**Rob and MarA alter susceptibility of *Escherichia coli* to antibiotics
in presence of salicylate**

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11

12 **Abstract**

13

14 When exposed to stress, bacterial cells launch a diverse response to enhance their
 15 chances of survival. This response involves modulation of expression of a large
 16 number of proteins which help the cell counter stress. This modulation is facilitated
 17 by several transcription factors in bacteria and in *E. coli* three homologous
 18 regulators, MarA, Sox, and Rob are known to launch a coordinated response to
 19 combat various stress environments. MarA and SoxS are known to control multiple
 20 antibiotic resistance and superoxide regulon respectively. Rob has been observed to
 21 control similar downstream targets as MarA and SoxS. However, physiological
 22 relevance of Rob is not understood. We show that Rob along with MarA, in presence
 23 of inducer salicylate, can help cell survive in presence of lethal concentration of wide
 24 range of antibiotics.

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26

27 **Keywords:** Salicylate, Rob, MarA, Antibiotics.

28 Introduction

29

30 Transcriptional regulation of cellular targets like efflux transporters or outer
 31 membrane porins help a bacterial cell combat stress [1-4]. In *E. coli*, three
 32 homologous transcriptional regulators – MarA (encoded by *multiple antibiotic*
 33 *resistance marRAB* operon), SoxS (encoded by superoxide stress *soxSR* regulon),
 34 and Rob (right origin binding protein), together known as *mar/sox/rob* regulon, are
 35 known to regulate these processes [1,5-11]. For e.g., the TolC efflux pump is known
 36 to be upregulated by MarA, SoxS, and Rob [4]. Production of porin protein OmpF is
 37 regulated by small RNA *micF* [1], which is regulated by MarA, Sox, and Rob. Many
 38 of the cellular enzymes mentioned like Zwf, DeoB, SodA, etc. are also known to be
 39 controlled by either of MarA, SoxS, or Rob [12-14]. MarA and SoxS have been
 40 reported to play a role in conferring antibiotic resistance and combating superoxide
 41 stress [5,15-21]. Rob is known to act on an overlapping set of targets as MarA and
 42 SoxS [7,22-24], however, its role in regulating cellular physiology is not clearly
 43 understood [25,26].

44 The *marRAB* operon is known to be induced in presence of compounds such as
 45 salicylate, phenolic compounds [4,5,8,27-29]. *marRAB* system encodes for a
 46 transcriptional activator MarA, repressor MarR, and protein of unknown function
 47 MarB [8,15-17,29,30]. MarR repressor, in absence of inducers, remain bound to
 48 *Pmar* promoter and represses expression from the *Pmar* promoter [15-17]. In
 49 presence of inducers, MarR preferentially binds to the inducer molecule, hence
 50 relieving the repression of the *Pmar* promoter [15-17,23]. Thereafter, MarA binds to
 51 the *Pmar* and target genes promoters and activates transcription [1,4-6,23,27,28,31].

52 Rob, (right origin binding protein), was first discovered to bind to a site close to DnaA
 53 binding site to the DNA, [25]. However, its precise role in controlling DNA replication
 54 is not known [25]. Crystal structure of Rob further revealed structural homology with
 55 transcriptional regulators MarA and Sox and has been classified as AraC/XylS type
 56 transcriptional regulator family [7,12,13,22-24,32]. However, unlike MarA and SoxS,
 57 transcription from the *rob* promoter is constitutive (the protein in its uninduced state
 58 is present in the cell as agglomerate) and the protein amounts are regulated post
 59 translationally, in presence of cognate inducer bile salts [12,13,33,34]. Salicylate is

60 also known to control gene expression via Rob, however, the molecular mechanism
61 for the same is not understood [35,36].

62 Previous work from our lab demonstrated that in presence of salicylate, MarA and
63 Rob together control downstream targets like InaA by forming a Feed Forward Loop
64 [35]. MarA protein is important in altering MIC value of a large number of antibiotics
65 in gram-negative bacteria. However, physiological significance of Rob in this context
66 is not well understood. In this work, we show that Rob plays a similar role as MarA in
67 altering the MIC of antibiotics in bacteria in presence of the canonical inducer
68 salicylate. Since salicylate is the active molecule of aspirin (acetyl salicylic acid), a
69 clinically important molecule, it is important to understand the molecular mechanism
70 of this action and its further impact on antibiotic resistance.

71

72 **Methods**

74 **Growth Kinetics**

76 Cells were grown in LB media overnight with shaking at 37°C. The overnight cultures
77 were diluted 1:250 in fresh media. Cells were thereafter allowed to grow till an OD
78 0.2 upon attaining which and respective inducer was added. Growth dynamics was
79 captured for – wild type, $\Delta marA$, and Δrob in induced and uninduced conditions. A
80 range of inducer, salicylate (Sigma Aldrich) concentration - 1mM, 5mM, and 10mM)
81 was taken to capture sub-lethal effect by inducers. Experiments were performed for
82 a range of inducer concentrations to capture their precise physiological effect at
83 different concentrations.

84 **Minimal Inhibitory Concentration (MIC) determination**

86 Broth microdilution method was used to determine MIC of an antibiotic. Antibiotic
87 stocks and the concentration range used were prepared as per the guidelines given
88 by National Committee for Clinical Laboratory Standards, NCCLS [37]. Antibiotic
89 concentration range was taken one fold higher than the required range to count for
90 dilution because of inoculum addition. The inoculum was prepared using an
91 overnight culture (LB media) by diluting to have 10^6 cells per ml. A sterile 96 well
92 plate was taken and 100μL of inoculum was added to the well containing 100μL of
93 fresh media with appropriate antibiotic concentration. Thus, the final cell density for
94 the experiment was kept at 5×10^5 cells per ml. Inoculum density was cross checked
95 every time by counting the colony forming unit (CFU) on LB agar plate. The plate
96 was sealed using breathe easy membrane (Sigma Aldrich) and incubated at 37°C for
97 20 hours. The concentration of the lowest antibiotic concentration well showing no
98 visible growth was interpreted as the MIC for a particular antibiotic.

99 **Live-Dead Cell Assay using Propidium Iodide (PI)**

102 Cells were exposed to antibiotics in presence and absence of salicylate to track the
103 percentage of dead cells using propidium iodide assay (Sigma Aldrich). Propidium
104 iodide is known to rapidly penetrate cells with compromised membrane or dead cells.

Once in touch with DNA it fluoresces at 617nm. The reason for choosing this dye is its minimal interference with inducers or antibiotics used in this study.

An overnight culture of wild type and mutants was sub-cultured in 1:250 dilution. After 1.5 hours of growth at 37°C with shaking, OD 600 was monitored every 10 minutes using OD density meter. When the OD reached ~0.4, the cells were exposed to salicylate at a concentration of 7.5mM. After an hour of salicylate exposure ($OD \leq 0.65$), cells were exposed to varying concentration of antibiotics. The antibiotic range was decided as depending on the MIC values for each antibiotic. For the purpose of this study, the antibiotic range was chosen to consider both sub-lethal and lethal concentrations of antibiotics; i.e., both < MIC and > MIC concentration. The percentage of dead cells was estimated after three hours of antibiotic exposure, roughly in mid-exponential phase. Samples were collected and stored in PBS. Propidium iodide was used at a concentration 10µg/ml. Samples were immediately observed at single-cell resolution using a Flow Cytometry (Millipore Guava System and BD FACS Aria SORP). 50,000 events were recorded and FSC/SSC were plotted. Care was taken to not expose samples to light after adding Propidium Iodide. All single-cell expression experiments were repeated thrice on different days and found to show similar trend.

Results

***mar* and *rob* systems are associated with a phenotype in *E. coli*.**

To characterize the physiological role of MarA and Rob, growth dynamics were performed for wild type and mutants (in presence and absence of inducers) and growth dynamics compared. These downstream targets control several aspects of cellular physiology and consequently have an effect on growth dynamics of the bacterium. Previous reports suggest that MarA and Rob control a number of common downstream cellular targets [4,6,22,23,38-40]. Hence, the changes in growth phenotype arising due to absence of these systems were explored by measuring the growth defect associated with deletion of *marA* or *rob*.

Kinetic experiments were carried out to see the difference in growth dynamics in regulatory mutants (Δrob and $\Delta marA$) in presence and absence of salicylate, and compared to that of wild type. Our results show that, compared to wild-type *E. coli*, growth of mutants $\Delta marA$ and Δrob is inhibited in presence of sub-inhibitory concentrations of salicylate. This effect is especially pronounced at lower concentrations of salicylate. Defect in growth is calculated as percentage growth difference in induced cells as compared to uninduced conditions (Figure 1). At low concentration of salicylate (1mM), the wild-type cells show growth defect of around 10% as compared to 15-20% for mutants Δrob and $\Delta marA$ (Figure 1B and 1C). For higher concentrations of salicylate (5mM and 10mM), wild type and mutants show comparable growth defects. Since *mar/sox/rob* systems are thought to have evolved to combat sub-lethal stress [28,29,41], it is not surprising that absence of either regulator leads to growth defect when exposed to low inducer concentration (1mM) as compared to wild-type.

Salicylate alters Minimal Inhibitory Concentration (MIC) of antibiotics via both, MarA and Rob.

Another approach to analyze the phenotype of *mar/rob* is to study their role in changing the Minimal Inhibitory Concentration (MIC) of antibiotics. MIC is defined as the lowest concentration of the antibiotic at (and beyond) which the organism cannot

grow. One of the first published reports regarding role of salicylate and other similar compounds in altering MIC of known antibiotics was by Rosner in 1985 [41]. Since then a number of reports focus on the role of salicylate and acetyl salicylic acid in reducing susceptibility of the bacterium to antibiotics like fluroquinolone, carbenecillin, carbapenem, etc. [42,43]. However, there is no systematic work establishing role of inducers of Rob in changing MIC.

To understand the role of salicylate in altering MICs, we selected three antibiotics, commonly used in clinical practice against gram-negative bacteria. Carbenecillin is a β -lactam antibiotic having bactericidal activity, generally used against gram-negative infections. It acts by inhibiting cell wall synthesis process by interfering with the final transpeptidation step [44-46]. Cefotaxime is a broad spectrum antibiotic belonging to cephalosporin (third generation β -lactam group) active against both gram-positive and gram-negative bacteria. The mechanism of action of cefotaxime is similar to that of carbenecillin [44,45,47]. Ciprofloxacin is a fluroquinolone having bactericidal activity against gram-negative bacteria, and acts by inhibiting the process of DNA synthesis by hindering topoisomerase activity [48-51]. In this section, we focus on understanding the fold changes in MICs of antibiotics Carbenecillin, Cefotaxime, and Ciprofloxacin when both wild-type and regulatory mutant cells are grown in presence of inducers of *mar/rob* systems. We used salicylate as inducer.

Broth microdilution method was used to determine the MIC. Antibiotic stocks and the concentration range used were prepared as per the guidelines given by National Committee for Clinical Laboratory Standards, NCCLS [37].

To assess the effect of inducer salicylate, using MIC determination method, we first determined the sensitivity of *E. coli* to salicylate. MIC of salicylate was determined to be 4mg/ml which corresponds to a concentration of 25mM. We found that similar values of MIC have been reported previously for enterotoxigenic *E. coli* strain [52]. In our work, while using as an inducer, we have used lower concentration, 7.5mM of salicylate (represents both salicylate and acetyl salicylic acid) to prevent cell damage.

We performed broth microdilution assay for carbenecillin, cefotaxime, and ciprofloxacin both in absence and presence of inducers salicylate and paraquat to determine MIC. The concentration range of antibiotic screened and MIC values obtained are given in Table 1.

For wild-type cells, MIC for carbenecillin, was determined to be 32µg/ml. For cefotaxime, MIC was determined to be 0.06µg/ml, and for ciprofloxacin 0.032µg/ml. All three MIC values were found to be in the reported range of MIC for *E. coli* ATCC 25922 strain. Next, we repeated the same exercise by determining the respective MICs in presence of 7.5mM salicylate. We observed a two-fold change in MIC of carbenecillin in presence of salicylate. For cefotaxime and ciprofloxacin there was more than four-fold change when exposed to salicylate (Table 1). This suggests that inducer (salicylate) helps the cell survive even when exposed to lethal antibiotic concentration.

Next, we were interested in determining the MIC of the three antibiotics for single and double mutants of $\Delta marA$ and Δrob . Deleting either of MarA or Rob did not lead to significant change in MIC. Hence, we chose double mutant for further characterization of the role of salicylate in altering MIC via both MarA and Rob. For $\Delta marA \Delta rob$, for carbenecillin, cefotaxime, and ciprofloxacin, MIC was found to be half of that of wild type, i.e., 16µg/ml for carbenecillin, 0.016µg/ml for ciprofloxacin, and 0.032µg/ml for cefotaxime. The deletion of these regulators was observed to lead to at least 50% decrease in MIC when observed in uninduced condition. This suggests that the regulators might also be regulated by other metabolic intermediates as reported by Chubiz and co- workers [28].

We then repeated the same exercise in presence of 7.5mM salicylate. In absence of both MarA and Rob MIC was observed to be two-four fold less as compared to wild type MIC in identical conditions. The comparison of uninduced and induced values of MIC of all three studied antibiotics in $\Delta marA \Delta rob$, however, reveals around two-fold higher MIC in salicylate induced condition as compared to uninduced condition. Salicylate is known to act via MarA and Rob. The change in MIC in presence of salicylate and absence of MarA and Rob suggests that other regulators like SoxS might also be able to play a role in regulating MIC. In fact, closely related species like *Salmonella* are known to have another regulator (homologous to MarA), RamA,

which works together with *mar/sox/rob* towards conferring resistance [53]. Existence of such an additional regulator in *E. coli* is a likely possibility.

This study confirms the role of salicylate in altering the MIC of all three studied antibiotics via MarA/Rob respectively. We also studied if absence of either of MarA or Rob has the same effect on cells as absence of both. We observed that MIC in absence of either of MarA and Rob was same as that of wild-type cells (both in presence and absence of inducers) suggesting in absence of one, the other compensates for the loss of one of the regulators. Such redundancy in genetic network is quite ubiquitous and it is not surprising that it exists for a role as critical as stress response in bacterium *E. coli*.

Cell density effect and quantification of cell death using Propidium Iodide (PI) assay.

The previous section helped us understand the role of inducers in altering MIC. However, in absence of only one regulator (either MarA or Rob) it was difficult to capture any difference in MIC as the inducer salicylate acts via both MarA and Rob. In the double mutant, $\Delta marA \Delta rob$ we notice maximum difference in MIC in presence as well as absence of salicylate. To further confirm the independent role of MarA and Rob, we quantified in response to exposure to antibiotics (Propidium Iodide fluoresces when in contact with DNA, thus is used to quantify fraction of cells which are dead). This will help us in understanding cell density effect in higher antibiotic exposure and also help quantify the independent roles of MarA and Rob.

Cells at higher densities (around 10^8 cells per ml) were exposed to antibiotics in presence and absence of salicylate to track the percentage of dead cells using propidium iodide assay. We observed that in presence of salicylate, wild-type cells survive lethal antibiotics dosage for all three antibiotics studied (Figure 2, 3, and 4). As shown in Figure 2-4, the double mutant $\Delta marA \Delta rob$ cells are susceptible to antibiotics even in presence of salicylate. In presence of carbenecillin antibiotic, wild-type cell showed survival in presence of salicylate till 40µg/ml of carbenecillin (Figure 2). $\Delta marA$ cells were also able to survive exposure of antibiotics with the help of salicylate. However, $\Delta marA \Delta rob$ cells showed ~50% percent dead cells, when exposed to 10 – 40µg/ml of carbenecillin in both absence and presence of salicylate. Δrob cells also showed similar behavior as $\Delta marA \Delta rob$, however, percentage of

dead cells was lower (20-30%) as compared to the double mutant. Similar results were obtained for antibiotics cefotaxime and ciprofloxacin (Figure 3 and 4). When exposed to 2µg/ml cefotaxime, the percentage of dead cells in absence of MarA/Rob or MarA and Rob was 20-30%. However, at lower concentration of cefotaxime, presence of Rob helped in cell survival in absence of MarA (Figure 3) (cell death >10%). In case of ciprofloxacin, absence of MarA/Rob show similar effect, however, absence of both together renders cell susceptible to 1µg/ml ciprofloxacin similar to uninduced condition. Our result suggests that Rob plays an important role (more than that of MarA) in helping the cells survive antibiotic exposure.

Discussion

Our results suggest that Rob, like MarA, is an important player in responding to inducer salicylate. We quantify the phenotype associated with MarA and Rob in three different ways – growth kinetics, MIC, and PI assay. Growth experiments suggest that these systems are associated with a strong growth phenotype. MICs assays highlight the importance of chemicals like salicylate in altering the MICs of wide variety of clinically relevant antibiotics. PI assays help us understand the role of specific regulator in helping cells survive in an inducer dependent manner. MarA and Rob seems to act in coordination to help the cells survive wide range antibiotic stress in presence of salicylate. Rob being constitutively present in cell may enable cell to launch immediate response to harsh environment. The different mode of regulation of Rob as compared to MarA might be crucial to cell survival. Role of salicylate in independently acting via Rob can be of clinical importance as salicylate is the active component of medicines like aspirin.

Rob is reported to be present in 3-4 loci in cell with number varying from 5000-1000 molecules per cell [25,54]. In a single cell study, however, the number of Rob molecules in a cell was observed to be very low [55]. The variation in the number of protein molecules per cell suggest there could be cell-cell variation. Unlike other members of Arac/XylS regulators, the *Prob* is known to be regulated at post translational level [12,13]. It is present as agglomerate and dispersed to single molecule in presence of bipyridyl salt [34]. The transcriptional regulation of *Prob* in presence of salicylate is speculated but molecular mechanism of the same is not understood [35,36]. Moreover, the mechanism associated with transcriptional regulation of *Prob* by MarA, if any, is not understood [56,57]. Role of Rob protein, along with MarA and SoxS, in regulating cellular targets has been reported [7,12,13,22-24,32]. It is also speculated to be expressed in stationary phase under glucose and phosphate starvation [25,26]. What is the physiological relevance of Rob remains to be understood. It was first reported as right origin binding protein [25]. However, till date its role, if any, in DNA replication is not established. Our work attempts to understand role of Rob in cellular physiology, when exposed to wide variety of antibiotic stress. Further exploration of the mechanism involved in Rob mediated control of cellular physiology, in presence of inducers, can give new insights about intrinsic resistance mechanism.

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452

Figure Captions

Figure 1. Growth defect in wild-type *E. coli* and mutants in presence of varying concentrations of salicylate. (A) Wild type (B) Δrob , and (C) $\Delta marA$. Salicylate concentration used: 0mM, 1mM, 5mM, and 10mM.

Figure 2. Percentage of dead cells in wild-type *E. coli* and mutants in presence of varying concentration of carbenecillin, with and without salicylate (A) wild type (B) $\Delta marA$ Δrob (C) $\Delta marA$ (D) Δrob . Salicylate concentration used is 7.5mM and carbenecillin concentration used is 10 μ g/ml, 20 μ g/ml, and 40 μ g/ml.

Figure 3. Percentage of dead cells in wild-type *E. coli* and mutants in presence of varying concentration of cefotaxime, with and without salicylate (A) Wild type (B) $\Delta marA$ Δrob (C) $\Delta marA$ (D) Δrob . Salicylate concentration used is 7.5mM and cefotaxime concentration used is 0.5 μ g/ml, 1 μ g/ml, and 2 μ g/ml.

Figure 4. Percentage of dead cells in wild-type *E. coli* and mutants in presence of varying concentration of ciprofloxacin, with and without salicylate (A) Wild type (B) $\Delta marA$ Δrob (C) $\Delta marA$ (D) Δrob . Salicylate concentration used is 7.5mM and ciprofloxacin concentration used is 0.01 μ g/ml, 0.5 μ g/ml, and 1 μ g/ml.

470

471 **Table 1. MIC values for wild type and double mutants cells in presence and absence**
 472 **of inducer salicylate.**

473

MIC in µg/ml					
Antibiotics	Range of concentration checked in µg/ml	Wild type		<i>ΔmarA Δrob</i>	
		W/O Salicylate	W/ Salicylate	W/O Salicylate	W/ Salicylate
Carbenecillin	.25-128	32	>64	16	64
Cefotaxime	0.004-128	0.064	0.512	0.032	0.064
Ciprofloxacin	0.004-128	0.032	0.512	0.016	0.128

474

475

Figure 1

Figure 2

Figure 3

Figure 4