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# DELECIÓN DE UN ARN REGULADOR PEQUEÑO (SdsR) EN DOS CEPAS PATÓGENAS DE *ESCHERICHIA COLI* (CFT073 Y EDL933) Y SU INFLUENCIA EN LA RECUPERACIÓN DEL CRECIMIENTO Y VIABILIDAD BACTERIANA

# DELETION OF NON-CODING SMALL RNA (SdsR) OF TWO PATHOGENS *ESCHERICHIA COLI* STRAINS (CFT073 & EDL933) AND HOW THIS AFFECTS THEIR GROWTH RECOVERY & BACTERIAL VIABILITY

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## RESUMEN


El pequeño ARN no codificante llamado SdsR, es transcrito en bacterias que se encuentran en fase estacionaria debido a la falta de nitrógeno, un nutriente indispensable para su crecimiento. SdsR regula los procesos metabólicos llevados a cabo por *Escherichia coli* para volver a entrar en fase exponencial cuando entran nuevos nutrientes al medio. La función principal de este pequeño ARN es unirse mediante un emparejamiento de bases a ARN mensajeros inhibiendo o activando su traducción, dependiendo de la necesidad de producir dichas proteínas. En este trabajo, se han producido las versiones Δ*sdsR* de dos cepas de *E. coli* patógenas CFT073 (uropatogénica) y EDL933 (enterohemorrágica). De esta manera, el objetivo de este TFG fue comprobar si SdsR tiene la misma influencia en las cepas patógenas que en la cepa de *E. coli* no patógena MG1655 a la hora de recuperar el crecimiento y sobrevivir tras una larga fase estacionaria (24 h) sin o con inanición de nitrógeno (N-24).

La producción de las versiones Δ*sdsR* se llevó a cabo mediante métodos de clonación y recombinación bacteriana. Tras secuenciar sus genomas, éstos fueron los correctos. Para evaluar la influencia de SdsR durante la recuperación del crecimiento tras una larga fase estacionaria, tanto las bacterias *wild-type* (forma silvestre más común) como las bacterias Δ*sdsR*, crecieron durante 24 h en medio de cultivo LB. Después fueron inoculadas en nuevo LB y su turbidez ($OD_{600nm}$) fue medida cada 15 min. Los resultados mostraron que SdsR no es requerido para una recuperación del crecimiento óptimo en LB. Además, se determinó la viabilidad de las bacterias detenidas en crecimiento durante 24 h en LB o con inanición de nitrógeno (N-24) en Gutnick medio mínimo, midiendo unidades formadoras de colonias (UFC) en placas de LB agar. Los resultados mostraron que SdsR no es requerido para una viabilidad bacteriana óptima tras una larga fase estacionaria (24 h) sin o con inanición de nitrógeno. Por último, se evaluó la influencia de SdsR en la recuperación del crecimiento en bacterias N-24. Los resultados obtenidos mostraron dos fenotipos diferentes, en la cepa MG1655, SdsR es requerido para una recuperación del crecimiento óptimo en Gutnick medio mínimo, mientras que en la cepa EDL933, SdsR no es requerido. Este trabajo aumenta el conocimiento de las redes de regulación postranscripcional relacionadas con la recuperación del crecimiento y la viabilidad de *Escherichia coli*, así como la influencia en dicha regulación del sRNA SdsR.

## ABSTRACT

The small non-coding RNA called SdsR is transcribed in bacteria that are in stationary phase due to nitrogen deficiency, an essential nutrient for their growth. SdsR regulates the metabolic processes carried out by *Escherichia coli* to recover the exponential phase when new nutrients enter in the medium. The main function of this small RNA is to bind through base pairing to messenger RNAs, inhibiting or activating their translation, depending on the need to produce these proteins. In this work, the Δ*sdsR* versions of two pathogenic strains of *E. coli*, CFT073 (uropathogenic) and EDL933 (enterohaemorrhagic), have been produced. Thus, the aim of this work was to verify if SdsR has the same influence on pathogen strains as on the non-pathogen *E .coli* MG1655 when recovering growth and surviving after a long stationary phase (24 h) without or with nitrogen starvation (N-24).

The production of Δ*sdsR* versions was carried out using cloning and bacterial recombination methods. After sequencing their genomes, they were correct. To evaluate the influence of SdsR during growth recovery after a long stationary phase, both wild-type bacteria (most common wild form) and Δ*sdsR* bacteria grew for 24 h in LB culture medium. They were then inoculated into fresh LB and their turbidity ($OD_{600nm}$) was measured every 15 min. The results showed that SdsR is not required for optimal growth recovery in LB. In addition, the viability of bacteria arrested in growth for 24 h in LB or with nitrogen starvation (N-24) in Gutnick minimal medium was determined by measuring colony forming units (CFU) on LB agar plates. The results showed that SdsR is not required for optimal bacterial viability after a long stationary phase (24 h) without or with nitrogen starvation. Finally, the influence of SdsR on growth recovery in N-24 bacteria was evaluated. The results obtained showed two different phenotypes: in MG1655 strain, SdsR is required for optimal growth recovery in Gutnick minimal medium, while in the EDL933 strain, SdsR is not required. This work increases our understanding of the post-transcriptional regulation networks related to growth recovery and viability of *Escherichia coli*, as well as the influence of the sRNA SdsR on such regulation.

## 1. INTRODUCTION.

Since the discovery of antibiotics, they are some of the most important drugs. However, soon after their initial discovery, it became clear that bacteria could develop resistance to them (Martinez, 2014) due to natural selection (Santos-Lopez *et al.*, 2021). In addition, their unregulated use has accelerated this bacterial evolution. To address this issue, a strategy employed for several decades involved consistently introducing new antibiotics (Martinez, 2014).

Currently, antibiotic resistance is a serious problem because new antibiotics are not being discovered and the current treatments to fight bacterial infections are not unlimited. This has caused a global health threat because the antibiotic resistant pathogens are causing a high morbidity and mortality (Frieri *et al.*, 2017) (1.27 million deaths in 2019 (Murray *et al.*, 2022)). Although bacterial evolution via natural selection is inevitable (Santos-Lopez *et al.*, 2021), we must be aware of this global problem and try to mitigate the increase of antibiotic resistance with new antibacterial treatments (Frieri *et al.*, 2017).

Understanding the genetic basis of bacteria that are in dormancy state, when they are more resistant to antibiotics (Fleischmann *et al.*, 2021), it is important to understand how and why antibiotics can fail. Bacterial gene regulation is highly dependent on small RNAs (sRNAs). They are non-coding polynucleotides with 50-500 bp which are transcribed by bacteria to regulate the translation of their genes (Stork *et al.*, 2007). They are found throughout the bacterial chromosome (Hör *et al.*, 2018) and have three major functional domains: at least one to bind a mRNA or another sRNA by base-pairing, one to interact with RNA chaperones (proteins that assist the conformational folding or unfolding of large proteins) and a Rho-independent terminator to provide stability to the transcript (Papenfort & Melamed, 2023).

sRNAs act at post-transcriptional level, *i.e.* the control of RNA processing, that occurs between the stages of gene transcription and gene translation. They are involved in hundreds of translational regulatory chains (Papenfort & Melamed, 2023), such as regulation of bacterial virulence (Stork *et al.*, 2007), biofilm formation (Bak *et al.*, 2015), motility (Babitzke & Romeo, 2007), cell structure (Guillier *et al.*, 2006), carbon metabolism, envelope stress, iron homeostasis and quorum sensing (Papenfort & Melamed, 2023).

sRNAs bind to the mRNAs through base pairing (Huber *et al.*, 2020) sometimes to activate the expression of the gene and sometimes to inhibits that expression (Waters & Storz, 2009). One sRNA can regulate many mRNAs and one mRNA can be regulated by many sRNAs. They are

classifieds depending on the mRNA that it regulates. There are two types: cis-acting sRNA and trans-acting sRNA. Cis-acting sRNA can only interact with mRNAs encoded in the same genetic locus, having full interaction complementary. Trans-acting sRNA can interact with mRNAs encoded in different genetic locus, having partial interaction complementary (Guillet *et al.*, 2013). The interaction between sRNAs and their target mRNAs is often facilitated by RNA binding protein Hfq, a ring-shaped hexamer that is abundant in Gram-negative bacteria (Melamed *et al.*, 2016).

It is known from previous studies that sRNAs can activate the translation of a mRNA by two main mechanisms. If the mRNA has a stem-loop structure in the 5′ UTR (untranslated region), the sRNA releases that structure and the mRNA can be translated (Papenfort & Vanderpool, 2015). In the second mechanism, the binding site of sRNA to mRNA is also the ribonuclease cleavage site. Therefore, when the sRNA is bound, the ribonuclease cannot cleave the mRNA and this one can be translated (Papenfort *et al.*, 2013). There is another mechanism in which a sRNA changes the secondary structure of an mRNA and that cause the dissociation of Hfq chaperone. Therefore, the ribosome binding site (RBS) will be free to start the translation (Prévost *et al.*, 2007).

sRNAs are also able to inhibit the translation of an mRNA. The main mechanism is by binding to the mRNA by base-pairing in the RBS, so the ribosome cannot translate it. It is possible that the resulting translation block is sufficient to promote the rapid degradation of the targeted mRNA (Prévost *et al.*, 2011). There are also translation repression mechanisms based on binding to the mRNA in the upstream of RBS, as well as the upstream open reading frame (uORF) repression (Večerek *et al.*, 2007), masking of a standby site (Darfeuille *et al.*,2007) or masking of an enhancer site (Azam & Vanderpool, 2020).

The bacterial growth is classically divided in four main phases. The first one is lag phase, during this phase the bacterial population adapts to the new characteristics of the environment (Swinnen *et al.*, 2004) and grow slowly. Then, the bacteria move into the exponential phase, when the cell division is maximal with a constant rate (Rolfe *et al.*, 2012). There comes a point where conditions change, or the population becomes too dense. This causes the decrease of the growth rate, and the bacteria enter the stationary phase. During this phase, the mortality rate starts to increase (Akerlund *et al.*, 1995). Finally, the death phase take part when the mortality rate is higher than the growth rate because the conditions are not favourable. Then overall the population decreases (Al-Qadiri *et al.*, 2008).

Nitrogen, as a building block of vital biomolecules, is a key nutrient for bacterial growth and essential cellular functions (Brown, 2019). The genes involved in the bacterial response to nitrogen starvation play pivotal roles in optimizing nitrogen utilization, scavenging for nitrogen sources, and conserving energy under nutrient-limited conditions. By regulating the expression of these genes, bacteria can adapt to varying levels of nitrogen availability in their ecological niches, underscoring their remarkable capacity for survival (Monostori *et al.*, 2017).

As well as being able to survive in conditions of nutrient starvation by remaining stationary phase. It is important for the bacteria to be able to start their growth again rapidly when new nutrients enter in the environment (McQuail, 2021). There are many abiotic and biotic factors that can affect the ability of bacteria to recover (Swinnen *et al.*, 2004). sRNA SdsR is highly expressed in *Escherichia coli* during the stationary phase. It is part of the RpoS regulon and acts as both cis-acting and trans-acting (Choi *et al.*, 2018).

SdsR has been shown to affect the growth recovery of *E coli*. This sRNA interacts with 124 mRNA targets in *E. coli* subjected to prolonged nitrogen starvation. The bacteria's growth recovery after a long-term N starvation is significantly impaired in the absence of SdsR, being the lag phase longer than in wild type (WT) bacteria (McQuail *et al.*, 2023).

The main aims of this work are: i) produce Δ*sdsR* versions of *Escherichia coli* CFT073 and EDL933 by cloning and recombination methods, *i.e.* bacteria without *sdsR* in their genome; ii) Test if the same phenotype is observed in *Escherichia coli* pathogens strains CFT073 and EDL933 as in non-pathogen strain K12 MG1655. The wild type and Δ*sdsR* bacteria phenotypes were compared by measuring growth recovery and viability of the bacteria.

In this work, three *E. coli* strains were used. *E. coli* K12 MG1655, which is a non-pathogen laboratory strain (Biryukova *et al.*, 2010). The sRNAs of this strain have been studied in a lot of works (Bak *et al*, 2015) as well as their interactions with mRNAs (McQuail *et al.*, 2023). The other two *E. coli* strains are pathogens. A uropathogenic strain (CFT073), which is P-fimbriated pyelonephritogenic and hemolysin-positive. It has a high cytotoxicity and cause acute pyelonephritis (Mobley *et al.*, 1990). Finally, an enterohemorrhagic strain (EDL933), which is an O157:H7 serotype and cause haemorrhagic colitis (Wells *et al.*, 1983) was used. In both, the influence of SdsR has never been studied before. In terms of genetics, the genome of CFT073 is 590,209 bp longer than MG1655's genome and similar in size to EDL933. In terms of proteins that they synthesise, both pathogens have genes to synthesise about 750 more proteins than the non-pathogen strain (EDL933: 5,063; CFT073: 5,016; MG1655; 4,288) (Welch *et al.*, 2002).

## 2. MATERIALS AND METHODS.

All the experiments were carried out between 10/07/2023 and 20/10/2023 in the molecular microbiology laboratory of the infectious diseases department of the Centre for Bacterial Resistance Biology (CBRB) at Imperial College London.

### 2.1. Bacterial strains, plasmid and growth conditions.

All bacterial strains and plasmid used in this study are listed in Table 1. Strains were routinely grown in either lysogeny broth (LB) or Gutnick minimum medium (33.8 mM $KH_2PO_4$, 77.5 mM $K_2HPO_4$, 5.74 mM $K_2SO_4$, 0.41 mM $MgSO_4$), supplemented with Ho-LE trace elements (Atlas, 2010), 0.4% glucose, and $NH_4Cl$ at either 10 mM (for precultures) or 3 mM (for growth). In all growth assays and studies, overnight cultures were subcultured in 7.5ml of supplemented Gutnick media (3 mM $NH_4Cl$) or LB at a starting $OD_{600nm}$ of 0.05. Antibiotics were added at the following concentrations: Kanamycin – 50 µg/ml and Ampicillin – 100 µg/ml.

**Table 1.** Bacterial strains and plasmid used in this study.

| Bacterial strains | Antibiotic resistance | Reference |
|---|---|---|
| ***Escherichia coli*** | | |
| MG1655 | None | *E. coli* Genetic Stock Centre |
| MG1655 Δ*sdsR* | Kanamycin | (McQuail *et al.*, 2023) |
| CFT073 | None | *E. coli* Genetic Stock Centre |
| CFT073 Δ*sdsR* | Kanamycin | This Study |
| EDL933 | None | *E. coli* Genetic Stock Centre |
| EDL933 Δ*sdsR* | Kanamycin | This Study |
| **Plasmid** | | |
| pKD46 | Ampicillin | (Datsenko & Wanner, 2000) |

### 2.2. Production of Δ*sdsR* versions.

Two mutant strains (*E. coli* CFT073 Δ*sdsR* and *E. coli* EDL933 Δ*sdsR*) were created from *E. coli* CFT073 and *E. coli* EDL933 cells, respectively. *E. coli* K12 MG1655 Δ*sdsR* was created previously by Dr. Josh McQuail (McQuail *et al.*, 2023).

#### 2.2.1. Pathogens wild type DNA purification.

Genomic DNA extractions of CFT073 and EDL933 WT strains were performed following the INVITROGEN PureLink® Genomic DNA Kit. This protocol consists of four main phases. Cell lysis, binding the DNA to a column, washing the DNA and eluting the DNA from the column.

#### 2.2.2. PCR amplifications.

The specific exchange of our gene of interest (*sdsR*) was replaced by an antibiotic resistance cassette performing several PCRs (kanamycin was selected to obtain the CFT073 Δ*sdsR* and EDL933 Δ*sdsR* strains). The recombination DNA fragment was composed with the $kan^R$ gene, the

upstream region of *sdsR* and the downstream region. These regions were selected in order to provide homology for the recombination (Figure 1).

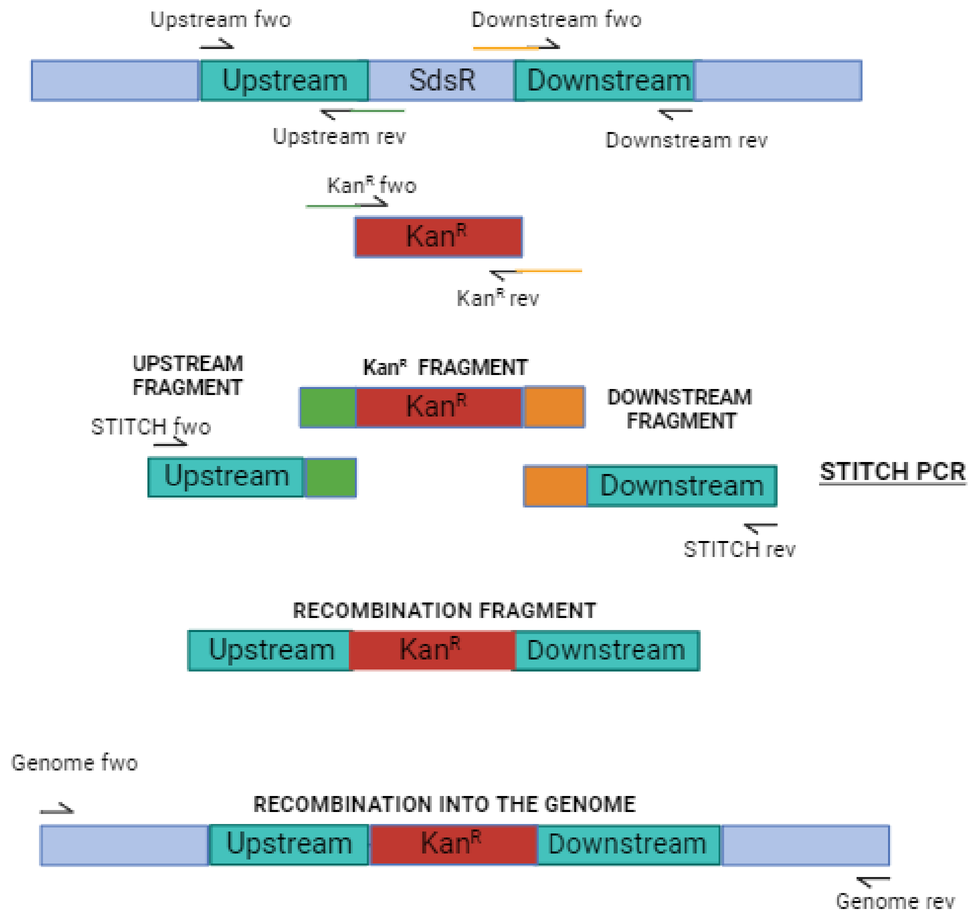



**Figure 1.** Illustration of *sdsR* deletion with PCRs.

The upstream and downstream regions of *sdsR* were amplified using PCR as follows: 1 units of Q5 DNA Polymerase (New England Biolabs), 0.2 mM dNTPs, 1x Phusion HF reaction buffer (New England Biolabs), 0.5 µM primers, 1 µL of genomic DNA (template) and up to 50 µL of molecular water. An initial denaturation of 30 s at 98°C, 30 cycles of 10 s denaturing at 98 °C, 30 s annealing at 55-75 °C (dependent on the tm of the primers) and 30 sec $kb^{-1}$ extension at 72 °C, and a final extension of 5min at 72°C were performed.

The *kan*$^R$ cassette was amplified using the same DNA Polymerase and the same PCR's protocol described above. The genomic Kan fragment DNA was already purified by Dr. Josh McQuail. Then, all the PCR products were purified after upscaling them using the MinElute PCR Purification Kit (QIAGEN). Gel electrophoresis was performed on purified PCR products by loading the product on 1% agarose gel containing SYBR safe (Figure S1) to confirm the correct size. All the designed primers are described in the Supplementary Table 1.

To assemble it, the same DNA Polymerase and the same PCR's protocol described above were used. But with the three purified PCR products amplified previously as a template, to assemble them into a single fragment without the *sdsR* gene. This process is called "STITCH PCR" (Figure 1). The primers used in this PCR are also listed in Table S1. Then, the recombination fragment was purified using the MinElute PCR Purification Kit (QIAGEN). Gel electrophoresis was performed on purified recombination fragment by loading the product on 1% agarose gel containing SYBR safe (Figure S1) to confirm that the correct size DNA was produced.

### 2.2.3. λ red recombination.

To recombine the recombination fragment with the pathogen's genome, pKD46 was used. This plasmid has genes to produce enzymes to help that recombination. This plasmid has resistant ampicillin genes, and it is easy to remove as it is thermosensitive (Datsenko & Wanner, 2000). All the procedures described below were performed for both pathogen strains (CFT073 & EDL933).

First, the cells were prepared for the electroporation in this way: 500 µL of overnight culture were added to 50 mL of LB media with 50 µL of ampicillin and incubated at 37 ºC for 2:30 h (OD600 ≈ 0.5). Then, it was chilled on ice for 30 min, after this, all the subsequent steps were performed under ice-cold conditions. Then, it was centrifuged at 4000 rpm for 10 min in 4 ºC and the supernatant was discarded. The pellet was re-suspended with 25 mL of 15% glycerol and centrifuged again with the same conditions as described above. This step was repeated with 12.5 mL 15% glycerol. Finally, the pellet was re-suspended with 80 µL 15% glycerol.

Thereafter, the electroporation of the cells with pKD46 was performed: 1 µL of pKD46 was added to the prepared strains for the electroporation previously. Then, a pulse was given by the electroporator, and all the sample was added to 1 mL of Super Optimal broth with Catabolite repression (SOC) media. Then, it was incubated at 30 ºC 180 rpm for 2 h. Finally, 200 µL of this culture were plated on LB agar with ampicillin Petri's plate and incubated at 30 °C for 15 h.

Second, transformed bacteria were prepared for a second electroporation with the recombination DNA fragment: 500 µl overnight culture of the bacteria transformed with pKD46 were added to 50 mL of LB media with 50 µL of ampicillin and incubated at 30 °C for 2:30 h ($OD_{600}$ ≈ 0.5). After this time, 50 µl of arabinose was added to the culture for induce the lambda-red recombinase gene expression, it was incubated for 30 min. The next steps were the same as described above.

Then, the electroporation of the cells with the recombination DNA fragment was performed: 80 µL of the recombination DNA fragment were added to the prepared strains for the electroporation previously. The next steps were the same as described above except the incubation, it was at 37 °C. As regards plating, 100 µL of this culture were plated in LB agar with kanamycin. Then, the remaining culture was centrifuged at 8000 g for 3 min and discarded 700 µL of supernatant. The pellet was re-suspended and plated on another LB agar with kanamycin plate.

**2.2.4. Colony PCR and sequencing.**

Colony PCR is a method to verify the presence of the desired gene construct in bacteria grown on selective media after a transformation step. 8 colonies of each transformed strain were added to 20 µL of molecular water separately. 5 µL of each mixture were inoculated to 5 mL LB media with 5 µL kanamycin, performing 8 cultures. The remaining 15 µL were incubated at 95 ºC for 5 min and centrifuged at 13,000 rpm for 3 min. Then, the PCR was performed as follows: 5u $\mu L^{-1}$ of GoTaq DNA Polymerase, 10 mM dNTPs, 5x X Colourless GoTaq buffer, 1 µM primers, 5µL of previous supernatant (template) and up to 50 µL of molecular water. An initial denaturation of 2 min at 95°C, 30 cycles of 30s denaturing at 95 °C, 30s annealing at 55 °C and 1 min $kb^{-1}$ extension at 72 °C, and a final extension of 5 min at 72 °C were performed. Forward primer was primer Genome fw, 950 bp upstream of *sdsR*, and reverse primer was primer Mid Kan, which corresponds to the middle of the $kan^R$ cassette, both listed in table S1. Gel electrophoresis was performed on Colony PCR products by loading the product on 1% agarose gel containing SYBR safe (Figure S1).

Cultures were incubated at 42 ºC overnight to remove the plasmid (pKD46). Then, DNA was purified from two samples of each strain following the INVITROGEN PureLink® Genomic DNA Kit described above. PCRs were performed as Q5 PCR described above, using this purified DNAs as a template and primers Genome fw and Genome rev (Table S1). Finally, PCR products were purified with MinElute PCR Purification Kit (QIAGEN) and sequenced by Genewiz.

**2.3. Growth recovery assay.**

Overnight cultures were subcultured in LB or Gutnick minimum medium 3 mM $NH_4Cl$ and bacteria were growing for 24 hours after reaching stationary phase. Then, they were subcultured in in a fresh medium, either LB or Gutnick minimum medium, in a 24-well plate for a starting $OD_{600nm}$ of 0.05. Finally, $OD_{600nm}$ was measured every 15 min in a SPECTROstar OMEGA plate reader for 24 hours.

**2.4. Bacterial viability assay.**

The proportion of viable cells in the 24 h growth arrested or 24 h nitrogen starved bacterial population was determined by measuring colony forming units (CFU), indicator of the amount of living micro-organisms in a liquid medium, from serial phosphate-buffered saline (PBS) dilutions on lysogeny broth agar plates.

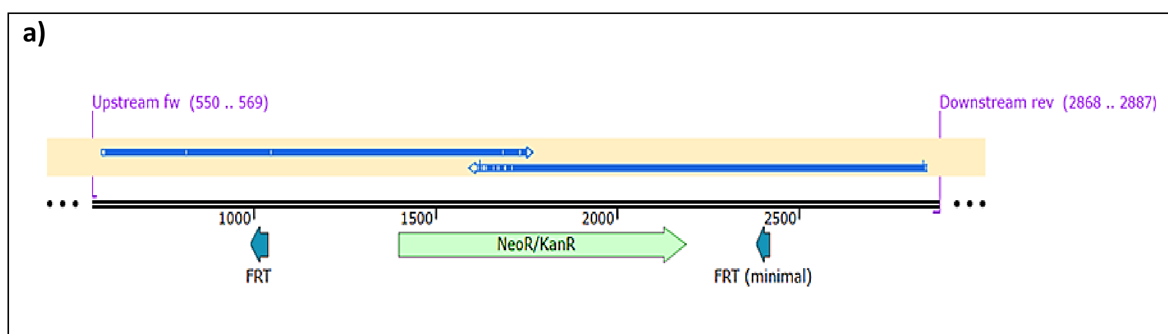
**2.5. Statistical analyses.**

Statistical results obtained for lag time (LT) and growth rate (GR) of WT and Δ*sdsR* bacteria were compared by student's t-tests. Statistical analyses were performed with Rstudio (version 4.3.1) software.

**3. RESULTS.**

**3. 1. Production of *Escherichia coli* CFT073 Δ*sdsR* and EDL933 Δ*sdsR*.**

The production of CFT073 Δ*sdsR* and EDL933 Δ*sdsR* was carried out in accordance with the procedures outlined in the materials and methods section. The productions were confirmed by colony PCRs performed on 8 different colonies of each strain. The forward primer was Genome fw, 950 bp upstream of *sdsR* gene, and the reverse primer was Mid Kan, which corresponds to the middle of the $Kan^R$ gene cassette, both listed in table S1. These primers were used to confirm the presence of the *kan* gene, instead of the *sdsR* gene. Gel electrophoresis was performed on Colony PCR products by loading the product on 1% agarose gel containing SYBR safe. The sizes were as expected, ~1500 bp (Figure S1). DNA from one colony of each strain was extracted and purified. Then, they were sequenced by Genewiz using the primer upstream fw and primer downstream rev, both listed in table S1. The strains were confirmed to be the expected genotype (Figure 2). The terminations of sequencing often exhibit irregularities, which explains the presence of white stripes at the end of the sequences (Cheng *et al.*, 2023).



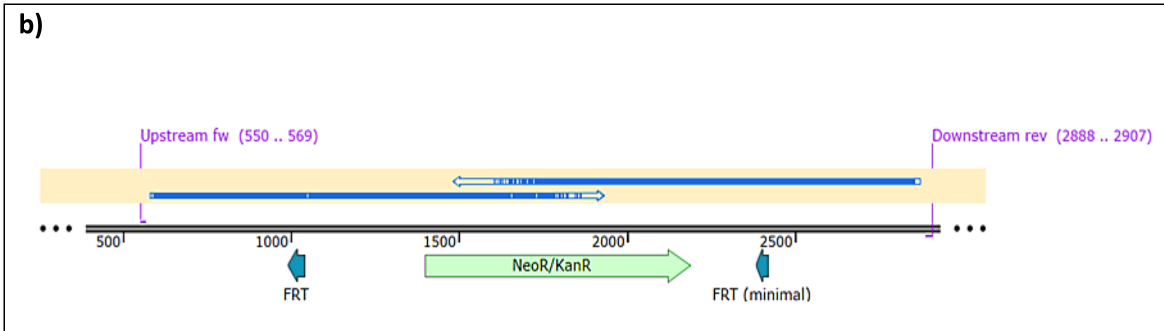



**Figure 2.** Comparison of real (thin blue arrows) and expected (black lines) sequences of (a) *E. coli* CFT073 Δ*sdsR* & (b) *E. coli* EDL933 Δ*sdsR.* White stripes in the real sequences mean that it is not the same nucleotide as in the expected sequences. Figures obtained as an output from SnapGene software.

**3.2. Growth recovery and bacterial viability of pathogens and non-pathogen *E. coli* WT and Δ*sdsR* strains following a long stationary phase (24 h) in Lysogeny Broth (LB).**

In this work, the growth recovery of wild-type (WT) and Δ*sdsR*, of all strains, was assessed when they were grown and recovered after a long stationary phase (24 h) in a nutrient-rich medium, LB. *E. coli* strains, both WT and Δ*sdsR*, were grown in LB medium until reaching the stationary phase. Then, an additional 24-h incubation period was implemented. Finally, the cultures were used to inoculate fresh LB, and the growth recovery of the respective strains was measured.

Upon introducing 24 h growth arrested Δ*sdsR* bacteria into fresh culture media, we did not observe a consistent extension in the lag phase, which is defined as the time from inoculation to the doubling of the $OD_{600nm}$ reading in the culture. We also did not observe a reduction in growth rate compared to WT bacteria (Figure 3). The differences in lag time and growth rate between WT and Δ*sdsR* bacteria of each strain were statistically compared. In all the t-student tests performed, a value of $p>0.05$ was obtained (Table 2). Therefore, in all strains, SdsR is not required for optimal growth recovery after 24 h growth arrest in LB.

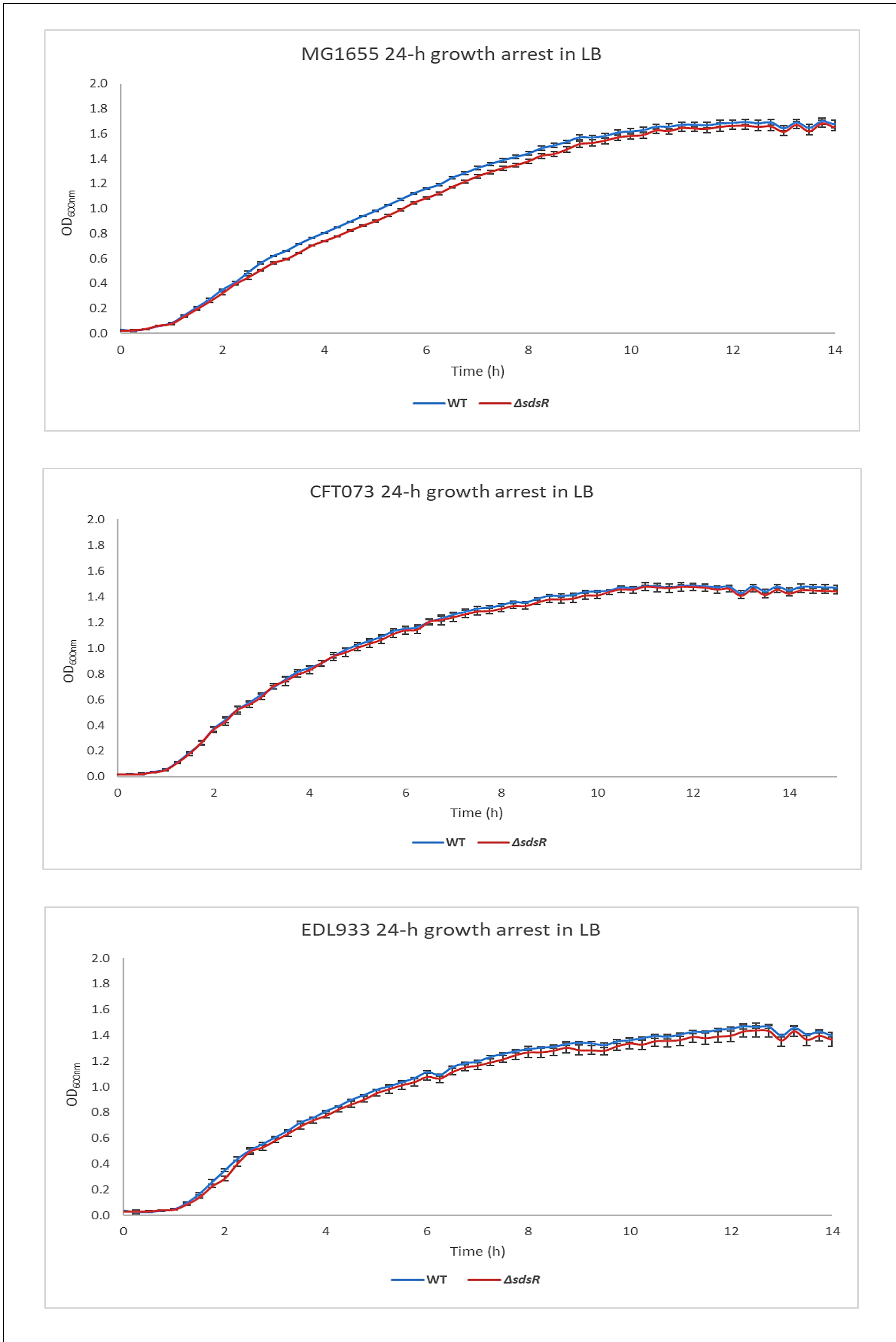



**Figure 3.** Growth recovery of wild-type and Δ*sdsR E. Coli* MG1655, CFT073 & EDL933 following sub-culturing of 24 h growth arrested bacteria into fresh LB.

**Table 2.** Lag time (LT) and growth rate (GR) with standard deviation of wild type and Δ*sdsR E. coli* MG1655, CFT073 & EDL933 following sub-culturing of 24 h growth arrest bacteria into fresh LB. Statistical analysis performed by Student T-test.

| Strain | Parameters | WT | Δ*sdsR* | p-value |
|---|---|---|---|---|
| **MG1655** | LT (h) | 0.77±0.09 | 0.72±0.07 | >0.05 |
| | GR ($OD_{600nm}$/h) | 0.18±0.00 | 0.18±0.00 | >0.05 |
| **CFT073** | LT (h) | 0.95±0.04 | 0.98±0.05 | >0.05 |
| | GR ($OD_{600nm}$/h) | 0.16±0.00 | 0.16±0.00 | >0.05 |
| **EDL933** | LT (h) | 0.99±0.04 | 1.05±0.05 | >0.05 |
| | GR ($OD_{600nm}$/h) | 0.16±0.00 | 0.16±0.00 | >0.05 |

In addition, the viability of WT and Δ*sdsR* 24 h growth arrested bacteria, of all strains, was assessed. Colony forming units (CFU) were measured from serial PBS dilutions on lysogeny broth agar plates.

When bacterial viabilities were measured after a 24 h growth arrest, no significant differences were observed in bacterial viability between WT and Δ*sdsR* bacteria in any strain (Figure 4). The differences in CFU/mL between WT and Δ*sdsR* bacteria of each strain were statistically compared. In all the t-student tests performed, a value of $p > 0.05$ was obtained. Therefore, in all strains, SdsR is not required for optimal bacterial viability after a 24 h growth arrest in LB.

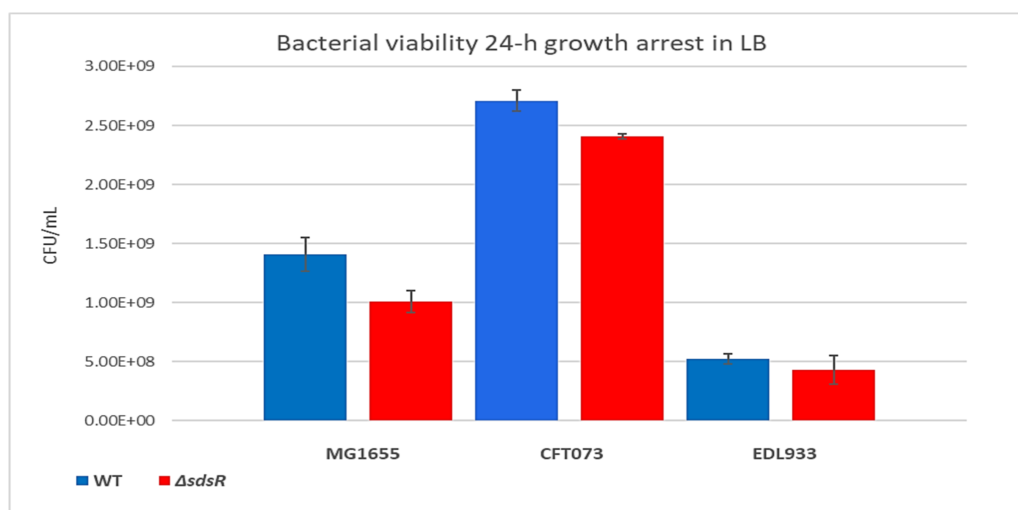



**Figure 4**. Bacterial viability of wild-type and Δ*sdsR E. Coli* MG1655, CFT073 & EDL933 24 h growth arrested bacteria in LB.

**3.3. Bacterial viability of pathogens and non-pathogen *E. coli* WT and Δ*sdsR* strains following a long N starved stationary phase in Gutnick minimum medium.**

Then, the viability of WT and Δ*sdsR* N-24 bacteria, of all strains, was assessed. Bacteria were grown in Gutnick minimum medium supplemented with glucose and a limiting amount of

ammonium chloride ($NH_4Cl$), such that growth arrest corresponded with nitrogen depletion (McQuail *et al.*, 2023). Cultures were grown until the bacteria reached stationary phase, and then for a further 24 hours. WT and Δ*sdsR* CFUs were measured from serial PBS dilutions on lysogeny broth agar plates.

When bacterial viabilities were measured after a 24 h N starvation, no significant differences were observed in bacterial viability between WT and Δ*sdsR* bacteria in any strain (Figure 5). The differences in CFU/mL between WT and Δ*sdsR* bacteria of each strain were statistically compared. In all the t-student tests performed, a value of $p>0.05$ was obtained. Therefore, as stated by McQuail *et al* (2023), SdsR is not required for optimal bacterial viability after a 24 h N starvation in the non-pathogen strain MG1655. In addition is also not required in the 2 pathogen strains tested (CFT073 & EDL933).

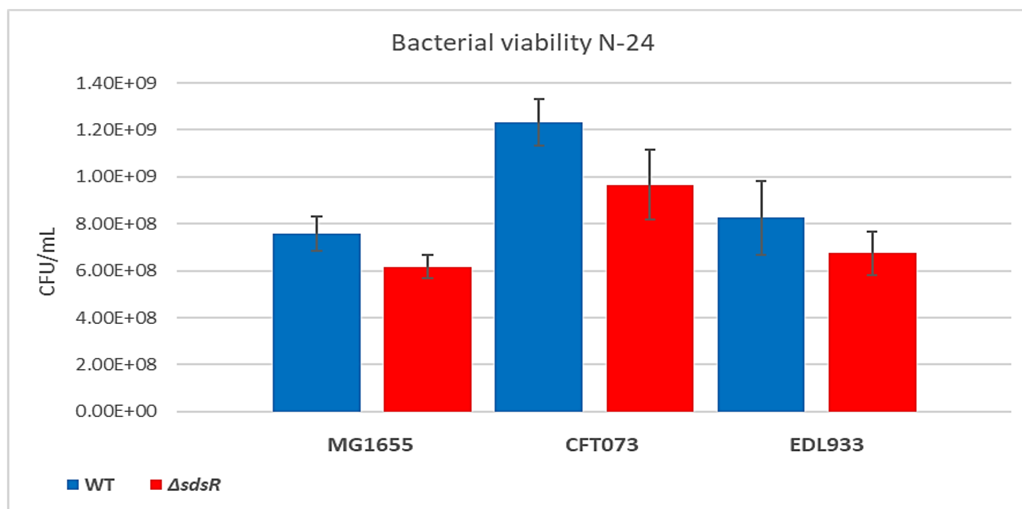



**Figure 5**. Bacterial viability of wild-type and Δ*sdsR E. Coli* MG1655, CFT073 & EDL933 24 h N starved bacteria in Gutnick minimum medium.

### 3.4. Growth recovery of pathogens and non-pathogen *E. coli* WT and Δ*sdsR* strains following a long N starved stationary phase in Gutnick minimum medium.

Finally, the growth recovery of wild-type (WT) and Δ*sdsR*, of all strains, was assessed when they were grown and recovered after a long stationary phase (24 h) in a N starved medium, Gutnick minimum medium. *E. coli* strains, both WT and Δ*sdsR*, were grown in Gutnick minimum medium supplemented with glucose and a limiting amount of ammonium chloride ($NH_4Cl$), such that growth arrest corresponded with nitrogen depletion (McQuail *et al.*, 2023). Cultures were grown until the bacteria reached stationary phase, and then for a further 24 hours. Finally, the cultures were used to inoculate fresh Gutnick minimum medium, and the growth recovery of the respective strains was measured.

Upon introducing N-24 MG1655 Δ*sdsR* bacteria into fresh culture media, we observed a consistent extension in the lag phase. This extension amounted to approximately 44 minutes longer than observed in WT bacteria. We also observed a reduction in growth rate compared to WT bacteria of 81% (Figure 6). The differences in lag time and growth rate between WT and Δ*sdsR* bacteria were statistically compared, for the lag time a p-value <0.05 was obtained and for the growth rate a p-value <0.001 was obtained (Table 2). Therefore, in MG1655 strain, SdsR is required for optimal growth recovery after a 24 h N starvation.

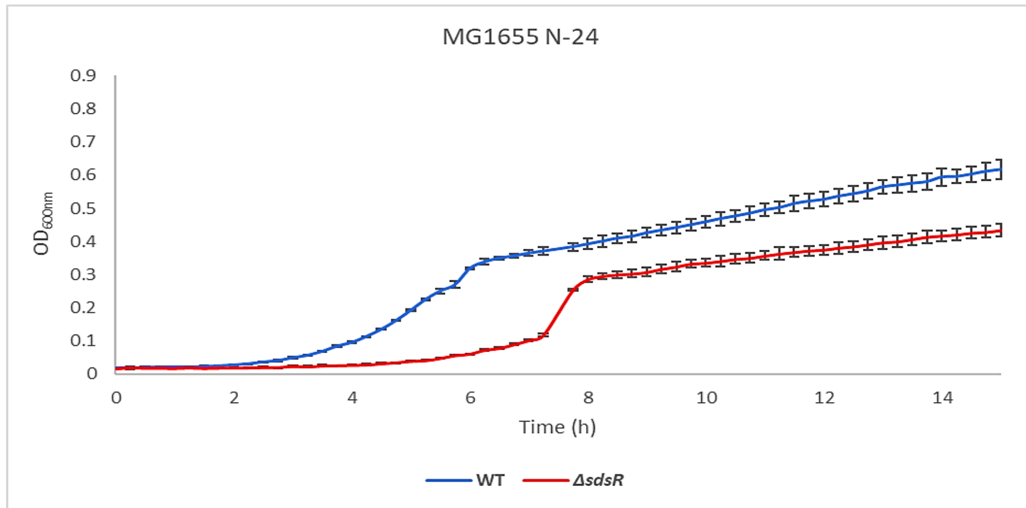



**Figure 6.** Growth recovery of wild-type and Δ*sdsR E. Coli* MG1655 following sub-culturing of 24h N starved bacteria into fresh Gutnick minimum medium.

**Table 3.** Average lag time (LT) and growth rate (GR) with standard deviation of WT and Δ*sdsR E. coli* MG1655 following sub-culturing of 24 h N starved bacteria into fresh Gutnick minimum medium. Statistical analysis performed by Student T-test.

| Parameters | WT | Δ*sdsR* | p-value |
|---|---|---|---|
| LT (h) | 3.29±0.19 | 4±0.15 | <0.05 |
| GR ($OD_{600nm}$/h) | 0.11±0.004 | 0.02±0.001 | <0.001 |

The results obtained with EDL933 strain were inconsistent. The same experiment was performed three different times, and three different results were obtained. In addition, both lag times increased as the experiment was repeated (Figure 7). Regardless, an increase in lag time in Δ*sdsR* bacteria was not observed. Therefore, we considered that EDL933 does not require SdsR for an optimal growth recovery after a 24 h N starvation.

The results of the experiment with CFT073 could not be obtained because this strain grew clustered in Gutnick minimum medium. Therefore, the plate reader did not provide accurate $OD_{600nm}$ measurements.

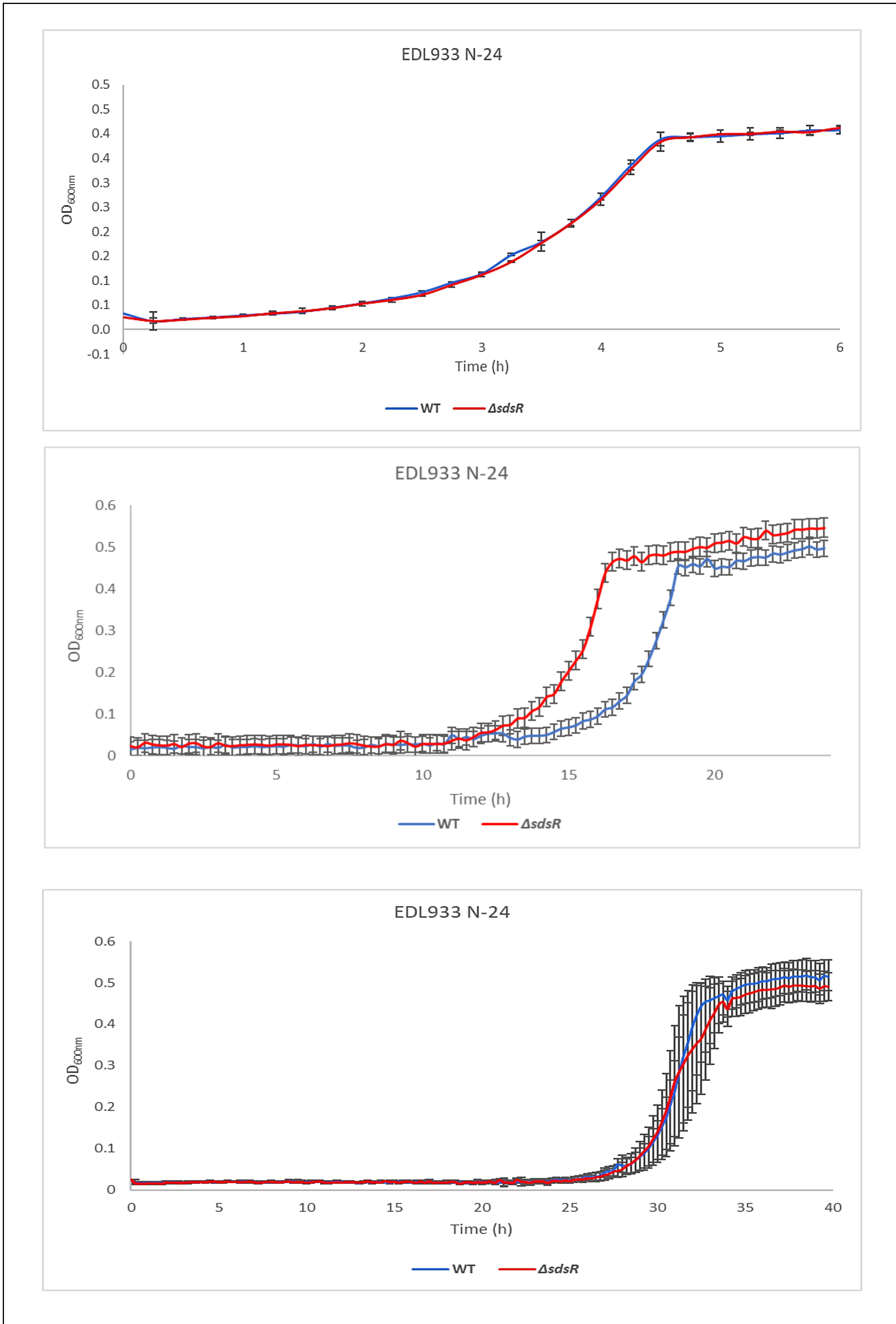



**Figure 7.** Growth recovery of wild-type and Δ*sdsR E. Coli* EDL933 following sub-culturing of 24h N starved bacteria into fresh Gutnick minimum medium. Each graphic represents the results of the same experiment performed three different times.

**4. DISCUSSION.**

In recent years, there has been a notable increase in the development of new approaches aimed at identifying post-transcriptional regulatory networks in bacteria on a global level (Melamed, 2020). However, this work aims to gain insight into post-transcriptional regulatory networks in starved bacteria, i.e. bacteria in stationary phase. When bacteria are in this growth phase, they are more resistant to antibiotics (Fleischmann *et al.*, 2021), so studying the regulatory mechanisms used during this phase will allow us to understand how and why antibiotics can fail. Specifically, we wanted to understand the influence of the sRNA SdsR on bacterial growth recovery and viability and comparing the phenotypes obtained from a non-pathogen strain with two pathogen ones.

To assess the phenotypes, we had to achieve one of the main objectives of this work, the production of *E. coli* CFT073 Δ*sdsR* and *E. coli* EDL933 Δ*sdsR*. These productions were performed through the cloning recombination method. The strains were confirmed to be the expected genotype through sequencing (Figure 2). This is consistent with previous *E. coli* mutant productions results (Jian *et al.*, 2010). These deletions make it possible to assess the influence that a gene has on a particular bacterial function.

The results revealed that both WT and Δ*sdsR* bacteria, in all strains, non-pathogen and pathogens, were equally able to regrow and survive after sustained growth arrest (Figures 3, 4 & 5) (Table 2), except for regrow after long nitrogen starvation (N-24) in Gutnick minimum medium, presenting two different phenotypes (Figures 6 & 7) (Table 3).

Despite the extensive participation of SdsR in various cellular processes in growth-arrested *E. coli* (McQuail *et al.*, 2023), the results from experiments involving WT and Δ*sdsR* bacteria, which were subjected to a 24-hour growth arrest and subsequently recover growth upon subculture into fresh LB medium, suggest that the presence of SdsR may not be necessary for optimal growth recovery in LB medium (Figure 3) (Table 2). Both WT and Δ*sdsR* bacteria in all strains, non-pathogen and pathogens, were able to have an optimal growth recovery. All lag times were around 1 h, as in previous studies in MG1655 strain (Cuny *et al.*, 2007). Growth rates declined when bacteria were subcultured into fresh LB after a long stationary phase, which is consistent with other works (Arunasri *et al.*, 2014). LB medium is used by most microbiological laboratories, as it contains an abundance of nutrient that are important for bacterial growth (Baev *et al.*, 2006). The use of this medium could be the reason why no differences are observed between WT and Δ*sdsR* bacteria, as they may no longer be nutritionally stressed when are subcultured on fresh LB and they do not need to control the functions related to nutritional stress. Therefore,

SdsR may not play an important role when they recover growth in LB. Further research is needed to confirm this assumption.

As discussed above, SdsR participates in a large number of bacterial processes during stationary phase (McQuail *et al.*, 2023). However, both WT and Δ*sdsR* bacteria in all strains, non-pathogen and pathogens, were equally able to survive sustained growth arrest, *i.e*. the same bacterial viabilities following a 24-hour stationary phase in LB and in a N starved medium (Gutnick minimum medium) are observed (Figures 4 & 5). This likely indicates that SdsR is not required for optimal bacterial viability, which is consistent with previous bacterial viability results after long N starvation (McQuail *et al.*, 2023). Bacterial viability may therefore be regulated by other sRNAs at the post-transcriptional level. Understanding the sRNAs that influence the bacterial viability of pathogen strains may be the topic of a future study that would help to develop new antimicrobial strategies based on post-transcriptional regulatory networks.

After comparing this work with the work of McQuail *et al* (2023), there is a clear similarity in the results of growth recovery in Gutnick minimum medium following long-term nitrogen starvation obtained with strain MG1655. In both works, a longer lag time (LT) and a lower growth rate (GR) (Figure 6) (Table 3) were observed when Δ*sdsR* bacteria were introduced into fresh Gutnick minimum medium compared to WT ones. The use of this medium leads to conditions of increased nutritional stress, as it has a limiting source of nitrogen (McQuail *et al.*, 2023). This stress may lead to an increased requirement to control the post-transcriptional regulation of different bacterial functions related with nutritional stress in order to recover their growth. One likely mechanism by which bacteria achieve this is through increase SdsR production and activity. Consistent with the previous work by McQuail *et al* (2023), we have demonstrated a greater role of SdsR in *E. coli* MG1655 during growth recovery in Gutnick minimum medium. It is likely that SdsR is important in other conditions, given it wide array of targets (McQuail *et al.*, 2023).

As stated above, the results of growth recovery in Gutnick minimum medium following long-term N starvation (Figures 6 & 7) (Table 3) revelated that the non-pathogen strain MG1655 and the pathogen strain EDL933 did not presented the same phenotype. Although the results with EDL933 were inconsistent, an increase in LT and a decrease in GR in Δ*sdsR* bacteria was not observed (Figure 7). This suggest that SdsR is not required for *E. coli* EDL933 during growth recovery in Gutnick minimum medium. In other works, with EDL933, Δ*ler* bacteria (*ler* codifies a regulatory protein that controls its virulence) exhibit the same LT and GR as the WT bacteria. However, Δ*hfq* EDL933 show lower LT and GR than WT EDL933 (Shakhnovich *et al.*, 2009). This

demonstrated that most of the sRNAs are not able to regulate without the enhance of Hfq, as SdsR. Probably, Δ*sdsR* EDL933 achieved optimal growth recovery by using other sRNAs for its regulation.

It is known that SdsR interacts with 124 mRNA in N-24 *E. coli* MG1655. In addition, an optimal growth recovery of N-24 *E. coli* MG1655 requires the translational downregulation of the conserved operon *yeaGH* by SdsR. This operon is also present in EDL933 strain (McQuail *et al.*, 2023). However, the other targets are likely to be different. Moreover, *E. coli* has a spontaneous mutation rate of $\sim 1\times10^{-3}$ per genome per generation (Lee *et al.*, 2012), resulting in a high probability that a mutant cell that can tolerate such conditions will emerge and rapidly proliferate to dominate the entire population. Further studies are needed to find out the real cause of this difference in phenotypes. A work like that of McQuail *et al* (2023) would be interesting, so that the real targets of the sRNA SdsR in *E. coli* EDL933 could be known.

In addition, it would be interesting to assess the influence of SdsR in the colony growth of *E. coli*. As we detected a large difference in size between the WT and Δ*sdsR* bacteria colonies when we seeded them on LB agar plates. The growth dynamics are influenced by both the microorganism's doubling time and any delay in growth when transitioning between conditions, known as the lag. The ScanLag method allows for the assessment of these two distinct properties. Petri dishes are positioned on scanners, which periodically capture images. An automated application analyses colony growth, providing data on the time of appearance and growth rate for each colony (Levin-Reisman *et al.*, 2014).

As mentioned above, bacteria are more resistant to antibiotics during their stationary phase (Fleischmann *et al.*, 2021) and when they recover growth, a long lag phase can cause greater persistence to antibiotics (Levin-Reisman *et al.*, 2014). Therefore, the study of how bacteria regrow after a long growth arrest and their protagonists, is important to understand how and why antibiotics can fail. On the other hand, these studies in laboratory strains may not be very useful if the interactions and phenotypes are very different in pathogen strains.

## 5. CONCLUSIONS.

From the work of deletion of the sRNA SdsR from two pathogen *Escherichia coli* strains (CFT073 and EDL933), we have confirmed a correct production of *E. coli* CFT073 Δ*sdsR* and *E. coli* EDL933 Δ*sdsR*. Next, we have compared the growth recovery in LB after a long growth arrest (24 h) of WT and Δ*sdsR* bacteria; we observed that both WT and Δ*sdsR* bacteria in all strains, non-pathogen and pathogens, were able to have an optimal growth recovery, indicating that SdsR is

not required for an optimal growth recovery in LB. Further, we have demonstrated that SdsR is not required for an optimal bacterial viability following a long growth arrest in LB or a long nitrogen starvation (N-24) in Gutnick minimum medium. In addition, we have compared the growth recovery in Gutnick minimum medium of WT and Δ*sdsR* N-24 bacteria; we have shown that in *E*. *coli* MG1655 SdsR is required for an optimal growth recovery. However, in *E. coli* EDL933 SdsR is not required, indicating that MG1655 strain and EDL933 strain did not presented the same phenotype. This may indicate that SdsR has different roles in pathogen strains.

This work has demonstrated that both WT and Δ*sdsR* bacteria, regardless of their pathogenicity, exhibited equal capacity for regrowth and survival after sustained growth arrest, except for regrow after long nitrogen starvation (N-24) in Gutnick minimum medium, where *E. coli* MG1655 and *E. coli* EDL933 showed two distinct phenotypes. This work holds a variety of potential applications and may be important for future studies of post-transcriptional regulation of nitrogen stress in bacteria.

**5. CONCLUSIONES.**

Tras el trabajo de deleción del sRNA SdsR de dos cepas patógenas de Escherichia coli (CFT073 y EDL933), hemos confirmado una correcta producción de *E. coli* CFT073 Δ*sdsR* y *E. coli* EDL933 Δ*sdsR*. A continuación, hemos comparado la recuperación del crecimiento en LB tras una detención prolongada del crecimiento (24 h) de bacterias WT y Δ*sdsR*; hemos observado que tanto las bacterias WT como las Δ*sdsR* en todas las cepas, tanto patógenas como no patógena, fueron capaces de tener una recuperación óptima del crecimiento, lo que indica que SdsR no es necesario para una recuperación óptima del crecimiento en LB. Además, hemos demostrado que SdsR no es necesario para una viabilidad bacteriana óptima tras una detención prolongada del crecimiento en LB o con inanición prolongada de nitrógeno (N-24) en el medio mínimo de Gutnick. Además, hemos comparado la recuperación del crecimiento en medio mínimo Gutnick de bacterias WT y Δ*sdsR* N-24; hemos demostrado que en *E. coli* MG1655 SdsR es necesario para una recuperación óptima del crecimiento. Sin embargo, en *E. coli* EDL933 no se requiere SdsR, lo que indica que la cepa MG1655 y la cepa EDL933 no presentaron el mismo fenotipo. Esto puede indicar que SdsR tiene diferentes funciones en las cepas patógenas.

Este TFG ha demostrado que tanto las bacterias WT como las Δ*sdsR*, independientemente de su patogenicidad, presentaban la misma capacidad de recuperar su crecimiento y sobrevivir tras una detención prolongada del crecimiento, excepto cuando recuperaron su crecimiento tras una inanición prolongada de nitrógeno (N-24) en medio mínimo Gutnick, en el que *E. coli* MG1655 y *E. coli* EDL933 mostraron dos fenotipos distintos. Este trabajo tiene diversas aplicaciones potenciales y puede ser importante para futuros estudios sobre la regulación postranscripcional del estrés por nitrógeno en bacterias.

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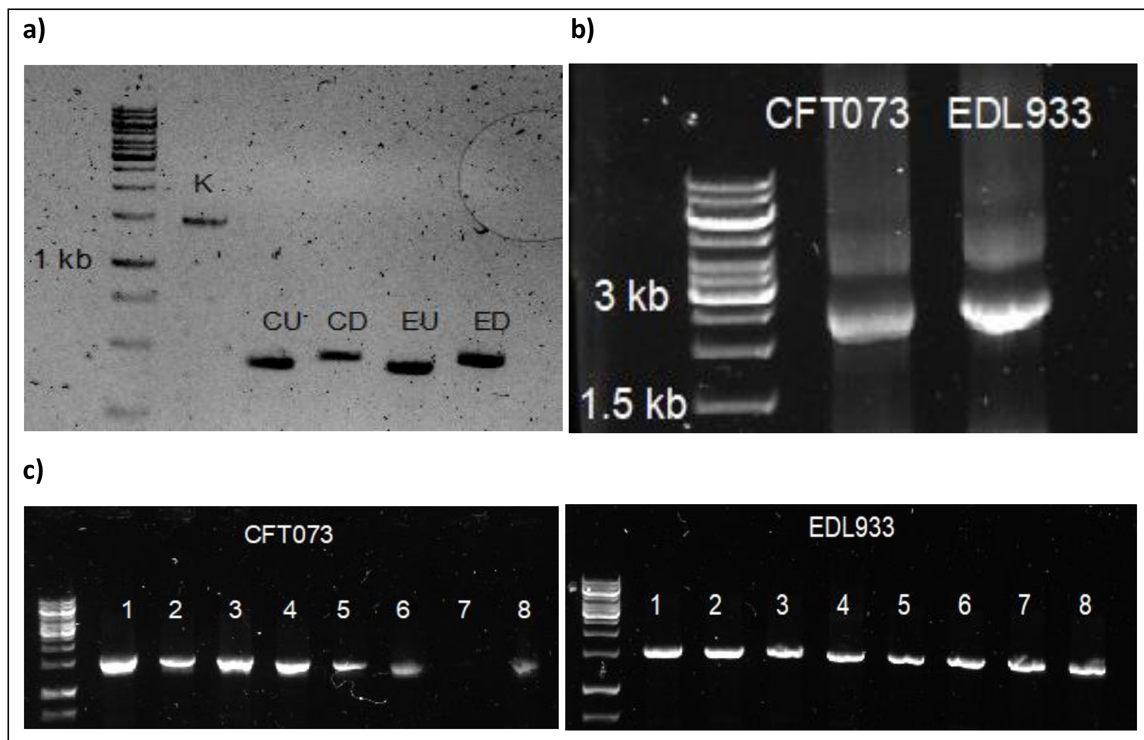
**SUPPLEMENTARY MATERIAL.**

**Supplementary Table 1**. Primers used in this study.

| | |
|---|---|
| **Upstream fw** | ACTTGCGGCGCAATCAGCCTGCTTGCGACTC |
| **Upstream rev** | CTCCAGCCTACACTTTAAGAATAGATGACGACGCCAG |
| **Downstream fw** | ATATTCATATGGATCTGTTAAAAGCCAGAAG |
| **Downstream rev** | GTGCGAAGAGGTCATCATCTGGATAATCGG |
| **Kan$^R$ fw** | TCTATTCTTAAAGTGTAGGCTGGAGCTGCTTC |
| **Kan$^R$ rev** | GCTTTTAACAGATCCATATGAATATCCTCCTTAGTTCCTATTC |
| **STITCH fw** | CGTCAGCCGTTTTGAAGTGG |
| **STITCH rev** | GTCGCCGCCATTTATCAACC |
| **Genome fw** | GAATGAAGGGGTAACGGCGA |
| **Genome rev** | GGATGAAATGTTCGCGCTGG |
| **Mid Kan** | GGACAGGTCGGTCTTG |




**Supplementary Figure 1.** Agarose gel electrophoresis of PCR products. a) Purified PCR products of each fragment. Kan$^R$ gene (K), CFT073 upstream region (CU), CFT073 downstream region (CD), EDL933 upstream region (EU), EDL933 downstream region (ED). b) Purified recombination fragment of each strain. c) 8 different colony PCR products of each strain.