignment also shows high similarity (Fig: 6D). So, we hypothesized that MinD may interact with AtoC to control the expression of flagellar genes in *E. coli*.

We examined MinD's interaction with AtoC using Y2H assay to test our hypothesis, but we were unable to detect any direct connection. Rather, we discovered that MinD interacts with AtoS of AtoSC complex (Fig: 7A). The pulldown assay demonstrated a direct interaction of His-tagged cytoplasmic AtoS and GST-MinD (Fig 7B).

MinD regulates *fliA* promoter activity via interact with AtoSC:

The AtoSC complex is known to positively regulate *E. coli* motility (27). We used an eGFP reporter assay to investigate the effects of MinD-AtoS interaction on *fliA* promoter activity in order to better understand how motility is regulated by both the protein. We found that in presence of AtoS the *fliA* promoter activity increased, whereas in presence of MinD the *fliA* promoter expression decreased it (Fig. 7C), indicating that MinD interacts with AtoS and negatively regulate flagellation.

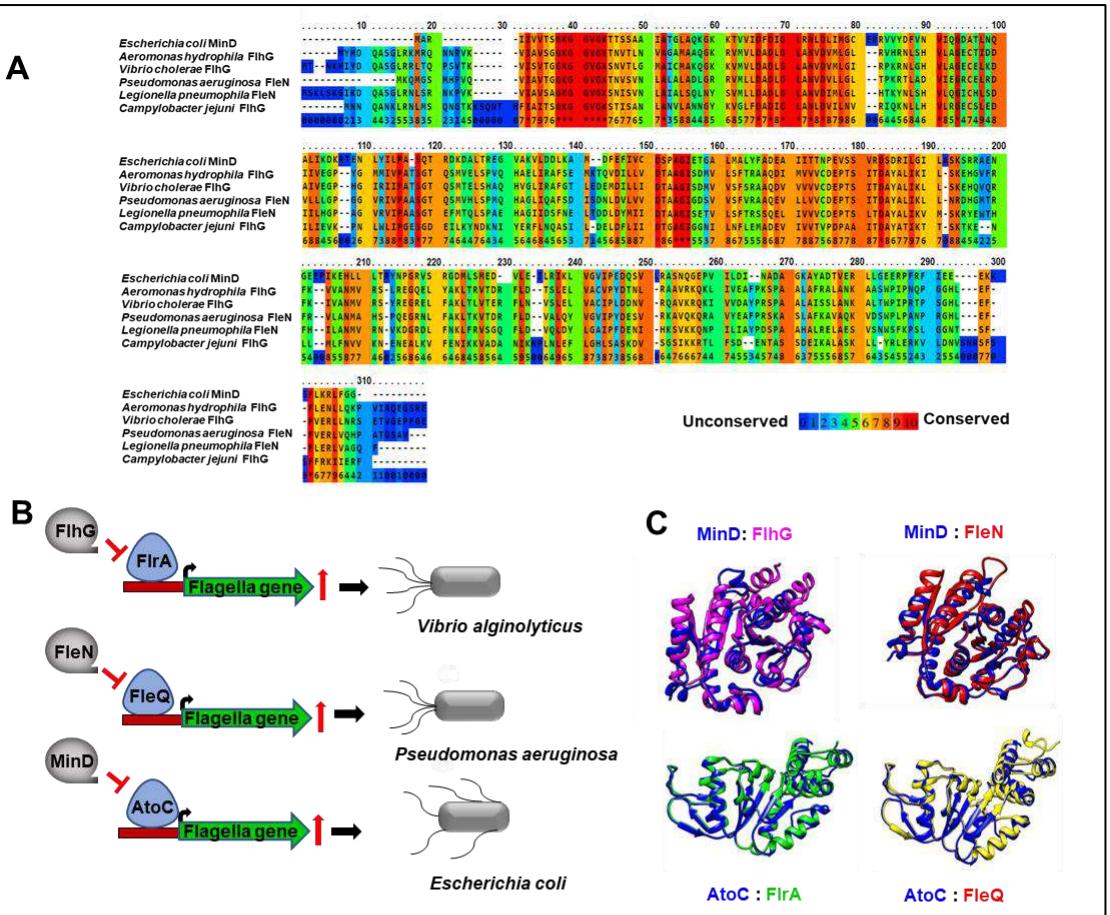

Figure: 6

Figure 6: *Escherichia coli* MinD homologs in bacteria and MinD binding partners for flagella synthesis. ***Panel A*** displays the multiple sequence alignment of MinD homologs from various bacteria using the Praline program. ***Panel B*** provides a schematic of MinD homologs and their regulatory partners involved in flagellation across different species. ***Panel C*** shows structural alignments of MinD (PDB: 3Q9L), FlhG (PDB: 4RZ2), and FleN (PDB: 5J1J), as well as structural comparisons of FleQ, FlrA, and AtoC using Chimera and PROCHEK. Panel D presents the sequence alignment of FleQ, FlrA, and AtoC.

MinDE inhibits AtoSC phosphorylation:

Previously it was reported that AtoC phosphorylation regulates of motility in *E. coli*. And phosphorylation of AtoSC is also necessary for *atoDAEB* promoter activity (20). After AtoS autophosphorylates and transfers phosphate it phosphate to AtoC which in turn activates *atoDAEB* promoter. This promoter cannot be activated by AtoS mutants that are impaired in ATP-binding and phosphorylation. According to our reporter assay, AtoSC increases *atoDAEB* promoter activity, whereas MinDE decreases it, indicating that MinDE inhibits the phosphorylation of AtoS (Fig: 7D). Since AtoC phosphorylation promotes flagellation and MinDE inhibits flagellation, MinDE effect on flagellation is likely due to its inhibition of AtoSC phosphorylation.

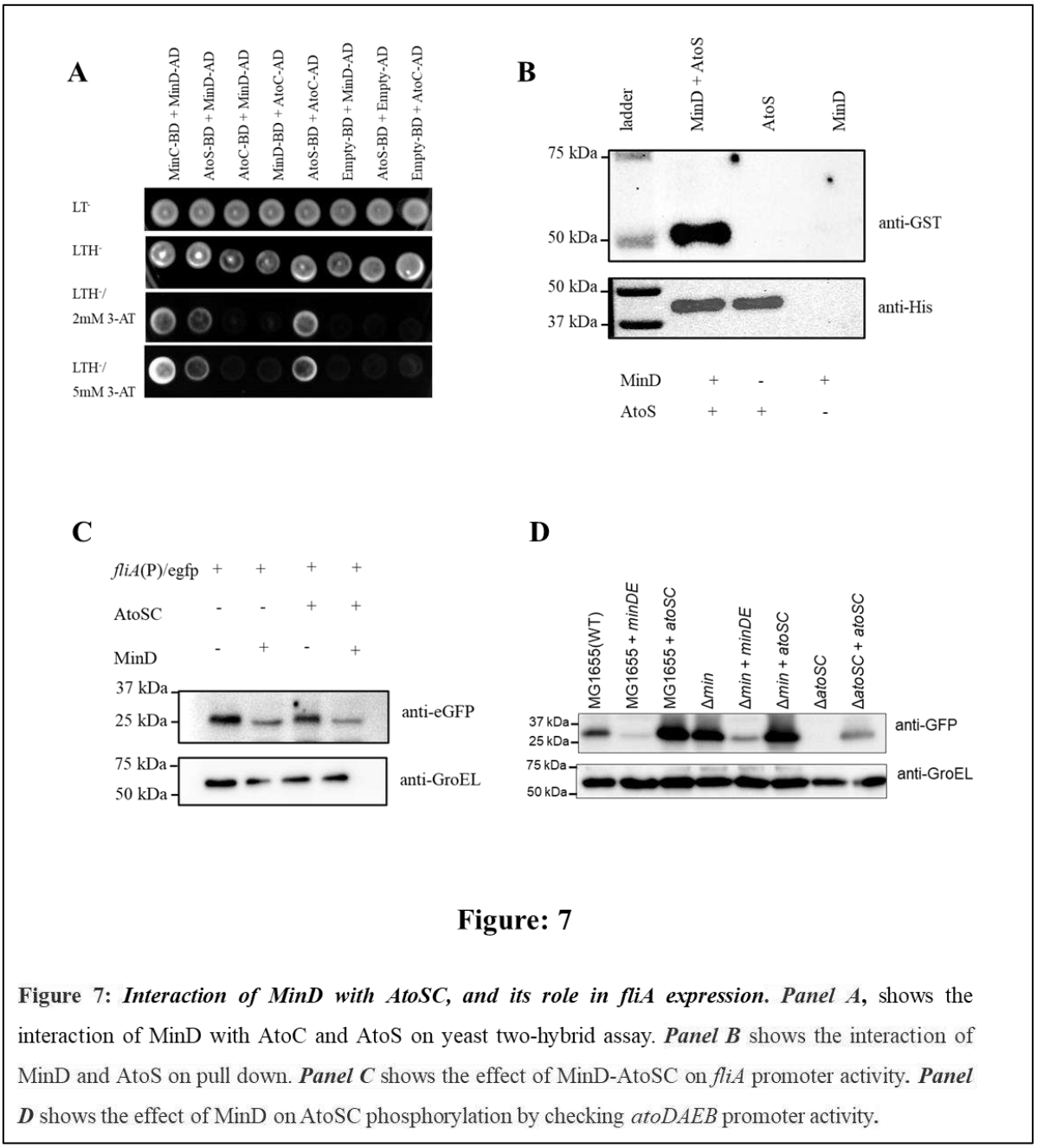

Figure: 7

Figure 7: ***Interaction of MinD with AtoSC, and its role in fliA expression. Panel A*****,** shows the interaction of MinD with AtoC and AtoS on yeast two-hybrid assay. ***Panel B*** shows the interaction of MinD and AtoS on pull down. ***Panel C*** shows the effect of MinD-AtoSC on *fliA* promoter activity. ***Panel D*** shows the effect of MinD on AtoSC phosphorylation by checking *atoDAEB* promoter activity.

G. Statistical Analysis:

In all cases we used an unpaired t-test to compare the sampling process and error bars show the ± SEM of three replicates and *p* values were <0.05.

H. Discussion:

Bacterial flagella-mediated locomotion is a complex process and the assembly of the flagellum is synchronized with cell division so each round of division daughter cell receives an equal number of flagella. Studies show that different bacteria use different strategies to coordinate these two cellular processes. Like *Myxococcus xanthus* stops its motility during division and restarts it after completion of division, whereas *Caulobacter crescentus* changes its flagella into a stalk-like structure before division, and after division assuring daughter cells receive functional polar flagella. The complicated relationship between cell division and motility is not fully understood in the model organism like *E. coli*. We here demonstrated that molecular mechanisms that link cell division and motility.

The *E. coli* Min system is previously known for its role in cell division we here uncover its role in motility and flagellation. When we looked *E. coli* Δ*min* strains under the microscope, we found that these cells are more motile than *E. coli* wild-type cells. Soft agar motility assay validated the hypermotility seen in Δ*min* cells is caused by the lack of the Min system. The wild-type non-motile in Δ*min* cells phenotype was restored when *minCDE* was complemented on it confirming that the lack of Min proteins is the cause of the observed hypermotility.

Further examination demonstrated that Δ*min* cells were hyperflagellated, with dense flagella covering the whole cell surface. This was verified by transmission electron microscopy and fluorescence microscopy. In Δ*min* cells, there was higher expression of flagellar genes, such as *fliA, motA, motB,* and *fliC*, so the hyperflagellation is due to this. In *E. coli*, the expression of flagellar genes is regulated in a three-tiered manner: class I flagellar protein FlhDC influences the expression of class II flagellar protein FliA, which in turn controls the expression of class III genes. Δ*min* cells exhibit increased expression of *fliA* and other FliA-controlled genes, which is correlated with their enhanced motility and flagellation, according to our complementation studies and qRT-PCR results. Nevertheless, the absence of DNA binding motifs in MinD and MinE indicates that they do not bind to the *fliA* promoter directly but they may interact with some other regulatory protein to control the *fliA* expression.

We further investigate the potential connections between MinD and flagellar regulatory proteins to understand the regulatory process. According to our yeast two-hybrid assay there was no direct interaction found between MinD and FlhDC or other flagellar master regulators. From bioinformatics analysis, we found that MinD homolog FlhG and FleN control motility and flagellar expression. Both of FlhG and FleN has lack of DNA binding domain they interact with transcriptional regulators FlrA and FleQ and control flagella gene expression. But interestingly this regulatory system is absent in *E.*

coli and AtoC is the homologue of FlrA -and FleQ in this organism. Our results suggest that instead of directly interacting with flagellar regulators, MinD interacts with AtoS, a part of the AtoSC complex like its homolog. The *fliA* promoter is more active when AtoSC is present, but MinD decreases this activity, suggesting that MinD inhibits AtoS phosphorylation, which in turn adversely affects flagellar gene expression. It is believed that this suppression hinders AtoC binding to the *fliA* promoter, which in turn regulates *E. coli* flagellation.

I. Impacts and Future direction:

The knowledge gained from this study about the synchronization of bacterial motility with cell division may prove to be extremely beneficial to humankind as both motility and cell division are important for the full pathogenesis of bacteria. The understanding of *E. coli* Min system on bacteria motility provides new insights into the regulation of flagellar gene expression by MinDE, which may be used to target the flagella and the motility of bacteria to develop novel antibacterial techniques. Improving treatment efficacy and tackling antibiotic resistance may require the use of such focused strategies. These findings also improve our understanding of fundamental bacterial processes, which can inform the design of innovative biotechnological applications. Moreover, understanding these bacterial processes can also benefit technology and the environment. For example, we could use this knowledge to design new tools for cleaning up pollutants or creating bacteria-based sensors. In short, this research not only improves our grasp of basic bacteriology but also has the potential to lead to practical solutions that could enhance public health and technological applications.

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