Identification and analysis of Rob, a transcriptional regulator from *Serratia marcescens*

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Thesis Abstract

Serratia marcescens, a member of *Enterobacteriaceae* family, is a causative agent of nosocomial and opportunistic infections. Numerous reports show that the multidrug resistance among *S. marcescens* is growing. This organism has high-level intrinsic resistance to a variety of antimicrobial agents, which makes the treatment of infections caused by this bacterium very difficult. The major mechanism for antibiotic resistance, especially to fluoroquinolones, in Gram-negative organisms is the active efflux of the antibiotic molecule mediated by efflux pumps belonging to the Resistance-Nodulation-Cell Division (RND) family. It was previously shown that the SdeAB and SdeXY multidrug efflux pumps are important for conferring the intrinsic drug resistance in *S.marcescens*. In *Escherichia coli*, the up-regulation of transcriptional activators, such as MarA, SoxS and Rob, affect transcription of ac*rAB*, *tolC* and *micF. O*ver-expression of Rob results in increased expression of the *E. coli* AcrAB-TolC efflux pump and decreases outer membrane permeability through up-regulation of *micF,* resulting in multidrug, organic solvent and heavy metal resistance. In the present study, we report the identification of a *rob* gene in *S. marcescens* which has a 70% identity at the DNA level and 71% identity at the amino acid level to that of *E. coli*. Moreover, the *S. marcescens rob* demonstrated similar properties to the *E. coli rob* including having an effect on expression of outer membrane protein F (OmpF) and over-expression of SdeAB and SdeXY, conferring antibiotic resistance to divergent antibacterial agents and tolerance to organic solvents. We performed *rob* promoter evaluations using transcriptional fusions to the Green Fluorescence Protein (GFP) in the vector pGlow-

TOPO and constructed a *rob* knock-out using the TargeTron Gene Knockout System. Promoter activity assessment, using the pGlow-TOPO reporter plasmid, showed that *rob* had higher promoter activity at 37°C than 30°C. In the presence of 2,2'-dipyridyl, *rob* promoter activity was observed to be slightly increased in the early and mid-log phase by 1.4 and 1.1 fold, respectively. We also showed that sodium decanoate and sodium salicylate can reduce the transcription of *rob* at 30°C and 37°C. This reduction was observed more potently when *rob* was exposed to sodium decanoate at 30°C. Minimum inhibitory concentration (MIC) for various antibiotics of the *S. marcescens rob* knock-out demonstrated a decrease in susceptibility to nalidixic acid, tetracycline, chloramphenicol, ciprofloxacin, norfloxacin, and ofloxacin. Over-expression of *rob* resulted in an increased resistance by 4, 2, and 2-fold to nalidixic acid, tetracycline and chloramphenicol, respectively. In addition, *rob* over-production displayed 8, 4, and 4-fold increase in resistance to ciprofloxacin, norfloxacin, ofloxacin, respectively. To discover the role of *rob* in the efflux mechanism, we performed ethidium bromide accumulation assays on over-expressing and knock-out strains. Organic solvent tolerance assays were carried out using n-hexane to determine if *rob* is involved in expression of efflux pumps. We found the *rob* null mutant to be sensitive to n-hexane while the over-expression of *rob* resulted in resistance to n-hexane. RT-PCR of the *rob* knock-out strain showed a decrease in expression of *micF*, *ompC*, *sdeXY*, *sdeAB* and *tolC*, respectively, and an increase in the expression of *ompF*. To conclude, we identified a *rob* homolog in *S. marcescens* which contributes to resistance to multiple antibiotics and tolerance to organic solvent.

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Table of Contents

Thesis Abstract i

Acknowledgements iii

Table of Contents iv

List of Tables vii

List of Figures viii

List of Abbreviations ix

CHAPTER 1: Introduction and Literature Review 1

1.1. Introduction 1

1.2. *Serratia marcescens* 2

1.3. Antibiotic Resistance in *S. marcescens* 5

1.3.1 β-lactam resistance 5

1.3.1.1. β-lactamase 6

1.3.2. Fluoroquinolone resistance 8

1.3.2.1. Mutations in DNA gyrase and topoisomerase IV 9

1.3.2.2. Outer membrane permeability and active efflux changes 10

1.4. The Role of Porins in Permeability 11

1.4.1. *Serratia marcescens* porins 14

1.5. Active Drug Efflux Pumps 15

1.5.1. Major Facilitator Superfamily (MFS) 16

1.5.2. ATP Binding Cassette (ABC) superfamily 17

1.5.3. Small Multidrug Resistance (SMR) family 17

1.5.4. Multidrug and Toxic Compound Extrusion (MATE) family 17

1.5.5. Resistance-Nodulation-Cell-Division (RND) family 18

1.6. Efflux Pumps in *S. marcescens* **19**
1.6.1. MFS pumps 19
1.6.2. ABC pumps 19
1.6.3. SMR pumps 19
1.6.4. RND pumps 20

1.7. Regulation of Multidrug Efflux Pumps and Porins **21**
1.7.1. Positive regulation by global transcriptional activators 21
1.7.1.1. MarA 25
1.7.1.1.1. SdeR, the *S. marcescens* MarA homolog 26
1.7.1.2. SoxRS 26
1.7.1.3. Right Origin Binding protein (Rob) 27
1.7.1.3.1. Structure of *E. coli* Rob 32
1.7.1.4. Other global regulators 33
1.7.2. Negative regulators 34
1.7.2.1. OmpX 34
1.7.2.2. Small Non-coding RNAs (sRNA) 34
1.7.3. Efflux pump repressors 35
1.7.4. Two-Component Systems (TCS) 36

1.8. Hypothesis and Objectives **37**

CHAPTER 2: Materials and Methods **38**

2.1. Bacterial strains, plasmids, and growth conditions 38
2.2. Molecular biological procedures 38
2.2.1 Rapid plasmid preparation 38
2.2.2 PCR, plasmid purification, cloning and DNA sequencing 41
2.2.3 Transformation 41
2.3. Computer analysis of DNA and amino acid sequences 45
2.4. Promoter evaluation 45
2.4.1 Induction of *rob* promoters by 2, 2'-dipyridyl 47
2.4.2 Induction of *rob* promoters by sodium decanoate and salicylate at 30°C and 37°C 48
2.5. Construction of *rob* knock-out mutant strains 48
2.6. Minimum Inhibitory Concentration (MIC) 50
2.7. Ethidium Bromide (EtBr) accumulation assay 51
2.8. n-hexane tolerance test 51
2.9. RNA extraction 52
2.10. Real-time PCR 52

CHAPTER 3: Results and Discussion **54**

3.1. Computer analysis of DNA and amino acid sequences 55
3.2. Promoter evaluation 66
3.3. Induction of promoters of *rob* candidates by 2,2'-dipyridyl at 37°C 73
3.4. Induction of *rob* promoters by sodium salicylate (SSA) and sodium decanoate (SDE) at 30°C and 37°C 78
3.5. Construction of *rob* knock-out mutant strains 85
3.6. Minimum Inhibitory Concentrations (MICs) 88
3.7. Ethidium Bromide (EtBr) accumulation assay 91
3.8. n-hexane tolerance test 94
3.9. RT-PCR results 97

CHAPTER 4: Conclusion and Future Studies **101**

Appendix **105**

References **108**

List of Tables

Table 2.1. Bacterial strains used in present study. 39

Table 2.2. Plasmids used in present study. 40

Table 2.3. Primers used in present study. 42

Table 3.1. Percentage identity of genes flanking *E. coli rob* and *S. marcescens rob*3. 64

Table 3.2. Minimum Inhibitory Concentration (MIC) (µg/ml) for wild-type, *rob*-deficient and *rob* over-expressing strains. 89

Table 3.3. RT-PCR of wild-type *S. marcescens* UOC-67 and *rob3* knock-out strain (SMRD3) for the evaluation of the expression of various genes. 98

List of Figures

Fig. 3.1. Analysis of *S. marcescens* Rob domains. 57

Fig. 3.2. Alignment of *S. marcescens* Rob candidates with *E. coli* Rob at the amino acid level using BLAST2. 59

Fig. 3.3. Alignment of *S. marcescens* Rob3 with those of *E. coli*, *Shigella flexneri*, *Salmonella enterica* serovar Typhimurium, and *E. cloacae* using PRALINE multiple sequence alignment. 62

Fig. 3.4. Promoter activity of *S. marcescens rob*1, *rob*2, *rob*3 and *E. coli rob* at 30°C. 67

Fig. 3.5. Promoter activity of *S. marcescens rob*1, *rob*2, *rob*3 and *E. coli rob* at 37°C. 69

Fig. 3.6. Induction of the *S. marcescens rob*3 promoter by 2,2'-dipyridyl at 37°C. 75

Fig. 3.7. Induction of the *S. marcescens rob*3 promoter by sodium salicylate at 30°C and 37°C. 80

Fig. 3.8. Induction of the *S. marcescens rob*3 promoter by sodium decanoate (5mM) at 30°C and 37°C. 82

Fig. 3.9. PCR products from wild type *S. marcescens* and *rob1*, *rob*2 and *rob*3 knock-out strains SMRD1, SMRD2 and SMRD3. 86

Fig. 3.10. Ethidium bromide accumulation by wild-type *S. marcescens*, SMRD3 and SMRD3 complemented with *S. marcescens rob*3 (SMRD3/pUSROB3). 92

Fig. 3.11. n-hexane tolerance assay. 95

List of Abbreviations

ABC	ATP-Binding Cassette superfamily
Ala	Alanine
Ap	Ampicillin
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
ATCC	American Type Culture Collection
BES	Brazilian extended spectrum
bp	base pairs
CAMHB	Cation-adjusted Muller Hinton Broth
Cb	Carbenicillin
cbpB	Curved DNA-binding protein B
CCCP	Carbonyl cyanide m-chlorophenylhydrozone
CFU	colony forming unit
Cip	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
Cm	Chloramphenicol
Ct	Cycle threshold
CTD	Carboxy-terminal domain
C-terminal	Carboxy-terminal
CTX-M	Cefotaximase
Cys	Cysteine
DAPI	4,6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ESBL	Extended-spectrum β-lactam
EtBr	Ethidium bromide
GFP	Green fluorescent protein
Glu	Glutamic acid
H-NS	Histone-like Nucleoid structuring protein
hrs	hours
HTH	Helix-turn-helix
Ile	Isoleucine
kb	kilobase pairs
kDa	kiloDalton
Km	Kanamycin
L	Litre(s)
LB	Luria-Bertani
Lys	Lysine
mar	multiple antibiotic resistance locus

MATE	Multidrug and toxic compound extrusion family
MBL	Metallo-β-lactamase
MDR	Multi-drug resistance
MFP	Membrane fusion protein
MFS	Major facilitator superfamily
mg	milligram(s)
MHB	Muller Hinton Broth
MIC	Minimum inhibitory concentration
min	minute(s)
mm	millimeter
mM	millimolar
NaI	Nalidixic Acid
NICU	Neonatal intensive care unit
NO	Nitric oxide
NTD	Amino-terminal domain
OM	Outer membrane
OMP	Outer membrane protein
ORF	Open reading frame
PBP	Penicillin binding protein
PCR	Polymerase chain reaction
QRDR	Quinolone resistance determining region
qRT-PCR	Real-time quantitative polymerase chain reaction
RBS	Ribosomal binding site
RNAP	RNA polymerase
RND	Resistance-nodulation-cell division family
RNP	RNA-protein complex
Rob	Right origin binding protein
rpm	revolution per minute
Sdi	Suppressor of division inhibition
SDS	Sodium dodecyl sulfate
Ser	Serine
SHV	Sulfhydryl variable
SME	*Serratia marcescens* enzyme
SMR	Small multidrug resistance family
Sp	Spectinomycin
sRNA	Small Non-coding RNA(s)
SsmE	*Serratia* small multidrug efflux pump
Str	Streptomycin
TCS	Two-component system
Tet	Tetracycline
TPCCI	Tetraphenyl phosphonium chloride
TSB	Trypticase Soy Broth
Tyr	Tyrosine

UTI	Urinary tract infection
v/v	volume/volume
w/v	weight/volume
µg	microgram(s)
µl	microlitre(s)
µM	micromole(s)

CHAPTER 1: Introduction and Literature Review

1.1. Introduction

The sulfa drugs and penicillin were introduced to the clinical settings in the 1930s and 1940s, respectively. Treatment of the infectious diseases with these drugs resulted in the dramatic decrease in the mortality rate (Cohen 2000). After penicillin, many drugs have been discovered or synthesized; however, despite the development of many drugs to treat different infections, infectious diseases are the second major cause of the death across the world (Fauci 2001; Nathan 2004).

Public health systems have faced another challenging problem. Antimicrobial resistance and, even worse, multi-drug resistant bacteria have emerged from the extensive use of the antibiotics. By imposing a constant selection pressure, development of antibiotic resistance has become the outcome of excessive antibiotic usage (Leeb 2004; Palumbi 2001). Presently, antimicrobial resistance has become a growing worldwide problem. The emergence of resistance among pathogens causing hospital and community acquired infections has gained much attention. Spreading of resistant strains has imposed huge financial impact on the health systems and has led to high morbidity and mortality rates worldwide (Bosso 2005).

Certain bacterial genera such as *Serratia* (Collatz and Gutmann 1987; Goldstein et al. 1983; Traub et al. 1983; Collatz et al. 1983; Sanders et al. 1984; Gutmann and Chabbert 1984), *Enterobacter* (Quentin et al. 1987; Sanders et al. 1984; Gutmann et al. 1985; Then and Angehrn 1986), *Salmonella* (Gutmann et al. 1988) , *Klebsiella* (Sanders et al. 1984; Gutmann and Chabbert 1984), and *Pseudomonas* (Preheim et al. 1982;

Rella and Haas 1982) have more often than other genera developed multi-resistant phenotypes. Resistance to multiple drugs can arise from target modification, active efflux systems, plasmids and/or transposons, reduced porin permeability, and inactivation of drugs by enzymatic inactivation or modification (Ouellette and Kundig 1997; Mazel and Davies 1999; Hayes and Wolf 1990).

It is well known that porins facilitate the diffusion of some antibiotic classes such as β-lactams, aminoglycosides and quinolones through the outer membrane (Gutmann et al. 1985; Nikaido and Vaara 1985; Hooper et al. 1986), while efflux pumps eliminate these antibiotics, once they enter the cell. With this in mind, the study of the mode of regulation of porin and efflux pump expression by *S. marcescens* was undertaken.

1.2. ***Serratia marcescens***

S. marcescens is a Gram-negative bacillus classified as a member of *Enterobacteriaceae* family. *S. marcescens* is motile by peritrichous flagella. *S. marcescens* is a non-spore forming bacterium, with the exception of *S. marcescens* subsp. *Sakuensis* which is able to produce endospores (Ajithkumar et al. 2003). *S. marcescens* can be isolated from a wide variety of ecological niches including water, sewage, animals, plants, and air (Grimont and Grimont 1984). Even starchy foods, such as bread and communion wafers, can provide this bacterium a rich growth substrate (Hejazi and Falkiner 1997). In addition, *S. marcescens* is able to survive under extreme conditions such as in double-distilled water (Szewzyk et al. 1993), disinfectants (Marrie and Costerton 1981), and antiseptics (Nakashima et al. 1987).

This bacterium was thought to be a saprophytic non-pathogenic and innocuous microorganism. The first case of human disease attributed to this bacterium was identified in 1913. A healthy man with chronic cough and having a red colored sputum was diagnosed as the first case of *S. marcescens* infection (Woodward and Clark 1913). Presently, *S. marcescens* has become a prominent opportunistic bacterium of the hospitalized patients where the multi-resistance strains cause complications (Van Houdt et al. 2007).

Immunocompromised patients, heroin addicts and hospitalized patients are highly susceptible to *S. marcescens* infections (Grimont and Grimont 1992; Hejazi and Falkiner 1997). The first occurrence of nosocomial infections (hospital-acquired infection) caused by *S. marcescens*, occurred in patients who previously had urologic instrumentation (indwelling catheters) and were under antibiotic therapy (Wheat et al. 1951).

S. marcescens is a causative agent of various infections from simple wound infections to meningitis and septicemia. Urinary tract infections (UTI), respiratory tract infections (Acar 1986; Hejazi and Falkiner 1997), endocarditis (Brouqui and Raoulet 2001), brain abscesses, and abdominal infections (Grimont and Grimont 1984) are some examples of diseases which can also be caused by *S. marcescens*.
S. marcescens has been recognized as a well-known hospital-acquired infection among infants in neonatal intensive care units (NICUs) (Smith et al. 1984; Christensen et al. 1982; Aygun et al. 2000). Complications including conjunctivitis, skin infections, pneumonia (Khan et al. 1997), septicemia, and meningitis (Sakata and Maruyama

1998) are common *S. marcescens* infections among NICU infants. During *S. marcescens* outbreaks in NICUs, the sources of infections are frequently sinks and respiratory therapy equipment (Berthelot et al. 1999; Archibald et al. 1997; Newport et al. 1985; Anagnostakis et al. 1981).

S. marcescens strains are able to produce certain enzymes including DNases, lipases, and gelatinases, which differentiate them from the other genera among *Enterobacteriaceae* family members (Anahory et al. 1998; Kawai et al. 1998; Li et al. 1995; Nestle and Roberts 1969). Moreover, *S. marcescens* has developed a number of other virulence factors such as haemolysin, protease, chitinase, chloroperoxidase and multiple isoenzymes of alkaline phosphatase (Anahory et al. 1998; Braun and Schmitz 1980; Hase and Finkelstin 1993; Hertle 2000; Hines et al. 1988; Yanagida et al. 1986). Pili, siderophores, proteases, cell wall antigens, and resistance to antibacterial action of the serum are also important virulence factors leading to the pathogenicity of *S. marcescens* (Old et al. 1983; Grimont and Grimont 1992).

S. marcescens is capable of producing two secondary metabolites (by-products) which have potential therapeutic application. Strain ATCC 39006 produces a carbapenem antibiotic (5R)-carbapen-2-em-3 carboxylic acid (Parker et al. 1982) which is active against both Gram-positive and Gram-negative bacteria (Kropp et al. 1980, 1985).

The other secondary metabolite is prodigiosin, a linear tripyrrole and non-diffusible red pigment, which gives the *S. marcescens* colonies dark red to pale pink color. The age of the colony is a factor which determines the color of the colony (Harris

et al. 2004). The role of the mysterious prodigiosin still remains unknown, although there are some studies indicating that it has an antibacterial, antifungal, antiprotozoan or immunosuppressant activity (Han et al. 1998; Slater et al. 2003). Groscop and Brent (1964) reported that this pigment is toxic to protozoa which might be an ecological advantage in soil and water; however, pigmented isolates are more commonly found in unpolluted water such as wells and springs. Clinical isolates of *S. marcescens* usually do not produce prodigiosin (Hejazi and Falkiner 1997) while environmental isolates are typically able to produce it (Gaughran 1968). This differential pigment production has resulted in misidentification of the isolates, especially among clinical isolates. Unpigmented isolates tend to be more resistant to antibiotics (Gargallo-viola 1989) than pigment producers. The expression of the prodigiosin is regulated by various ecological and intracellular signals, for example, nutrient deprivation, temperature, and pH (Lai et al. 2005; Slater et al. 2003; Williamson et al. 2006).

1.3. Antibiotic Resistance in *S. marcescens*

Multi-drug resistance (MDR) against many drugs including β-lactams, aminoglycosides and fluoroquinolones has developed in *S. marcescens* (Stock et al. 2003; Traub 2000). The emergence of the MDR phenotypes has made *S. marcescens* infections difficult to treat, particularly in hospitalized patients (Livermore 1998a, and b).

1.3.1. β-lactam resistance

There are three main mechanisms for β-lactam resistance (Jones 1998):

1) Production of inactivating enzymes (e.g. β-lactamases)

2) Penicillin binding protein (PBP) target alteration

3) Diminished permeability of outer membrane proteins

1.3.1.1. β-lactamase

Clinical strains of *S. marcescens* typically confer their resistance through β-lactamase production. β-lactam antibiotics bind to inner membrane penicillin binding proteins (PBPs), which play an important role in the cell wall synthesis. The binding of the β-lactams to PBPs results in the lysis of the cell wall, dramatic changes in cell wall shape, and prevention of cell wall division during multiplication (Dever and Dermody 1991). β-lactamase cleaves the β-lactam ring of the antibiotic molecule. Genes encoding β-lactamse production can be either transferred genetically to the off-spring cells or via plasmids to another bacterium.

β- lactamases produced by pathogenic bacteria are usually categorized into two different schemes: the molecular (Ambler) classification based on their amino acid sequences (Ambler 1980) and the functional (Bush-Jacoby-Medeiros) classification based on enzyme functional properties such as substrate specificity and being inhibited by β-lactamase inhibitors such as clavulanate (Bush and Jacoby 2010).

According to the Ambler classification, there are four molecular classes of β-lactamases: A, B, C and D (Ambler 1980). Classes A, C and D contain serine at the active site and are mostly susceptible to clavulanic acid inhibition while class B are the metallo-enzymes having two zinc atoms in their structure which are resistant to β-lactamase inhibitors and inactivated by EDTA.

β-lactamases are classified in 3 broad categories based on functional classification including metallo- β-lactamases (MBLs), extended Spectrum β-lactamases (ESBLs), and other β-lactamases.

Many ESBLs contain a serine at the active site. Almost all the ESBLs fall into specific families: TEM (patient's initial), SHV (sulfhydryl variable), CTX-M (Cefotaximase), BES (Brazil extended spectrum), and SME (*Serratia marcescens* enzyme*)*. *S. marcescens* is able to produce TEM, SHV and SME enzymes (Luzzaro et al. 1998; Queenan et al. 2000) and also produces BES-1, another ESBL which has activity against alkyloiminocephems, monobactams, and β-lactamase inhibitors (Bonnet et al. 2000).

ESBLs strains are widespread especially in the hospital environment (Paterson and Bonomo 2005; Pfaller and Segreti 2006). Hospital-acquired infections through ESBL producer bacteria have been frequently reported in neonatal intensive care units (NICUs) (Villari et al. 2001; Gastmeier et al. 2003; Miranda et al. 2004; Bagattini et al. 2006; Linkin et al. 2004). *S. marcescens* strains which are able to produce ESBL were isolated from a neonate's blood sample for the first time in 2004 (Crivaro et al. 2007).

Extended spectrum β-lactam antibiotics such as aztroenam, ceftazidime, ceftriaxone, and cefotaxime are readily hydrolyzed by ESBL strains. The ESBL producers are very commonly multi-resistant. All *S. marcescens* ESBL producers are resistant to aminopenicillins, monobactams, third-generation cephalosporins, and penicillin associated clavulanic acid. These isolates also confer intermediate sensitivity to cefepime, piperacillin/tazobactam, chloramphenicol and tetracycline. One study

indicated that all isolates were also resistant to gentamycin, netilmicin and kanamycin but sensitive to amikacin (Crivaro et al. 2007).

1.3.2. Fluoroquinolone resistance

In 1962, nalidixic acid was the first quinolone introduced to the medicinal market for treatment of urinary tract infections (UTI) (Ball 2000). In the 1970s, adding fluorine to quinolone molecules at the C-6 position led to the production of norfloxacin (Emmerson and Jones 2003). New fluoroquinolones are among the most extensively used antibiotics (18% of total antibiotic consumptions) owing to their potent and wide range of antimicrobial activity for human or veterinary infections (Kresse et al. 2007). According to Acar and Goldstein (1997), ciprofloxacin was named as the most used antibiotic worldwide. Quinolones are active against Gram-positive and Gram-negative bacteria targeting DNA gyrase (Wolfson and Hooper 1985).

In the recent years, quinolone- and fluoroquinolone-resistance among *S. marcescens* strains has been greatly escalating (Martinez-Martinez et al. 1998; Kreskan and Wiedemann 1988; Wolfson and Hooper 1985). The first case of ciprofloxacin resistance in *S. marcescens* was from an endocarditis infection in 1994 (Korner et al. 1994).

There are two main resistance mechanisms to quinolones: 1) Mutations in DNA gyrase and topoisomerase IV (Hane and Wood 1969; Robillard and Scarpa 1988; Hooper et al. 1986) and 2) decrease in the accumulation of antibiotic in bacteria as a result of either the outer membrane permeability or decreased influx (Hirai et al. 1986a,

b) and/or overexpression of efflux pumps leading to increased efflux (Li et al.1994; Charvalos et al. 1995).

1.3.2.1. Mutations in DNA gyrase and topoisomerase IV

DNA gyrase is composed of two A and two B subunits (A_2B_2) which are encoded by *gyrA* and *gyrB*, respectively (Gellert 1981). DNA gyrase is a type II topoisomerase which alters the topology state of the DNA. Gyrase A is able to transform closed circular DNA in helix form into a negatively supercoiled form (Gellert et al. 1981) while the GyrB subunit hydrolyzes ATP and captures the DNA strand (Heddle and Maxwell 2002).

In *E. coli* and *S. marcescens*, the most frequent mutations fall between the Ala-67 and Glu-106 region which is called quinolone resistance determining region (QRDR) (Yoshida et al. 1990a; Fujimaki et al. 1989). QRDR from eight *Enterobacteriaceae* type strains and 60 fluoroquinolone-resistant clinical isolates of *Citrobacter freundii*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *E. coli*, *K. pneumoniae*, *K. oxytoca*, *Providencia stuartii*, and *S. marcescens* showed that the predominant alterations in GyrA are Gly-81→Cys, Ser-83 →Ile or Arg, and Asp-87→Asn, all of which are located near the active site of enzyme (Tyr-122) (Vila et al. 1994; Weigel et al. 1998). Mutations in the GyrA will lead to a 20-fold increase in resistance while if the mutation takes place in GyrB, a 4-fold increase in resistance would be expected (Heddle and Maxwell 2002). Unlike GyrA, the mutations in GyrB have been reported outside of the QRDR, including Lys-447→Glu and Asp-426→Asn (Hooper 2003).

Amino acid alteration in DNA topoisomerase IV also leads to quinolone resistance. Topoisomerase IV is composed of two C and two E subunits encoded by *parC* and *parE*, respectively. This enzyme is involved in chromosome segregation (Adams et al. 1992). Studies indicated that ParC is the secondary target for quinolones (Baucheron et al. 2002; Guerra et al. 2003; Hansen and Heisig 2003).

Compared with mutations in *parC* and *gyrA*, few mutations have been reported in *gyrB*. This is considered a rare phenomenon among fluoroquinolone resistant *E. coli*, *Staphylococcus aureus*, *Streptococcus pneumonia* and *Salmonella* Typhimurium (Piddock 1999). Moreover, amino acid alterations in ParC and ParE of *E. coli* could be found only when GyrA mutations were present (Breines et al. 1997; Heisig 1996; Kumagai et al. 1996).

To obtain high levels of resistance to fluoroquinolones, the presence of additional mutation(s) in *gyrA* and/or in other targets such as *parC* is required (Vila et al. 1994; Ruiz et al. 2002). In Gram-negative bacteria, DNA gyrase is more sensitive to quinolone activity than topoisomerase IV, while in Gram-positive bacteria, topoisomerase IV is the prime target (Jacoby 2005).

1.3.2.2. Outer membrane permeability and efflux changes

In *E. coli*, OmpF and OmpC are two major outer membrane proteins. There is evidence showing that *ompF* deficient *E. coli* exhibited a decreased sensitivity to quinolones while the *ompC* deficient *E. coli* did not show the same properties (Cohen et al. 1989; Gutmann et al. 1985). Therefore, the alteration in the quinolone uptake via OmpF led to a decline in antibiotic accumulation in the cell (Hooper et al. 1989; Piddock

1991). *S. marcescens* has similar outer membrane proteins to *E. coli,* including OmpF and OmpC (Hutsul and Worobec 1994, 1997). *ompF* mutants of *S. marcescens* exhibited antibiotic resistance phenotypes similar to that of *E. coli ompF* mutant strains (Berlanga et al. 2000a).

Efflux pumps are considered to have a major role in acquired and intrinsic resistance of many bacteria to fluoroquinolones (Tseng et al. 1999). Thus, the over-production of proteins involved in efflux systems, such as AcrAB-TolC in *E. coli*, can pump out much more antibiotics and results in quinolone resistance (Hooper 2001a, b; Baucheron et al. 2004; Okusu et al. 1996).

There are at least two AcrA-like proteins in *S. marcescens* (Kumar and Worobec 2002), where increased efflux has been contributed to the fluoroquinolone resistance. SdeAB of *S. marcescens* is responsible for pumping out various substrates including fluoroquinolones, chloramphenicol, ethidium bromide, and n-hexane (Kumar and Worobec 2005a). The other AcrAB-like efflux pump in *S. marcescens*, SdeXY, also has a wide range of substrates including fluoroquinolones, tetracycline, acriflavine, and ethidium bromide (Chen et al. 2003).

1.4. The Role of Porins in Permeability

The cytoplasmic membrane is enclosed by the outer membrane (OM) and peptidoglycan layer in Gram-negative bacteria. As a semi-permeable barrier, the outer membrane is considered as the primary line of protection against the noxious agents (Nikaido 2003).

Porins form transmembrane pores which are found in the outer membrane. They function as a gate for the cell, making them important elements in the interaction of Gram-negative bacteria with their niches. Porins determine the extent of the permeability (Hirai et al. 1986a; Aoyama et al. 1987), allowing some hydrophilic molecules such as nutrients and β-lactam antibiotics to enter the cell (influx) (Nikaido and Nakae 1979).

Two antibiotic classes, β-lactams and fluoroquinolones, are considered as important groups among the all antibacterial agents (Bryskier 2005). Among the β-lactam class of antibiotic, penicillins and cephalosporins are the most consumed drugs for treatment of *S. marcescens* infections. This class of antibiotic needs to have access to outer membrane to be efficient. The antibacterial functions of these classes are modified by the influx barrier in clinical strains. Thus, the porins play a crucial role to allow them to enter the cell (Bryskier 2005; Kohanski et al. 2007).

Crystallography has revealed a common structure for most bacterial porins consisting of a tight trimer of 16-strand anti-parallel β-barrels that transverse the outer membrane (Cowan et al. 1992; Garavito et al. 1983; Walian and Jap 1990; Weiss and Schulz 1992). Both β-barrels and α-helices have hydrogen bonds but hydrogen bonds in β-barrels are much stronger, making these structures more stable than α-helix bundles (Schulz 2004).

Porins can be classified into two groups: specific and general diffusion or non-specific porins. Specific porins allow only certain molecules to pass through the outer membrane, having specific binding sites for their substrates. LamB of *E. coli* is the

prototype of the specific porins. LamB allows the passage of maltodextrin through the outer membrane (Jap and Walian 1996).

Non-specific or general diffusion porins lack specific binding sites for any particular solutes and have poor substrate selection (Nikaido 2003; Delcour 2003). The majority of known porins belong to the non-specific porin class (Benz 1988; Benz and Bauer 1988). In *E. coli*, there are three major non-specific porins, OmpF, OmpC and PhoE (Jap and Walian 1996). OmpF and OmpC serve to facilitate passive diffusion across the outer membrane. It has been suggested that OmpF is a crucial pore allowing cells under nutritionally poor media to absorb more nutrients while OmpC, having a smaller pore size (Ferrario et al. 1995).

In *E. coli*, there is a slight preference for the passage of cations over anions for OmpF and OmpC. OmpC is slightly smaller than OmpF based on the diffusion of organic molecules (Nikaido and Rosenberg 1981). Molecules with up to 600 Da in molecular weight are able to pass through OmpF (Nikaido and Vaara 1985), such as ions, amino acids, small-sized carbohydrates (Nikaido 2003) and β-lactams antibiotics (Nikaido and Rosenberg 1983). Other findings showed that more charged residues exist in the OmpC pore (Schulz 2002). Although, the total amount of the OmpC and OmpF in the cell is constant, their ratio might change depending on the environmental changes such as osmolarity. For instance, in hypo-osmotic medium, OmpF is the dominant porin while OmpC is the major porin in hyperosmolic medium and *ompF* expression becomes suppressed (Alphen and Lugtenberg 1977). The same findings were reported in *S. marcescens* (Hutsul and Worobec 1997).

There are several reports correlating antibiotic resistance to porin malfunction or porin loss in some bacteria including *E. coli*, *P. aeruginosa*, *Neisseria gonorrhoeae*, *E. aerogenes* and *K. pneumoniae* (Nikaido 2003; Achouak et al. 2001; Poole 2002, 2004). These studies showed that OmpF and OmpC expression is important for innate resistance (Delcour 2009).

1.4.1. *Serratia marcescens* porins

To date, two general porins, OmpF and OmpC have been characterized in *S. marcescens*. OmpF is a 41 KDa protein while OmpC is 40 KDa (Hutsul and Worobec 1997). OmpF and OmpC have 68% - 71% identity at the amino acid level to those of *E. coli*, respectively (Hutsul and Worobec 1997). The third external loop is vital for porin constriction by folding down into the pore lumen (Cowan et al. 1992). In *S. marcescens* the third external loop (L3) motif of OmpF shows high amino acid sequence consensus with those of other enterobacterial porins, whereas, the L3 of OmpC *S. marcescens* is less well conserved, having amino acid alterations at positions 112, 114, and 124 (Hutsul 1996). To investigate the role of these amino acids, site-directed mutagenesis was performed by replacing Asp112→ Gly, Met114→ Asp and Gln124→ Gly. This study showed that the amino acids in position 114 had no role in pore constriction or ionic selection but amino acid 112 was involved in the size selection and amino acid 124 was important for ionic selection and pore constriction (Begic and Worobec 2007).

Environmental stimuli, such as pH, temperature, and osmotic changes can affect both OmpF and OmpC expression (Begic and Worobec 2006). OmpF is the main porin produced at low temperature, in basic pH, and in the absence of the salicylate while

OmpC forms the major porin in high temperature, alkaline pH, and presence of the salicylate (Begic and Worobec 2006).

S. marcescens OmpF is unusual in that it contains two cysteines (Hutsul and Worobec 1997). The amino acid alignment of *E. coli* and *S. marcescens* OmpF suggested these cysteines could be positioned in the sixth external loop (Cowan et al. 1992). To date, cysteines have only been found in three other porins, OprF and OprB of *Pseudomonas aeruginosa* (Duchene et al. 1988; Wylie and Worobec 1994) and LamB of *E. coli* (Clement and Hofnung 1981). The presence of cysteines in *E. coli* LamB and *P. aeruginosa* OprB porins appear not to have an important role (Ferenci and Stretton 1989 ; Wylie and Worobec 1994), while four cysteines in OprF of *P. aeruginosa* may have a functional role (Hancock 1987) but this has not been completely studied.

1.5. Active Efflux Pumps

There are five mechanisms for resistance to drugs in bacteria including: 1) inactivation of drugs by degradation or modification; 2) alteration of drug targets; 3) emergence of a bypass that is not inhibited by drugs; 4) changes in membrane permeability for drugs; and 5) drug efflux from cells. Among these mechanisms, drug efflux is the major mechanism for multidrug resistance in bacteria (Nikaido 1988). The term Multidrug resistance (MDR) is applied to explain innate resistance mechanisms which are encoded by the genes that belong to the native genome of the cell (George 1996).

In bacteria, according to the similarities in amino acid sequences, size of proteins and types of energy coupling, multidrug resistance efflux pumps are classified into five

distinctive families (Putman et al. 2000) including: 1) Major Facilitator Superfamily (MFS) (Marger and Saier 1993); 2) ATP Binding Cassette (ABC) superfamily (Van Vean and Konings 1998); 3) Small Multidrug Resistance (SMR) family (Paulsen et al. 1996); 4) Resistance-Nodulation-Cell-Division (RND) family (Saier et al. 1994); 5) Multidrug and Toxic Compound Extrusion (MATE) family (Brown et al. 1999). The two superfamilies, MFS and ABC, make up approximately 50% of all transporters (Pao et al. 1998).

In another type of classification, based on the number of components which form the efflux pumps, the transporters can be categorized into two groups: single- and multi-component pumps. The single component pumps pass their compounds through the cytoplasmic membrane while a multi-component pump is composed of a MFP (membrane fusion protein) which is in the periplasm and an OMP (outer membrane protein). Multi-component pumps are typically found in Gram-negative bacteria.

An individual bacterium is able to express more than one family type of MDR efflux pump. The substrate for each efflux pump is variable, based on the efflux pump and bacterial strains (Poole 2004, 2005). The substrates of individual efflux pumps are very diverse including antibiotics such as tetracycline, fluoroquinolones, chloramphenicol; dyes such as acriflavin and ethidium bromide; detergents, and biocides such as cetrimide and triclosan (Piddock 2006).

1.5.1. Major Facilitator Superfamily (MFS)

MFS pumps take part in transportation of sugars, metabolism, anions and antibiotics (Saier 2000). NorA of *S. aureus* (Yoshida et al. 1990b), EmrAB-TolC of

E. coli (Lomovskaya and Lewis 1992), and QacA of *S. aureus* (Kaatz et al. 1993; Neyfakh et al. 1993; Lowy 2003) are some examples of MFS pumps.

1.5.2. ATP Binding Cassette (ABC) superfamily

The prominent characteristic of ABC proteins is their capability of transporting compounds such as hydrophobic drugs and antibiotics, and taking up small nutrients including histidine, maltose, peptides, or ribose (Ehrmann et al. 1998; Holland and Blight 1999). Certain ABC transporters are also involved in antimicrobial resistance (Klokouzas et al. 2003; Lage 2003; Mckeegan et al. 2004). Lmr of *Lactococcus lactis* (Bolhuis et al. 1996) and MsbA of *E. coli* (Chang and Roth 2001) are two members of the bacterial ABC superfamily.

1.5.3. Small Multidrug Resistance (SMR) family

Among the efflux pumps, the SMR family has the simplest structure. Smr of *Staphylococcus aureus* (Grinius et al. 1992) and EmrE of *E. coli* (Schuldiner et al. 1997) are two well-characterized members of SMR family. The substrates of the SMR family are restricted to lipophilic cations such as antiseptics and disinfectants (Li and Nikaido 2004).

1.5.4. Multidrug and Toxic Compound Extrusion (MATE) family

NorM of *Vibrio haemolyticus* and YdhE of *E. coli* are two examples of this family. These two pumps confer resistance to aminoglycosides, fluoroquinolones, kanamycin, streptomycin and cationic dyes such as acriflavin and ethidium bromide (Morita et al.

1998& 2000; Huda et al. 2001). To date, no common substrate has been identified between bacterial and mammalian MATE transporters (Kuroda and Tsuchiya 2009).

1.5.5. Resistance-Nodulation-Cell-Division (RND) family

RND pumps are ubiquitous efflux pumps found in bacteria, archaea and eukaryotes. These are the most frequently found efflux pumps among Gram-negative bacteria. Although the RND pumps are usually encoded by the bacterial chromosome, new findings indicate that there is a transporter that is expressed from a plasmid (Droge et al. 2000). RND transporters are capable of catalyzing substrate efflux by means of a substrate–H^+ antiport process. RND systems play a prominent role in conferring innate and acquired resistance to different antibiotic classes (Nishino et al. 2006; Baucheron et al. 2004; Eaves et al. 2004; Giraud et al. 2000). A wide range of substances are considered as substrates of RND efflux pumps (e.g. AcrAB-TolC) including acriflavin, ethidium bromide, β-lactams, bile salts, chloramphenicol, fluoroquinolones, macrolides, rifampin, SDS, crystal violet, novobiocin, organic solvents and fatty acids (Ma et al. 1993; Fralick 1996). In addition, RND pumps are important for virulence. For example, wild type *Salmonella* Typhimurium and *P. aeruginosa* RND deficient strains exhibited remarkably less virulent phenotypes (Nishino et al. 2006; Piddock 2006; Buckley et al. 2006; Burse et al. 2004; Hirakata 2002).

RND pumps are a tripartite system. For example, the AcrAB-TolC efflux pump of *E. coli* consists of a transporter protein (AcrB) located inside the inner membrane, a helper protein (AcrA) in periplasmic space and an outer membrane protein (TolC)

(Koronakis et al. 2004). The expression of *acrAB* is modulated locally by its repressor AcrR (Mallea et al. 2002). AcrR mutations result in an increase in efflux by AcrAB (Wang et al. 2001). At the global level, *acrAB* expression is controlled by regulators such as MarA, SoxS and Rob (Khandekar et al. 2003; Schweizer 1998).

1.6. Efflux Pumps in *S. marcescens*

1.6.1 MFS pumps

S. marcescens produces Smfy, a MFS pump (Shahcheraghi et al. 2007). This pump possesses between 37- 41% identity with KmrA of *K. pneumonia* (Ogawa et al. 2006), SmvA of *Salmonella* Typhimurium (Hongo et al. 1994), LfrA of *Mycobacterium smegmatis* (Takiff et al. 1996), SgcB of *Streptomyces globisporus* (Liu and Shen 2000), VarS of *S. virginiae* (Lee et al. 1999), and QacA of *Staphylococcus aureus* (Rouch et al. 1990). SmfY substrates include norfloxacin, benzalkonium chloride, acriflavin, ethidium bromide, tetraphenyl phosphonium chloride (TPPCI), methyl viologen, Hoechest 33342 dye and 4,6-diamidino-2-phenylindole or nylindole (DAPI) (Shahcheraghi et al. 2007).

1.6.2. ABC pumps

SmdAB is a heterodimer ABC efflux pump family produced by *S. marcescens*. This pump confers resistance to norfloxacin, tetracycline, DAPI and Hoechst 33342 dye. It consists of six putative transmembrane segments following a hydrophilic segment (Matsuo et al. 2008).

1.6.3. SMR pumps

In *S. marcescens*, another pump is SsmE (Serratia small multidrug efflux pump) which possess high similarity with SMR-type multidrug efflux pumps. Acriflavin and

ethidium bromide are found to be good substrates for SsmE. Moreover, this pump exhibits the high similarity with EmrE of *E. coli* and EmrE of *P. aeruginosa* at the amino acid level (Minato et al. 2008). Two other SMR-type pumps were detected in *S. marcescens*, called SsmD and SsmJK, which seem to be orthologs of SugE and YdgE of *E. coli*. So far, no substrates for these pumps have been found (Minato et al. 2008).

1.6.4. RND pumps

Berlanga first described the presence of an RND efflux pump activity in *S. marcescens* (Berlanga et al. 2000b). Kumar and Worobec identified the major RND pump as SdeAB, which was over-expressed in many fluoroquinolone resistant clinical isolates. Expression of SdeAB in type strains can be induced by exposure to fluoroquinolones (Kumar and Worobec 2002, 2005a).

Like SdeAB, SdeXY was found to be another RND-type efflux pump in *S. marcescens* (Chen et al. 2003). SdeXY confers norfloxacin and tetracycline resistance (Chen et al. 2003). SdeXY is suggested to also be a homolog of AcrAB-TolC of *E. coli* (Kumar and Schweizer 2005). Diverse compounds are considered as SdeXY substrates including acriflavin, ethidium bromide, fluoroquinolones and tetracycline (Chen et al. 2003).

In *S. marcescens*, a *tolC*-like (*hasF*) gene was characterized, with the translation product having approximately 80% identity with *tolC* of *E. coli* at the amino acid level. HasF was demonstrated to have very similar 3-dimensional structure to TolC in computer modeling simulations (Kumar and Worobec 2005b).

SdeCDE is another RND-type efflux pump (Kumar and Worobec 2005a) which is thought to be homolog the MdtABC system of *E. coli*. Novobiocin was found to be the only substrate for the SdeCDE pump (Begic and Worobec 2008) which is the same substrate for MdtABC (Nagakubo et al. 2002).

1.7. Regulation of Multidrug Efflux Pumps and Porins

The ability to orchestrate the proper action toward the aggressive external signals is very crucial for bacteria to survive. Among the porin and efflux pump regulators, the role of transcriptional regulators including *marA*, *soxS* and *rob* is very important in this regard. As *E. coli* and *S. marcescens* both belong to Enterobacteriaceae family, finding similar regulators is highly likely. The focus of my thesis was on the identification of a Rob regulator in *S. marcescens* and the study of its role in the regulation of porins and efflux pumps.

In *E. coli*, efflux pump and porin expression are controlled by: 1) positive regulation by means of general or specific transcriptional activators that harmonize the expression of some genes including *marA*, *soxS* and *rob*; 2) negative regulation through the porin or efflux pump suppressors; and 3) chemicals or drugs that stimulate sophisticated regulatory cascades (Davin-Regli et al. 2008).

1.7.1. Positive regulation by global transcriptional activators

To date, a number of global regulators have been characterized. The prominent examples of these regulators are MarA, SoxS, Rob, RamA and SdiA. MarA, SoxS, Rob and RamA are members of the AraC/XylS family. Historically, it is believed the transcriptional regulators, MarA, SoxS and Rob had enzymatic activity on specific amino

acids that were able to bind to their active sites but owing to active site disruption, they lost this function and they have gained a new function over the course of evolution (Grishin 2000). The over-expression of these genes results in low-level resistance to diverse and unrelated compounds (Ariza et al. 1995; Cohen et al. 1989; Greenberg et al. 1990).

The AraC/XylS family consists of over 1000 members (Alekshun and Levy 2004). Many of AraC/XylS members contain two highly specialized domains, a conserved carboxy-terminal domain (CTD) with two helix-turn-helix (HTH) DNA-binding motifs and a non-conserved amino-terminal domain (NTD) that binds to inducer molecules (Gallegos et al. 1997). In the HTH motif, the CTD α-helix is usually named as the recognition helix due to its binding to specific sequences in the targeted DNA through the DNA major groove. The members of AraC family possess a conserved region of 99 amino acids that could be usually observed at the C-terminus. Although in some cases, including Rob and CafR of *E. coli*, this region is found at N-terminal end of protein (Gallegos et al. 1997). Thus, the 99 residues of the N-terminal of Rob show high homology with the C-terminal of SoxS and MarA. SoxS and MarA could be considered as unique members among the transcriptional activators in terms of having only one DNA-binding domain, while Rob possesses a C-terminal and dual HTH motifs in the N-terminal (Gallegos et al. 1997; Kwon et al. 2000; Amabile-Cuevas and Demple 1991; Wu and Weiss 1991).

There is 42-59% identity at amino acid level among MarA, SoxS, Rob and TetD over the total 107 amino acids of the SoxS, one of the smallest members of AraC family

(Amabile-Cuevas and Demple 1991; Gambino et al. 1993). The presence of high homology among these proteins and their predicted DNA binding regions indicates that these proteins could have the same function (Jair et al. 1996b).

The AraC/XylS family members control various cellular functions including catabolism of sugars, bacterial virulence and response to external aggressive stimuli. A large number of the AraC/XylS members have been shown to be involved in bacterial virulence (Finlay and Falkow 1997). For example, triple knockouts of *marA*, *soxS* and *rob* made *E. coli* remarkably less pathogenic than the wild type. Deletions of *soxS* and *rob* were more critical than deletion of *marA* (Casaz et al. 2006).

In *E. coli*, MarA and SoxS were initially recognized for their capability to control multiple antibiotic resistance (Mar phenotype) (George and Levy 1983a, b). Studies on Rob, MarA and SoxS suggested that they might affect the expression of the different genes in a similar mechanism (Ariza et al. 1995). Several studies have shown that MarA, SoxS and Rob can control several target genes which are called *mar*/*sox*/*rob* regulon in *E. coli* (Barbosa and Levy 2000; Bennik et al. 2000; Pomposiello et al. 2001), *Salmonella*Typhimurium (Pomposiello and Demple 2000) and some other members of the Enterobacteriaceae.

The *mar*/sox/*rob* box is the DNA sequence in the promoter regions of regulons where SoxS, MarA and Rob bind, respectively. These sequences are degenerate (Martin et al. 2000; Jair et al. 1996a; Li and Demple 1996; Martin et al. 1999; Wood et al. 1999). These regions are classified as Class I and Class II regulon promoters (Jair et al. 1995, 1996a, b; Fawcett and Wolf 1994; Martin et al. 1999; Wood et al. 1999). There

are two possible orientations (Class I and II promoters) which are related to RNA polymerase binding sites (Martin and Rosner 2001). In Class I promoters, the *mar*/*sox*/*rob* box is upstream of the -35 hexamer in the backward direction (except the *zwf* promoter). In Class II, the *mar*/*sox*/*rob* box overlaps the RNA polymerase binding site and is in the forward orientation (Martin et al. 1999). The alignment of several of these regulon genes shows that they contain highly conserved -35 regions (Fawcett and Wolf 1994, 1995; Li and Demple 1994, 1996).

The global transcriptional activators MarA, SoxS, and Rob can respond to divergent and unrelated compounds including antibiotics belonging to various classes such as fluoroquinolones, tetracycline, tigecycline, chloramphenicol, biocides such as triclosan, disinfectants, uncoupling agent CCCP(carbonyl cyanide n-chlorophenyl hydrozone), cyclohexane, salicylate, acetaminophen, acetylsalicylate (aspirin), sodium benzoate, plumbagin, menadion, paraquat, and dinitrophenol (Seoane and Levy 1995). Upon exposure of the bacterium to extreme conditions, bacteria show the MDR phenotype and then after returning to its normal medium the bacterium displays its original phenotype due to the repression then re-establishment of global transcriptional regulator function.

There is a mechanism called “two-gene two-stage” which is employed by these transcriptional activators (Griffith et al. 2009). At the first stage MarA, SoxS and Rob become activated through binding to their inducers or effectors. In the second stage, the induced transcriptional activators establish a complex with the RNA polymerase, then this complex scans the chromosome for their corresponding binding sites within the

promoter region of their regulon and the regulons are activated (Griffith et al. 2009). The mechanism in which transcriptional activators and inducers are bound to each other and after scanning bind to their promoter of the regulon is called pre-recruitment or DNA Scanning (Griffith et al. 2009; Griffith and Wolf 2004). When the extreme condition is relieved, the repressor stops extending the mechanism and brings it to an end.

1.7.1.1. MarA

In *E. coli*, the *mar* (multiple antibiotic resistance) locus is a 1335 bp operon consisting of two divergent transcriptional units *marC* and *marRAB*, before which are separated by operator *marO* (McDermott et al. 2008; Alekshun and Levy 2004; Goldman et al. 1996). MarA plays a key role in aggressive conditions. Bacteria protect themselves against the stressful conditions by means of stimulating around 60 different genes (Barbosa and Levy 2000; Schneiders et al. 2004). 80% of these genes are up-regulated and 20% of them down-regulated by MarA (Barbosa and Levy 2000). MarA expression is suppressed by MarR and is de-repressed by some compounds such as salicylate, benzoate, dinitrophenol, and plumbagin, likely via binding to MarA (Seoane and Levy 1995).

The binding site for MarA, the *mar*box, is located at the *marO* promoter. This suggests that MarA stimulates its autoactivation (Martin et al. 1996). *marA* expression triggers a series of events that result in exhibiting a MDR phenotype through concurrent influx and enhanced antibiotic efflux. In *E. coli*, MarA is a small protein of 15.4 KDa molecular weight containing only one DNA-binding domain having two sets of HTH motifs. The MarA protein is composed of seven α-helices.

The *micF* promoter contains a *mar*box-like consensus sequence. Binding of MarA to this *mar*box results in up-regulation of *micF*, which inhibits *ompF* translation and restricts the influx of antibiotics. Likewise, MarA is capable of upregulating the AcrAB efflux pump. The net result of *marA* system activity is decreased quinolone influx via reduced porin production and increased efflux of the quinolone through the AcrAB-TolC efflux pump (Cohen et al. 1989; Alekshun and Levy 1997).

1.7.1.1.1. SdeR, the *S. marcescens* MarA homolog

To date, only one transcriptional activator, SdeR has been found in *S. marcescens.* SdeR is composed of 134 amino acids and exhibits 40% and 50% homology with MarA of *E. coli* at DNA and amino acids level, respectively. SdeR is found upstream of the *sdeAB* locus (Kumar 2004).

1.7.1.2. SoxRS

SoxS is the smallest member of global transcriptional activators, with a molecular weight of 13 KDa. SoxS functions as the effector of *soxRS*. SoxS exhibits 50% identity with MarA (Gaudu et al. 1997). In *E. coli*, the SoxRS regulon functions as the first line of protection against the superoxide anion (O_2^-) which is a radical oxygen produced as an aerobic respiration metabolite. O_2^- can also be generated through the reactions of redox compounds such as paraquat (Demple 1996). SoxS activates a similar set of promoters as the MarA regulon promoters for *acrAB*, *mar*, *micF*, *tolC* and *zwf* (Pomposiello et al. 2001; Martin and Rosner 2002). Moreover, to provide protection against oxidative stress, stimulation of the SoxRS regulon results in resistance to diverse antibiotics,

heavy metals and organic solvents (Demple 1996; Nakajima et al. 1995; Alekshun and Levy 1997, 1999; Ariza et al. 1994).

The presence of superoxide-producer agents such as hydrogen peroxide (H_2O_2), NO (nitric oxide) and paraquat is able to induce SoxS. It this process, exposing the reduced form of SoxR to certain compounds alters its chemical form to the oxidized form and re-activates SoxS expression (Demple 1996; Lu et al. 2005) and then this alteration allows the initiation of *soxS* transcription (Gaudu et al. 1997). SoxS activates the MDR phenotype in *E. coli* and *Salmonella enterica serovar* Typhimurium and is capable of inducing *micF* and *acrAB* transcription. *mar* boxes, which are located upstream of the MarA regulons, have the same binding sites for SoxS (Martin et al. 2000). SoxS can induce the expression of MarA and both SoxS and MarA expression would lead to activation of a large number of the genes which bacteria need to protect themselves against stressful conditions (Martin and Rosner 1997; Michan et al. 2002).

1.7.1.3. Right Origin Binding protein (Rob)

In *E. coli*, Rob (right origin binding protein) was initially identified by its capability to bind to DnaA located next to the rightmost binding sites for the replication initiator protein (Skarstad et al. 1993). Nevertheless, there is no evidence showing that Rob is related to DNA replication and no signal activation enhances the Rob cellular quantity for replication. In addition, *rob*-deficient strains of *E. coli* seem to have wild type phenotype under differential growth conditions in terms of replication rate (Skarstad et al. 1993). Two years later, a second group of investigators re-identified this gene and

called it *cbpB* (curved DNA-binding protein B) (Kakeda et al. 1995).They isolated this protein during a protein screening experiment.

Although Rob belongs to AraC family and possesses high homology with the other members, it exhibits important differences. First, Rob does not need to be produced *de novo* upon exposure to inducers, owing to a constant production of 5,000-10,000 molecules per cell (Skarstad et al. 1993; Ali Azam et al. 1999). The second difference is that pre-produced Rob molecules are not active but these molecules can potentially become active upon exposure to an inducer (Martin et al. 2000). The third difference is the intracellular position of Rob. Rob molecules are sequestered into three or four inclusion bodies or specific loci within nucleoid (Azam et al. 2000). The fourth, in contrast to MarA, SoxS and TetD which contain one domain, Rob has an additional C-terminal domain (CTD) domain with approximately 180 amino acids (Kwon et al. 2000; Skarstad et al. 1993).

As mentioned, the majority of the Rob protein is sequestered in inclusion bodies or the nucleoid (Azam et al. 2000). In this state, Rob has no access to DNA (Griffith et al. 2009) but upon the introduction of an inducer or effector such as 2,2'-dipyridyl which binds to C-terminal of Rob, promoters of the target regulon become activated (Rosner et al. 2002).

Despite the abundance of Rob in the cell, the basal level of Rob activity is low (Kakeda et al. 1995; Skarstad et al.1993). Constant expression of Rob is thought to saturate the overlapped regulons with MarA and SoxS and induce the multidrug resistance phenotype but this only happens after Rob becomes activated through the

binding of the effectors or inducers such as bile salts, decanoate and 2,2'-dipyridyl to the C-terminal region of Rob (Bennik et al. 2000; Rosenberg et al. 2003; Rosner at al. 2002).

The N-terminal domain (NTD) of Rob has homology with MarA and SoxS, unlike the other AraC/XylS family members which possess homology in their CTD (Martin and Rosner 2001). Surprisingly, the expression of only the Rob NTD, which contains 123 amino acids that have high homology with CTD of SoxS and MarA, is able to confer the same antibiotic resistance as the full-length Rob (Ariza et al. 1995). *In vivo* and *in vitro* findings indicated that over-expression of Rob without the CTD domain is adequate for Rob activity and its binding to regulon promoters (Ariza et al. 1995; Jair et al. 1996a). This finding is in line with structural data obtained from the Rob-*micF* complex where only the Rob NTD contacts the DNA (Kwon et al. 2000).

The global regulators, including Rob, are able to identify the similar asymmetric degenerate binding site as *mar/sox/rob* box in the regulon promoter region and trigger their expression. However, they can differentially activate each single promoter depending on the concentration of these transcriptional activators (Li and Demple 2006), and their affinity to the operator region (Martin et al. 2000; Martin and Rosner 2001). Having an effect on similar set of the genes (regulon) by means of Rob demonstrates that Rob provides the same protection responses for the cell to the external stimuli as SoxS and MarA (Ariza et al. 1995). Meanwhile, Rob has a restricted effect on the regulons at the expression level (Bennik et al. 2000).

Some regulon genes which become induced by Rob confer resistance to divergent antimicrobials, organic solvents and heavy metals (Ariza et al. 1995; Nakajima et al. 1995). Addition of organic solvents such as n-hexane, cyclohexane, n-pentane to growth media provides a harsh condition for bacteria. Although these organic solvents can not be found in the enteric tract, organic solvents are good example of toxic compounds that bacteria need to efflux (Ramos et al. 2002). In *E. coli*, a *rob*-deficient strain shows hypersensitivity to organic solvents (Nakajima et al. 1995). Consistent to this finding, over-expression of Rob led to tolerance of *E. coli* to organic solvents and a low-level antimicrobial resistance owing to up-regulation of *acrAB* (Jair et al. 1996b). Moreover, *in vivo* investigations showed that over-production of Rob, or even only its N-terminal segment, can transcriptionally induce the expression of some regulons including *sodA* (encoding mannose-containing superoxidase dismutase), *fumC* (encoding fumarase C), *inaA* (encoding a weak acid-inducible protein) and *micF* (encoding interfering mRNA) (Ariza et al. 1995). Interestingly, *galT* (encoding galactose-1-phosphate uridyltransferase) is the only gene that is suppressed by Rob (Bennik et al. 2000). The over-expression of activators including Rob enhances antibiotic efflux (*acrAB* and *tolC*) and diminishes outer membrane permeability (*micF*), escalates superoxide-resistance proteins for susceptible proteins (*acnA* and *fumC*), increases DNA repair (*nfo*), and controls some other genes with unknown functions (e.g. *inaA*).

In *E. coli*, *acrAB* and *micF* transcription is governed through MarA, SoxS and Rob as they are regulon members of these three global activators (Gallegos et al. 1997; White et al. 1997). These regulator proteins have different quantitative affects on

particular promoters (Ariza et al. 1995; Martin et al. 2008). Induction of *rob* results in *micF* activation (Ariza et al. 1995), and consequently, the expression of *micF* results in inhibition of *ompF* expression. The result of down-regulation of *ompF* results in resistance to β-lactams (Harder et al. 1981), fluoroquinolones, tetracycline, chloramphenicol and heavy ions (Nakajima et al. 1995).

tolC and *acrAB* are other members of *mar/sox/rob* regulon (Amabile-Cuevas et al. 1991; Fralick 1996; Ma et al. 1995; Martin and Rosner 2003; Okusu et al. 1996; White et al. 2005). As already mentioned, *acrAB* and *tolC* form a tripartite RND efflux pump, AcrAB-TolC of *E. coli.* A predicted *mar/sox/rob* box has been found upstream of the *tolC*. *tolC* expression is increased upon over-expression of the MarA, SoxS and Rob (Aono et al. 1998). In *tolC*-deficient strains, metabolites which are not sufficiently pumped out remain inside the cell and are able to interact directly or indirectly with the *marRAB* promoter, SoxS and Rob proteins (Rosner and Martin 2009). In these mutant strains, enhanced transcription of the *marA* and *soxS* and increased activity of Rob were observed (Rosner and Martin 2009).

At the lowest level, local regulators or repressors like AcrR (Ma et al. 1996) and at the higher level, global regulators such as MarA, SoxS and Rob are involved in AcrAB regulation (Ma et al. 1995, 1996). *acrAB* is over-produced when *E. coli* is in medium-chain fatty acids such as decanoate (Ma et al. 1995) and unconjugated bile salts (Rosenberg et al. 2003). These compounds are in high concentration in the enteric tract. These compounds bind to Rob as effectors and then Rob mediates the rest of the process (Rosenberg et al. 2003). *In vivo* experiments demonstrated that the absence of

the *mar* and *soxS* loci does not affect Rob induction by decanoate and bile salts which consequently led to enhancement of the *acrAB* expression (Rosenberg et al. 2003).

Rosenberg et al. concluded that the whole process begins with the induction of the AcrAB efflux pump by an inducer such as bile salts which is mediated through Rob. As AcrAB has to rid the bile salts as waste molecules from inside the cell, upon the binding of the inducer to Rob CTD, some conformational changes take place in already produced Rob. These conformational changes convert inactive Rob to the activated form (Rosenberg et al. 2003). Finally, the activated Rob binds to the regulon promoter and expresses the corresponding gene.

Another study by Bennik and coworkers demonstrated that among the tested Rob regulons, *marRAB* and *micF* promoters possessed the highest affinity to bind to Rob (Bennik et al. 2000). Based on this study Bennik and associates reported that 60% of *mar* expression is Rob-dependent (Bennik et al 2000) which is consistent with the former studies that approximately 65% of *marRAB* transcription is activated via Rob (Martin and Rosner 1997).

1.7.1.3.1. Structure of *E. coli* Rob

The molecular weight of the *E. coli* Rob protein is 33.14 KDa (Skarstad et al. 1993). Rob is twice as large of SoxS and MarA. Rob possesses 289 amino acid residues bearing an N-terminal domain functioning as DNA-binder and a C-terminal domain as an effector-binder. The Rob C-terminal is similar to GalT of *E. coli* (Kwon 2000). This subfamily is capable of binding to Class I and Class II promoters. As mentioned before, in Class I promoters, the Robbox (specific binding site) is located

upstream of the -35 element and transcription activity needs α-C-terminal domain (α-CTD) RNA Polymerase (RNAP), however, the Robbox in Class II promoters overlaps with the -35 element and does not need the α-CTD RNAP (Jair et al. 1996a, b; Martin et al. 1996).

Rob exhibits the same structure of MarA, however, there are some important differences which may explain the different ways that these two regulators bind to their targets (Kwon 2000). The crystallized form of Rob demonstrated that its C-terminal is located at the top of the N-terminal DNA binding domain, similar to GalT, and does not make any contact with DNA, indicating that the C-terminal is able to bind to small inducers or effectors (Kwon 2000; Bennik et al. 2000). The crystallization of the Rob in complex with the *micF* binding site revealed that two C-terminal HTH motif of NTD have only one contact with DNA (Kwon et al. 2000). Rob only employs one of its recognition helices into the DNA major groove, whereas two helices are placed into the DNA major groove in MarA context (Kwon et al. 2000; Rhee et al. 1998).

1.7.1.4. Other global regulators

H-NS (histone-like nucleoid structuring protein) governs approximately 5% of all E*. coli* genes. The majority of these genes are employed in virulence or adaptation of the bacterium to external environments (Pomposiello et al. 2001; Stancik et al. 2002; Stoorvogel et al. 1991). In *E. coli* and *Enterobacter aerogenes*, this protein controls the expression of porins and some MDR efflux pumps in various osmotic conditions (Stoorvogel et al. 1991; Suzuki et al. 1996; Thiolas et al. 2005; Viveiros et al. 2007).

Sdi (Suppressor of division inhibition) is the *E. coli* LuxR homologue which is regulated in a quorum-sensing-dependant manner (Sitnikov et al. 1996). Sdi positively regulates the expression of the AcrAB efflux pump in *E. coli*. Sdi can confer resistance to quinolones (Rahmati et al. 2002)

RamA is a global regulator belonging to AraC/XylS family in *E. coli*. It exhibits 45% identity with MarA. The over-expression of *ramA* results in an elevated MIC to various antibiotics such as chloramphenicol, tetracycline, tigecycline, fluoroquinolones, trimethoprim and down-regulation of OmpF and up-regulation of AcrAB (Schneiders et al. 2003; Keeney et al. 2007).

1.7.2. Negative regulators

1.7.2.1. OmpX

OmpX is an example of a suppressor of porin production. OmpX is a small, 18 KDa outer membrane protein (Barbosa and Levy 2000; Arnold et al. 2007; Atlung and Ingmer 1997). OmpX over-expression is related to a decline in Omp36, the main *Enterobacter aerogenes* porin, and an increased resistance to β-lactams (Bertin et al. 1994).

1.7.2.2. Small Non-coding RNAs (sRNAs)

To date, eight sRNA have been identified in *E. coli: invR, micA, micC, micF, omrAB, rseX* and *rybB* (Guillier et al. 2006). Small, non-translated regulatory RNAs (SRNA) are recognized as non-coding RNAs which exist in all kingdoms of life (Argaman et al. 2001). *micF* was the first sRNA recognized to be involved in porin regulation (Andersen et al. 1987; Mizuno et al. 1984). *micF* is capable of suppressing

OmpF expression. MicF is composed of 93 nucleotides of which 20-base pairs bind imperfectly with the translation-initiation region of the *ompF* mRNA (Schmidt et al. 1995) resulting in repression of *ompF* expression. Transcriptional regulators including OmpR, LrP, SoxS, Rob, MarA, and H-NS all bind to the promoter region of *micF* (Delihas and Forst 2001). This binding leads to over-expression of *micF.* Consequently, this event leads to base-pairing of *micF* with *ompF* mRNA to stop translation initiation. The net result is a reduction in OmpF production (Guillier at al. 2006).

In *E. coli* and *S. marcescens*, *micF* transcription is increased by high temperature, presence of salicylate and acidic pH (Ramani and Boakye 2001; Begic and Worobec 2006; Nikaido 2003). Unlike the *E. coli*, *micF* transcription remains constant in *S. marcescens* in different osmotic concentrations (Nikaido 2003; Begic and Worobec 2006).

In *E. coli, micC* is also involved in porin regulation. MicC consists of 109 nucleotides which are encoded in an intergenic region between *ompN* and *ydbK* (Chen et al. 2004). Expression of *ompC* is negatively regulated by MicC. *micC* base-pairs with *ompC* mRNA and does not allow *ompC* to initiate translation. Therefore, *micC* controls *ompC* expression at the post-transcriptional level (Chen et al. 2004).

1.7.3. Efflux pump repressors

These are local regulators which adjust efflux pump expression. For instance, in *E. coli, acrAB* transcription is divergently suppressed via *acrR,* a TetR-type repressor containing a HTH DNA-binding domain (Kumar and Schweizer 2005).

1.7.4. Two-Component Systems (TCS)

Some efflux pumps are controlled by TCS, such as BarSR for MdtABC pump of *E. coli* (Baranova and Nikaido 2002; Nagakubo et al. 2002), EvgAS for YhiUV pump of *E. coli* (Nishino and Yamaguchi 2002), and PhoPQ which regulates *E. coli tolC* expression (Eguchi et al. 2003).

In *E. coli*, one of the main porin regulators involving two-component systems is encoded by the *ompB* operon (Hall and Silhavy 1981), and is composed of a sensor kinase (EnvZ) and a response regulator (OmpR). EnvZ is an inner membrane osmosensor histidine kinase which senses environmental signals and then becomes phosphorylated by ATP. OmpR is a cytoplasmic response regulator and transcriptional factor of EnvZ. OmpR catalyzes the phosphotransfer of the phosphoryl group from EnvZ to OmpR (Robinson et al. 2000; Stock et al. 2000). OmpR regulates *ompF* and *ompC* expression and other cellular mechanisms including chemotaxis and pathogenicity (Brzostek et al. 2007; Chatfield et al. 1991; Garmendia et al. 2003; Lee et al. 2000; Park and Frost 2006; Shin and Park 1995; Slauch and Silhavy 1989).

Comparison of the upstream sequences of *ompF* of *E. coli* and *S. marcescens* revealed that the intergenic region in *S. marcescens* is 300 bp shorter than the comparable region of *E. coli* where EnvZ/OmpR binds to repress the *ompF* transcription. Therefore, osmoregulation mechanism through OmpR/EnvZ is not a likely mechanism in *S. marcescens* (Hutsul and Worobec 1997). Therefore, it seems that the other regulators such as Rob may have an important role in OmpF and OmpC regulation.

1.8. Hypothesis and Objectives

Hypothesis: *Serratia marcescens* produces a *rob*-like transcriptional activator that contributes to antibiotic resistance by controlling the expression of the SdeAB-TolC efflux pump and OmpF and OmpC porins.

Objectives:

1- Identify and clone a *rob*-like gene from *S. marcescens*.

2- Evaluate the *rob* gene promoter function by transcriptional fusion using a GFP reporter system.

3- Create a *rob* gene knock-out strain of *S. marcescens.*

4- Compare Minimum Inhibitory Concentration (MIC) for diverse antibiotics for wild type and knock-out strains of *S. marcescens.*

5- Study the effect of the *rob* knock-out on efflux pump expression.

6- Study the effect of the *rob* knock-out on outer membrane protein expression.

CHAPTER 2: Materials and Methods

2.1. Bacterial strains, plasmids, and growth conditions

All bacterial strains and plasmids used in this study are listed in Tables 2.1 and 2.2, respectively. UOC-67 was grown in Luria-Bertani (LB) medium (BD, Maryland, USA) at 37°C. Freezer stocks were prepared by the addition of 0.5 ml glycerol solution (65% v/v glycerol, 0.1 ml $MgSo_4$ and 25 mM Tris-Cl, pH 8) to 0.5 ml of an overnight culture and then stored at -60°C (Ausubel et al. 2002). Ampicillin (100 µg/ml), kanamycin (50 µg/ml), carbenicillin (25 µg/ml), chloramphenicol (25 µg/ml), and spectinomycin (100 µg/ml) were included in the growth media of strains or strains harboring plasmids containing Amp^r, Km^r, Cb^r, Cm^r, and Sp^r, respectively.

2.2. Molecular biological procedures

All molecular techniques including plasmid and genomic DNA isolation, restriction enzyme digestion, gel electrophoresis, DNA ligation, and PCR cloning were carried out by previously described standard protocols (Ausubel et al. 2002; Sambrook and Russel 2001).

2.2.1 Rapid plasmid preparation

For rapid plasmid preparation, the method described by Kado and Liu (1981) was carried out to screen transformants. Briefly, bacterial colonies were collected with a sterile toothpick and resuspended thoroughly in an Eppendorf tube containing 40 µl of STE buffer (20 mM Tris-HCl, pH 7.5, 10 mM EDTA, 100 mM NaCl). Consequently, 20 µl of Phenol: Chloroform: Isoamyl alcohol (25:24:1) were added and vortexed for 1 minute. The top layer was collected and centrifuged at 20,000 xg in a Thermo IEC Micromax

Table 2.1. Bacterial strains used in present study

Strain	Characterization	Source/Reference
S. marcescens UOC-67	Wild-type *S. marcescens* ATCC 13880	American Type Culture Collection
S. marcescens SMRD1	Wild-type *S. marcescens* UOC-67 *rob*1 deficient *(rob::kan*), Km^r	This study
S. marcescens SMRD2	Wild-type *S. marcescens* UOC-67 *rob2* deficient *(rob::kan*), Km^r	This study
S. marcescens SMRD3	Wild-type *S. marcescens* UOC-67 *rob*3 deficient *(rob::kan*), Km^r	This study
E. coli RA4468	GC4468 (wild type *E. coli* K-12) *rob* deficient (*robA*::*kan*), Km^r	Ariza et al. 1995
E. coli GC4468	Wild-type *E. coli* K-12, F^- Δ (*argF-lac*) *U169 rpsL*	Carlioz and Touati 1986

Km^r: Kanamycin resistant; Str^r: Streptomycin resistant

Table 2.2. Plasmids used in present study

Plasmid	Characterization	Source/Reference
pUS19	a high copy number plasmid, pUC19 derivative containing Sp^r	Benson and Haldenwang 1993
pGlow-TOPO	cycle 3 GFP, Promoterless vector for transcriptional fusion, linearized vector containing a TOPO cloning site with 3' thymidine (T) overhang, Sp^r	Invitrogen
pACD4K-C	expression vector pACD4K-C containing Cm^r for plasmid propagation and Km^r upon chromosomal insertion	Sigma-Aldrich
pAR1219	pBR322-based vector; expresses T7 RNA Polymerase under control of the IPTG inducible lac UV5 promoter, Cb^r, Amp^r	Sigma-Aldrich
pUSROB1	pUS19 carrying *rob*1 of *S. marcescens*, 980 bp *Hind*III fragment	This study
pUSROB2	pUS19 carrying *rob*2 of *S. marcescens,* 947 bp *Hind*III fragment	This study
pUSROB3	pUS19 carrying *rob*3 of *S. marcescens*, 923 bp *Hind*III fragment	This study
*rob*1	*S. marcescens rob*1 promoter region in pGlow-TOPO, 429 bp fragment	This study
*rob*2	*S. marcescens rob2* promoter region in pGlow-TOPO, 446 bp fragment	This study
*rob*3	*S. marcescens rob3* promoter region in pGlow-TOPO, 449 bp fragment	This study

Sp^r: Spectinomycin resistant, Cb^r: Carbenicillin resistant, Amp^r: Ampicillin resistant, Km^r: Kanamycin resistant

microcentrifuge (Needham Heights, MA, USA). The supernant was collected and was transferred to a new tube containing 1 µl of RNase (1 mg/ml). Samples were loaded on a 0.8% (w/v) agarose gel and the migration rate compared with plasmid without the insert.

2.2.2 PCR, plasmid purification, cloning and DNA sequencing

PCR primers used in this study are listed in Table 2.3. All primers were synthesized by Gibco (Gibco, Grand Island, USA). Thermal cycler Techne Model TC-312 (Burlington, NJ, USA) was used for all PCR amplifications. QIAquick ® PCR Purification Kit and QIAGEN ®Plasmid Midi Kit (Qiagen, Maryland, USA) were used to purify PCR products and prepare recombinant plasmids and regular plasmids without insert, respectively.

To verify the correct orientation and sequence of the PCR fragments in recombinant plasmids, DNA sequencing was performed by National Research Council/Plant Biotechnology Institute at Saskatoon, Saskatchewan, Canada using appropriate primers (Table 2.3.).

2.2.3. Transformation

To prepare the highly efficient electrocompetent and chemically competent cells standard protocols were followed (Ausubel et al. 2002). For electroporation, Eppendorf Electroporator Model 2510 was used (Eppendorf-Netheler-Hinz, Hamburg, Germany).

Table 2.3. Primers used in present study

Name	Sequence (5'→3')	Target
T7 promoter GFP Reverse	TAATACGACTCACTATAGGG GGGTAAGCTTTCCGTATGTAGC	sequencing primers for putative *rob* promoters in pGlow-TOPO
SP1-F SP1-R	AAAA**gacgtc**TTTCGTTCGTGAATACATGT AAAA**gacgtc**CAAGGGTTTATTGTTTTCTA	1.1 kb region of Spectinomycin cassette in pUS19, *Aat* II restriction site underlined
SP2-F SP2-R	GCCACCTGACGTCTTTCGTT ATTAGATCTCCCGATCCGTC	Spectinomycin cassette sequencing primers in pGlow-TOPO, 1.1 kb fragment
PRob1F PRob1R	CGAGGAAACTGATTATGTCA C**CAT**ATAATAACG**TCCT**CTGTTCAATCACCTGTTGCT	429 bp promoter region of *rob*1, ribosomal binding site (RBS) and initiation codon are underlined
PRob2F PRob2R	GTGATACCGGCCGGCCTGAACTTC C**CAT**ATAATAACG**TCCT**GATGTATCTCCACCCACTC	446 bp promoter region of *rob*2, ribosomal binding site (RBS) and initiation codon are underlined
PRob3F PRob3R	C**CAT**ATAATAACG**TCCT**CTTTCCAGCCAGCTAAGCAG CTGCTTAGCTGGCTGGAAAG	449 bp promoter region of *rob*3, ribosomal binding site (RBS) and initiation codon are underlined
PRobEF PRobER	C**CAT**ATAATAACG**TCCT**TAAAAGGTCGCGAATAATGC GCTCACATAACAGGTGACAT	417 bp promoter region of *E. coli rob,* ribosomal binding site (RBS) and initiation codon are underlined
Rob1F Rob1R	AAAA**aagctt**CTGAGCAAATAAGGGAATA AAAA**aagctt**AGTGTGAACCATTACCGATG	980 bp PCR fragment of *rob*1 in pUS19 for over-expression, engineered *Hind* III site underlined
Rob2F Rob2R	AAAA**aagctt**GTAGGTTACTATCGCGAACA AAAA**aagctt**GGTGCCGTTTAATCGATAGA	947 PCR fragment of *rob*2 in pUS19 for over-expression, engineered *Hind* III site underlined
Rob3F Rob3R	AAAA**aagctt**TGCCCGCGTTGGACGAAC AAAA**aagctt**CTACCCCCAAGCTTTACGAG	923 bp PCR fragment of *rob*3 in pUS19 for over-expression, engineered *Hind* III site underlined

M13F M13R	GTAAAACGACGGCCAGT CAGGAAACAGCTATGAC	M13 primers for sequencing of *rob* over-expression in pUS19, ~1 kb fragment
EBS universal IBS(1) EBS1d(1) EBS2(1)	CGAAATTAGAAACTTGCGTTCAGTAAAC AAAAAAGCTTATAATTATCCTTACTGACCCTGGACGTG CGCCCAGATAGGGTG CAGATTGTACAAATGTGGTGATAACAGATAAGTCCTG GACGATAACTTACCTTTCTTTGT TGAACGCAAGTTTCTAATTTCGGTTGTCAGTCGATAGA GGAAAGTGTCT	knock out primers for the *rob*1 re-target introns from Intron PCR template, 350bp fragment with engineered *Hind* III and *BsrG*I sites.
EBS Universal IBS(2) EBS1d(2) EBS2(2)	CGAAATTAGAAACTTGCGTTCAGTAAAC AAAAAAGCTTATAATTATCCTTACCAAACCT GGATGTGCGCCCAGATAGGGTGCAGATTG TACAAATGTGGTGATAACAGATAAGTCCTGGATGATAA CTTACCTTTCTTTGT TGAACGCAAGTTTCTAATTTCGGTTTTTGGTCGATAGA GGAAAGTGTCT	knock out primers for the *rob*2 re-target introns from Intron PCR template, 350 bp fragment with engineered *Hind* III and *BsrG*I sites.
EBS universal IBS(3) EBS1d(3) EBS2(3)	CGAAATTAGAAACTTGCGTTCAGTAAAC AAAAAAGCTTATAATTATCCTTACCATTCGGACCAGTG CGCCCAGATAGGGTG CAGATTGTACAAATGTGGTGATAACAGATAAGTCGGA CCAACTAACTTACCTTTCTTTGT TGAACGCAAGTTTCTAATTTCGGTTAATGGTCGATAGA GGAAAGTGTCT	knock out primers for the *rob*3 re-target introns from Intron PCR template, 350 bp fragment with engineered *Hind* III and *BsrG*I sites.
FompF RompF	AGCAAACGCAGCTGAAATCT CTGATCGCCGTCATTACCTT	RT-PCR primers for *S. marcescens ompF* amplification from genomic DNA, 135 bp fragment size
FmicF RmicF	GGCCAGAAGCCGGTTAAAG TGCACGCAGCCGCTATAAT	RT-PCR primers for *S. marcescens micF* amplification from genomic DNA, 100 bp fragment size

FompC RompC	AGTTCATGTTCCAGCGTTCC TTGGTCTCTTCGCCATTACC	RT-PCR primers for *S. marcescens ompC* amplification from genomic DNA, 124 bp fragment size
FsdeAB RsdeAB	ATCCAGTGGACCGATCTGAG CAGCGTCCAGCTTTCATACA	RT-PCR primers for *S. marcescens sdeAB* amplification from genomic DNA, 123 bp fragment size
FhasF RhasF	GATTACACCAACGGTTATCGTG GTCTTTTCCTGCAGCGTCAGT	RT-PCR primers for *S. marcescens hasF* amplification from genomic DNA, 125 bp fragment size
FsdeY RsdeY	TCCATCAACGAAGTGGTGAA AGTATCGCAAAGGTCCCCAG	RT-PCR primers for *S. marcescens sdeXY* amplification from genomic DNA, 135 bp fragment size
FrplU RrplU	GCTTGGAAAAGCTGGACATC TACGGTGGTGTTTACGACGA	RT-PCR primers for *S. marcescens rplU* housekeeping gene amplification from genomic DNA, 190 bp fragment size

2.3. Computer analysis of DNA and amino acid sequences

The *E. coli rob* sequence was extracted from GENBANK (Accession No.: M97495) and used to screen the *S. marcescens* genome for homology at DNA and amino acid level. (Sanger *Serratia marcescens* Blast Server: http://www.sanger.ac.uk/cgi-bin/ blast/ submitblast/s_marcescens. To find all ORFs (Open Reading Frames), these sequences were entered in the ORF FINDER program: http: //www.ncbi.nlm.nih.gov/ projects/gorf/. The deduced amino acid sequence for *rob* of *S. marcescens* was obtained from Expasy Proteomic Server-Translate Tool: http://www.expasy.ch/tools/dna.html. To compare two *rob* sequences, BLAST2 program was used: http://blast.ncbi.nlm.nih.gov/Blast.cgi. To align *rob* sequences from different bacteria, PREMIUM PRALINE program was used: http://www.ibi.vu.nl/programs/pralinewww/. All used program was used in their default settings.

2.4. Promoter evaluation

To assess promoter function, transcriptional fusions were performed using a Green Fluorescence Protein (GFP) in the vector pGlow-TOPO as the reporter gene. pGlow-TOPO (Invitrogen, Carlsbad, USA), contains a promoterless GFP ORF for transcriptional fusion. GFP was chosen due to its unique advantages, such as small size, low toxicity, autofluorescence (no substrate needed), no requirement for cofactors, and protein stability (Chalfie et al. 1994; Prasher et al. 1992; Tsien 1998). As *S. marcescens* is intrinsically resistant to ampicillin and pGlow-TOPO contains only an ampicillin resistance cassette, the spectionmycin cassette from pUS19 was amplified by

PCR using SP1-F and SP1-R primers engineered with *Aat* II at the both ends (Table 2.3) (Benson and Haldenwang 1993), and then ligated into digested pGlow-TOPO with the same restriction enzyme.

The promoter region sequences for putative *rob* genes, *rob*1, *rob*2, and *rob*3, were extracted from upstream of each start codon through the FTP database of Sanger *Serratia marcescens*: ftp://ftp.sanger.ac.uk/pub/pathogens/sm/. The promoter region sequence for *E. coli rob* was extracted from upstream of start codon through Ecogene website: http://ecogene.org/dnaSequence.php.

The promoter regions of each gene were amplified by PCR using the primers shown in Table 2.3., purified by QIAquick ® PCR Purification kit (Qiagen, Maryland, USA), and ligated into pGlow-TOPO. The resulting fusion for putative *rob* genes and *E. coli rob* were transformed into *S. marcescens* UOC-67 and *E. coli* K-12 (GC4468), respectively, and screened on LB plates containing spectinomycin (100 µg/ml). PCR screening was performed on recombinant colonies. To confirm the correct orientation and sequence of the inserts, DNA sequencing was carried out by the National Research Council/Plant Biotechnology Institute at Saskatoon, Saskatchewan, Canada using the primers in listed Table 2.3.

Bacterial strains harboring GFP plasmids were grown in LB medium containing spectinomycin (100 µg/ml), as required, overnight at 37 °C. Two µl of overnight culture was added to 148 µl LB fresh medium containing spectinomycin (100 µg/ml) in the wells of flat-bottomed sterile 96-well microplates (BD Falcon, Franklin Lakes, USA). Incubation was carried out in a Biotek Synergy2 Multi-Mode Microplate Reader

(Winooski, Vermont, USA) set at 30°C and 37°C with continuous shaking (medium speed) and assayed every 30 minutes with an automatically repeating protocol for fluorescence excitation at 395 nm and emission at 505 nm and OD measurement at 600 nm to monitor turbidity. Measurements of the triplicate wells of each experiment were averaged.

The normalized fluorescence for each experiment was calculated by subtracting the fluorescence of the control strain (containing the pGlow-TOPO plasmid without insert) grown under the same conditions, then dividing by the absorbance (OD) at the same time point.

2.4.1. Induction of *rob* promoters by 2, 2'-dipyridyl

Induction of *rob*1*,* *rob*2 and *rob*3 promoters cloned in pGlow-TOPO by 2,2'-dipyridyl was performed according to a previously described method (Rosner et al. 2002). Briefly, different stages of growth were determined based on the growth curve. When turbidity reached log-phase (OD_{600}= 0.2), mid-log phase (OD_{600}= 0.6), and stationary phase (OD_{600}=1.26), 75 µl of culture was diluted with the same amount of pre-warmed 10 mM 2,2'-dipyridyl in LB and/or LB spectinomycin (if needed) and incubated for 1 hour at 37°C with shaking at 200 rpm. The normalized fluorescence for each experiment was calculated by subtracting the fluorescence of the control strain (containing the pGlow-TOPO plasmid without insert) grown under the same conditions, then dividing by the absorbance (OD) at the same time point. All experiments were performed at least three times repeates.

2.4.2. Induction of *rob* promoters by sodium decanoate and salicylate at 30 °C and 37 °C

To study the effect of sodium salicylate and sodium decanoate on the induction of *rob* promoters, transcriptional fusion of the promoters for *rob*1, *rob*2 and *rob*3 in pGlow-TOPO were tested according to a previously described method (Hartog et al. 2008). Briefly, 3 ml of an overnight LB and/or LB spectinomycin (100 µl/ml) culture, was diluted 1:100 in a fresh LB broth and/or LB spectinomycin (100 µl/ml). 150 µl of the diluted cultures were dispensed into flat-bottomed sterile 96-well microplates (BD Falcon, Franklin Lakes, USA) and sodium salicylate and sodium decanoate (prepared in were added separately to a final concentration of 5 mM. Incubation was carried out in a Biotek Synergy2 Multi-Mode Microplate Reader (Winooski, Vermont, USA) set at 30°C and 37°C with continuous shaking (medium speed) and assayed every 30 minutes as described above. Measurements of the triplicate wells of each experiment were averaged.

The normalized fluorescence of each culture was calculated by subtracting the fluorescence of the control strain (containing the pGlow-TOPO plasmid without insert) grown under the same conditions, then dividing by the absorbance (OD) at the same time point.

2.5. Construction of *rob* knock-out mutant strains

To construct *S. marcescens* strains deficient for each *rob* candidate (*rob*1, *rob*2, *rob*3), the TargeTron Gene Knockout System was used following manufacturer instructions (Sigma-Aldrich, St. Louis, USA). The TargeTron Gene Knockout System

provides reagents and protocols for the rapid and site-specific disruption of bacterial genes by insertion of group II introns (Karberg et al. 2001). Briefly, target sites of *rob* candidates were determined using TargeTron Design website: http:// www. sigma-genosys.com/targetron/. Three primers were produced: IBS, EBS1d and EBS2 (Table 2.3). The EBS universal primer was already included in the kit. Using these primers, PCR was performed following manufacturer's cycling program to mutate (re-target) intron RNA from the provided template to obtain a 350 bp PCR fragment. This fragment was digested with *BsrG* I and *Hin*d III and then ligated into the linearized intron expression vector pACD4K-C containing the remaining intron components and chloramphenicol resistance for general selection. The recombinant vector was then transformed into *S. marcescens* UOC-67 to express the RNA-protein complex (RNP). RNP retargets insertion into the targeted gene on the bacterial chromosome.
S. marcescens does not have a source of T7 RNA Polymerase, hence pA1219 (Sigma-Aldrich, St. Louis, USA) was co-transformed into *S. marcescens* UOC-67. pAR1219 contains a carbenicillin resistance marker which allows for selection on LB agar containing carbenicillin (25 µg/ml). Upon integration of the of group II intron into the bacterial chromosome, the kanamycin marker becomes activated. The kanamycin marker is within the group II intron which is interrupted by the *td* group I intron. As group II introns are transcribed and spliced, the *td* group I intron is removed from the group II intron and the kanamycin marker is activated. Then the resulting *rob* mutant strains are able to grow on LB kanamycin (25µg/ml).

PCR primers used for gene disruption and confirming intron insertion (kanamycin

marker) are listed in the Table 2.3. Recombinant plasmids were grown on LB kanamycin plates and screened using colony PCR (Lu 2003). The presence of the kanamycin cassettes and intron insertion in the correct orientation was confirmed by National Research Council (Saskatoon, Saskatchewan) via DNA sequencing.

2.6. Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentrations (MICs) were performed using the macrodilution method according to the Clinical and Laboratory Standards Institute (CLSI M7-A7 2007). The final twofold dilutions of antibiotics were prepared in Muller-Hinton Broth (MHB). When the MIC tube was ambiguous, additional dilutions were prepared to obtain the definite turbidity or button at the bottom of tube. Cation-adjusted Muller Hinton Broth (CAMHB) was used for performing MIC for Tetracycline. To prepare the bacterial suspension, 3-5 colonies from each strain were picked and inoculated in 4 ml T-Soy broth (TSB) containing antibiotic (if needed) and incubated for 2-3 hours at 37ºC. Then the turbidity was compared with a 0.5 McFarland standard. To make sure all strains had the same turbidity, samples were adjusted to $OD_{625=}$ 0.08-0.13, equal to the 0.5 McFarland. These cultures were then diluted to 1:150 in fresh TSB containing the proper antibiotic if needed for those tested strains harboring recombinant plasmids, and 1 ml was added to all tubes. All tubes were incubated for 16-20 hours at 37°C without shaking.

MIC is defined as the minimal concentration of an antimicrobial agent which is able to inhibit visible bacterial growth in the tube (CLSI M7-A7 2007). Definite turbidity or ≥2 mm button at the bottom of the tubes were considered as bacterial growth. Antibiotics

tested included carbenicillin, chloramphenicol, gentamycin, cefoperazone, cefuroxime, novobiocin, cephalothin, streptomycin, cefotaxime, norfloxacin, ofloxacin, tetracycline, amikacin, nalidixic acid and ciprofloxacin (Sigma- Aldrich, St. Louis, USA).

2.7. Ethidium Bromide (EtBr) accumulation assay

Accumulation and efflux of ethidium bromide was carried out using a modified method (Neyfakh et al. 1991). Briefly, an overnight culture in TSB and appropriate antibiotic (if needed) was pelleted by centrifugation at 20,000 xg for 3 min, resuspended in 50 mM sodium phosphate buffer, pH 7, and adjusted to obtain OD_{600} =0.2. 150 µl of each culture was dispensed into flat-bottomed sterile 96-well microplates (BD Falcon, Franklin Lakes, USA). Ethidium Bromide (prepared in ddH_2O) was added to each well to obtain a final concentration of 2 µg/ml. Fluorescence intensities were recorded every 30 sec for 10 min at 530 nm as excitation and 600 nm as emission wavelength using Biotek Synergy2 Multi-Mode Microplate Reader (Winooski, Vermont, USA) set at 37°C with continuous shaking. After the first 5 minutes, CCCP (Cabonyl cyanide m-chlorophenyl hydrozone), a proton gradient uncoupler, which already prepared in DMSO (Dimethyl sulfoxide) was added to reach a final concentration of 100µM.

2.8. n-hexane tolerance test

Tolerance or resistance to organic solvents such as n-hexane is considered an efflux pump indicator. This experiment was carried out following a method previously described (White et al. 1997). Briefly, an overnight culture was inoculated in 3 ml TSB and appropriate antibiotic (if needed) with shaking at 37 °C. After 2 hrs, turbidity was checked and compared with a 0.5 McFarland standard. To make sure all strains have

the same turbidity, samples were adjusted with TSB to $OD_{625=}$ 0.08-0.13, equal to the standard turbidity (0.5 McFarland). Then 5 µl of the tested organism was spotted on LB agar (using 100 mm glass Petri plates) containing appropriate antibiotic (if needed) and allowed to dry for 20 min at 37ºC. The 100 mm glass plate was then flooded with approximately 20 ml of n-hexane, sealed with parafilm and incubated at 37°C for 24 hours. Any sign of growth was considered resistance to n-hexane.

2. 9. RNA extraction

Overnight LB cultures were diluted 1:100 in fresh LB broth and/or LB broth containing kanamycin (25 µg/ml). Cultures were incubated at 37ºC until mid-log phase (OD_{600}=0.6). Subsequently, the total RNA was extracted using the RNeasy Protect Bacteria Mini Kit (Qiagen, Maryland, USA). To further purify RNA and remove small amounts of DNA, an additional step was carried out using the RNase-free DNase set (Qiagen, Maryland, USA).

2.10. Real-time PCR

One-step reverse transcription and real-time quantitative PCR (qRT-PCR) was performed on RNA extracted from all test strains using the EXPRESS One-Step SYBR GreenER Universal Kit (Invitrogen, Carlsbad, USA). All experiments were performed on the SmartCycler System (Cepheid, Sunnyvale, USA) equipped with SmartCycler DX 3.0 Software.

To calculate any change in the gene expression, the cycle threshold (Ct) for housekeeping gene *rplU* was subtracted from the cycle threshold (Ct) of targeted genes. The resulting value, ΔCt, was calculated for the calibrator strain (UOC-67).

Subtracting ΔCt for the calibrator strain from the ΔCt for the tested strain resulted in the ΔΔCt. The formula ($2^{-\Delta\Delta Ct}$) was then used to calculate the fold change in gene expression relative to the calibrator strain (Livak and Schmittgen 2001).

CHAPTER 3: Results and Discussion

In *E. coli*, transcriptional activators, *mar*, *soxS* and *rob,* regulate a common set of the genes called *mar*/*sox*/*rob* regulons (Barbosa and Levy 2000; Bennik et al. 2000; Pomposiello et al. 2001), including *micF*, *tolC* and *acrAB*, which are important with regards to bacterial resistance. Like Mar and Sox, Rob controls the expression of the AcrAB-TolC efflux pump in *E. coli* and *micF,* the *ompF* regulator (Jair et al. 1996b; Ariza et al. 1995). Over-expression of *micF*, *acrAB* and *tolC*, under extreme conditions, results in resistance to multiple antibiotics, organic solvents and heavy metals (Ariza et al. 1995; Nakajima et al. 1995). We proposed that a similar process occurs for *S. marcescens*.

In *S. marcescens*, while no homolog has been identified for SoxS, another transcriptional activator, SdeR, has been reported to be a MarA homolog (Kumar 2005). *sdeR* is located upstream of *sdeAB*, which encodes the SdeAB efflux pump (Kumar 2005: Maseda et al. 2009). SdeR has 40% identity at amino acid level with MarA of *E. coli*, a transcriptional activator for the efflux pump AcrAB-TolC (Alekshun and Levy 1997; Hachler et al. 1991; Kumar 2005). MarA and SdeR were shown to have similar predicted three-dimensional structures and conserved DNA-binding domains (Kumar 2005). Our results suggest that Rob is also a regulator of *sdeAB* and *sdeXY*. As both SdeR and Rob are considered transcriptional activators, we expected to see similar results, to some extent, for their knock-out strains. *sdeR* null mutant strains showed an increased susceptibility to multiple antibiotics including fluoroquinolones and chloramphenicol (Begic and Worobec 2008). These are known substrates for efflux

pump SdeAB (Kumar and Worobec 2005). Similarly, we found the same sensitivity to these antibiotics in *rob* null mutant strains (SMRD3). Complementation of *sdeR* deficient mutant strains returned the cell to similar level of resistance as wild-type *S. marcescens* UOC-67 (Begic and Worobec 2008). Similarly, we found the same to be true for *rob* deficient mutant strains in this present study. The steps taken to characterize the role of Rob as a transcriptional activator and the importance of Rob in the antibiotic resistance of *S. marcescens* are outlined in this chapter.

3.1. Computer analysis of DNA and amino acid sequences

Identifying a Rob-like transcriptional regulator in *S. marcescens* and its role in over-expression of RND efflux pumps, SdeAB and SdeXY, will help in understanding of how antibiotic resistance is regulated in this medically relevant bacterium. A variety of software programs were used to find an *E. coli rob* homolog in *S. marcescens*. Three ORFs were found to have identity with *E. coli rob* using the Sanger *Serratia marcescens* genome Blast Server and ORF Finder. These ORFs, *rob*1, *rob*2 and *rob*3, were found to be 876, 894 and 870 bp in size, respectively. The translated amino acid sequences were obtained using Expasy Proteomic Server-Translate Tool, with Rob1, Rob2, and Rob3 having 291, 297, and 289 amino acids, respectively (See Appendix). All three sequences contained 2HTH (Helix-Turn-Helix) motifs at their C or N-terminal, with Rob1 and Rob3 having the HTH motif at their N-terminal domains and Rob2 at the C-terminal end (Fig. 3.1).

Rob1 had a homologous region to the AraC E-binding site superfamily at its C-terminal, indicating high similarity with the structure of the Rob of *E. coli* (Fig.3.1A). *rob*1

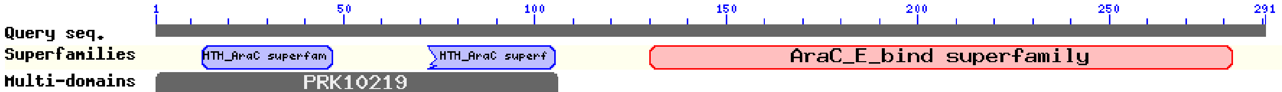
has 59% and 47% identity at DNA and amino acids level with that of *E. coli*, respectively (Fig. 3.2A). Rob2 had more similarity to *E. coli* SoxS than Rob in the context of having 2 HTH domains at its C-terminal and no conservative N-terminal domain (Fig. 3.2D); however, the length of *rob2* is as twice long as that of the *E. coli soxS*. *rob*2 showed 62% and 33% identity at DNA and amino acids level with *E. coli rob*, respectively (Fig. 3.2B). *rob*3 had highest similarity with *E. coli rob*, with 70% and 71% identities at DNA and amino acid level with that of *E. coli*, respectively (Fig. 3.3C). Alignment of *S. marcescens rob*3 with the other previously known *rob* genes from members of the *Enterobacteriaceae* family was performed using PRALINE multiple sequence alignment. The alignment showed high similarity between *S. marcescens rob*3 and the other sequences (Fig. 3.3). No significant identity was found at promoter regions of *S.marcescens rob*3 and *E. coli rob* promoter.

BLAST analysis of genes upstream and downstream of *E. coli rob* was performed using the BLAST2 program (Table 3.1). The Ecogene website was used to obtain the gene map of *E. coli* and Sanger *Serratia marcescens* genome Blast Server was used to locate the position of each gene in *S. marcescens* genome. This analysis revealed some similarities at the amino acid levels on upstream and down stream of *E. coli rob* in comparison to that of *S. marcescens rob*3 including *creA* (84%), *arcA* (93%), *vjjY* (48%), *vitD* (64%) and *thrA* (27%) at upstream of *E. coli rob* and *vtiC* (77%), *vjjX* (54%), *trpR* (62%), *slt* (63%), *vjjK* (91%), *nadR* (83%), *radA* (89%) and *serB* (74%) at the down stream of *E. coli rob*. No significant identity was found for *creB*, *creC* and *creD*.

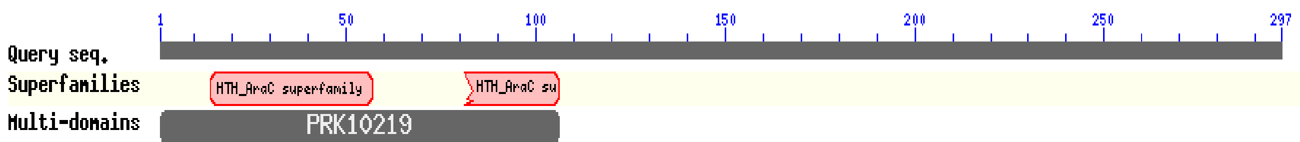
Computer analysis suggested that the *rob*1 and *rob*3 were structurally more

Fig. 3.1. Analysis of *S. marcescens* Rob domains. A) Rob1 domains from BLAST showing the N-terminal (PRK 10219) a DNA-binding domain containing two helix-turn-helix (HTH) domains and the C-terminal domain (AraC-E-bind superfamily), a ligand-binding domain, similar to those of *E. coli* Rob. B) Rob2 domains domains from BLAST showing the N-terminal (PRK 10219) a DNA-binding domain containing two helix-turn-helix (HTH) domains similar to that of *E. coli* but unlike *E. coli* Rob, no conserved N-terminal domain is observed. C) Rob3 domains from BLAST showing the N-terminal (PRK 10219) a DNA-binding domain containing two helix-turn-helix (HTH) domains and the C-terminal domain (AraC-E-bind superfamily), a ligand-binding domain, similar to those of *E. coli* Rob.PRK stands for Protein K(c)luster and its number 10219 corresponds to a certain protein group from the same family. PRK provides an easy access to annonated and domains. PRK 10219 contains dual HTH domains from AraC superfamily, which includes a DNA binding transcriptional regulator. The AraC-E-bind superfamily contains proteins with a putative effector binding domain.

A.



B.



C.

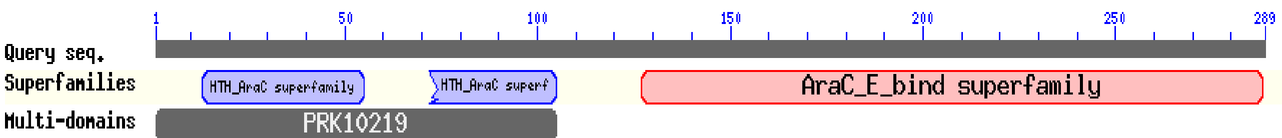


Fig. 3.2. Alignment of *S. marcescens* Rob candidates with *E. coli* Rob at the amino acid level using BLAST2. A) Rob1, B) Rob2, and C) Rob3. Query sequence at the top of each row is the *E. coli* Rob sequence and the bottom sequence is the subject sequence (sbjct) in *S. marcescens*. D) Alignment of *E. coli* SoxS with the first half sequence of *S. marcescens* Rob2 at amino acid level using BLAST2. As mentioned, Rob2 is twice in size as *E. coli* SoxS. The second half of Rob2 is not shown. Query sequence at the top of each row is *E. coli* SoxS sequence and the bottom sequence or subject (sbjct) is the first half of the *S. marcescens* Rob2 sequence.The middle sequence shows the similarities between two sequences at amino acid level. "+" indicates a conservative replacement. To obtain a better alignment, the program uses gap/s in the alignment which is represented by" –". Blank spots represent the presence of different amino acids in the same position of the two compared sequences.

A.

```
Score = 671 (241.3 bits), Expect = 4.8e-66, P = 4.8e-66
Identities = 137/289 (47%), Positives = 180/289 (62%), Frame = +2

Query:      1 MDQAGIIRDLLIWLEGHLDQPLSLDNVAAKAGYSKWHLQRMFKDVTGHAIGAYIRARRLS 60
              M Q  +I  LL W+E  LDQPL+LD++AAK+GYSKWHLQR+FK  TGH +G Y R RRL+
Sbjct:5019803 MHQQQVIEQLLAWIEQSLDQPLTLDDIAAKSGYSKWHLQRIFKQHTGHILGTYARRRLT 5019982

Query:      61 KSAVALRLTARPILDIALQYRFDSQQTFTRAFKKQFAQTPALYRRSPEWSAFGIRPPLRL 120
                +A  LRLT   +  IA  Y+FDSQQTFTR F+KQF   PA YRRS +WS++G++PPLRL
Sbjct: 5019983 AAARELRLTGTSVACIADTYQFDSQQTFTRCFRKQFGLPPASYRRSQDWSSYGLQPPLRL 5020162

Query:     121 GEFTMPEHKFVTLEDTPLIGVTQSYSCSLEQISDFRHEMRYQFWHDFLGNAPTIPPVLYG 180
                E  +P+   VTL    L+G TQ  S +L Q++D + E+R   W   L     +P V+YG
Sbjct: 5020163 TEAPLPQADIVTLPAMQLVGNTQRRSFTLGQLADSKCELRRHAWRQLL-RPQALPEVVYG 5020339

Query:     181 LNETRPSQDKDDEQEVFYTTALAQDQADGYVLTGHPVMLQGGEYVMFTYEGLGTGVQEFI 240
               L      + +     + YT AL  + A      G  V ++ GEY  FTY+G   G+Q FI
Sbjct: 5020340 LTSLEVDRQRRGCPRMAYTAALPDEGA-----MGERVTIEQGEYARFTYQGQAEGLQNFI 5020504

Query:     241 LTVYGTCMPMLNLTRRKGQDIERYYPAEDAKAGDRPINLRCELLIPIRR 289
               + +Y T MP +N  RR GQDIER+YPA++         +RCE LIPIRR
Sbjct: 5020505 VRLYDTAMPQMNAIRRPGQDIERFYPAQEGCCPLGGAAIRCEYLIPIRR 5020651
```

B.

```
Score = 338 (124.0 bits), Expect = 8.3e-29, Sum P(3) = 8.3e-29
Identities = 89/266 (33%), Positives = 136/266 (51%), Frame = +3

Query:       7 IRDLLIWLEGHLDQPLSLDNVAAKAGYSKWHLQRMFKDVTGHAIGAYIRARRLSKSAVAL 66
               I +L+ W+E HL++  +LD VA  +GYSKWHLQR FK +TG  +  YIR+R L+++AVAL
Sbjct: 1578381 IEELIEWVEIHLEKRPNLDEVARISGYSKWHLQRKFKRITGIQLATYIRSRILTRAAVAL 1578560

Query:      67 RLTARPILDIALQYRFDSQQTFTRAFKKQFAQTPALYRRSPEWSAFGIRPPLRL----GE 122
               R+T R I+DI+ +  FDSQQTFTR FK++F  TP  YR    W    + P        G
Sbjct: 1578561 RITRRSIIDISDELGFDSQQTFTRMFKQRFGTTPNRYRSMAHWDVKNLMPRFNFEASYGA 1578740

Query:     123 FTMPEHKFVTLEDTPLIGVTQSYSCSLEQISDFRH--EMRYQFWHDFLGNAPTIPPVLYG 180
                  PE K +TL D  L+G T+    + EQ  ++     M+ + ++DF          +Y
Sbjct: 1578741 GYYPEVKRLTLPDMQLVGFTRRLDFASEQELEYSSCMAMKDEIFNDFFKGLHVDCRRIYS 1578920

Query:     181 LNETRPSQDKDDEQEVFYTTALAQD-QADGYVLTGHPV---MLQGGEYVMFTYEGLGTGV 236
               +    P   + DE  +  T  +A D +    +L+ H +    L   E++   ++G
Sbjct: 1578921 IYS--PHAGEGDE--LSSTLVMAVDPEHKKDILSNHQIDTFHLPSREFISINHKGTAKEC 1579088

Query:     237 QEFILTVYGTCMPMLNLTRRKGQDIE 262
                +F   +    MP L    R   ++E
Sbjct: 1579089 LQFFGYLMSHVMPGLKDEVRGSMEME 1579166
```

C.

```
Score = 1113 (396.9 bits), Expect = 3.0e-111, Sum P(2) = 3.0e-111
Identities = 208/289 (71%), Positives = 243/289 (84%), Frame = -2

Query:       1 MDQAGIIRDLLIWLEGHLDQPLSLDNVAAKAGYSKWHLQRMFKDVTGHAIGAYIRARRLS 60
               MDQAGIIRDLL WLE HLDQPLSLDNVAAKAGYSKWHLQRMFKD+TG+AIGAYIRARRLS
Sbjct: 5110876 MDQAGIIRDLLSWLESHLDQPLSLDNVAAKAGYSKWHLQRMFKDITGNAIGAYIRARRLS 5110697

Query:      61 KSAVALRLTARPILDIALQYRFDSQQTFTRAFKKQFAQTPALYRRSPEWSAFGIRPPLRL 120
               K+AVALRLT+RPILDIALQYRFDSQQTFTRAFKKQFAQTPALYRR+ +W+AFGI PP+RL
Sbjct: 5110696 KAAVALRLTSRPILDIALQYRFDSQQTFTRAFKKQFAQTPALYRRAEDWNAFGICPPIRL 5110517

Query:     121 GEFTMPEHKFVTLEDTPLIGVTQSYSCSLEQISDFRHEMRYQFWHDFLGNAPTIPPVLYG 180
               G FT+P+ +FV+L D  L+G+TQSYSC+LEQI+  R E+R QFW  FLG+  T+PPVLYG
Sbjct: 5110516 GAFTLPQPEFVSLPDKHLVGLTQSYSCTLEQITTVRTELRSQFWRQFLGDVETLPPVLYG 5110337

Query:     181 LNETRPSQDKDDEQEVFYTTALAQDQADGYVLTGHPVMLQGGEYVMFTYEGLGTGVQEFI 240
               L+ +RPSQ+K +EQEV YTTAL  DQ    V  G P++L GGE+ MF+YEG    +Q+FI
Sbjct: 5110336 LHHSRPSQEKANEQEVLYTTALEPDQVPDKVQEGQPLVLPGGEFAMFSYEGPTENLQDFI 5110157

Query:     241 LTVYGTCMPMLNLTRRKGQDIERYYPAEDAKAGDRPINLRCELLIPIRR 289
               LTVYGTC+P L LTRRKG DIER+YP  + +    PI ++C+ LIPIRR
Sbjct: 5110156 LTVYGTCLPALQLTRRKGHDIERFYPKGERRPHQAPIEIKCDYLIPIRR 5110010
```

D.

```
Score = 260 (96.6 bits), Expect = 1.7e-20, Sum P(2) = 1.7e-20
Identities = 52/106 (49%), Positives = 76/106 (71%), Frame = +3

Query:       1 MSHQKI--IQDLIAWIDEHIDQPLNIDVVAKKSGYSKWYLQRMFRTVTHQTLGDYIRQRR 58
               M+++ I  I++LI W++ H+++  N+D VA+ SGYSKW+LQR F+ +T   L  YIR R
Sbjct: 1578357 MTNEDIFFIEELIEWVEIHLEKRPNLDEVARISGYSKWHLQRKFKRITGIQLATYIRSRI 1578536

Query:      59 LLLAAVELRTTERPIFDIAMDLGYVSQQTFSRVFRRQFDRTPSDYR 104
               L  AAV LR T R I DI+ +LG+ SQQTF+R+F+++F  TP+ YR
Sbjct: 1578537 LTRAAVALRITRRSIIDISDELGFDSQQTFTRMFKQRFGTTPNRYR 1578674
```

Fig. 3.3. Alignment of *S. marcescens* Rob3 with those of *E. coli*, *Shigella flexneri*, *Salmonella enterica* serovar Typhimurium, and *E. cloacae* using PRALINE multiple sequence alignment. The colors show the conservations scored from 0 to 10. Consistency refers to the consensus sequence. *= score of 10.

Results colour-coded for amino acid conservation

The current colourscheme of the alignment is for **amino acid conservation**.

The conservation scoring is performed by PRALINE. The scoring scheme works from 0 for the least conserved alignment position, up to 10 for the most conserved alignment position. The colour assignments are:

Unconserved 0 1 2 3 4 5 6 7 8 9 10 Conserved

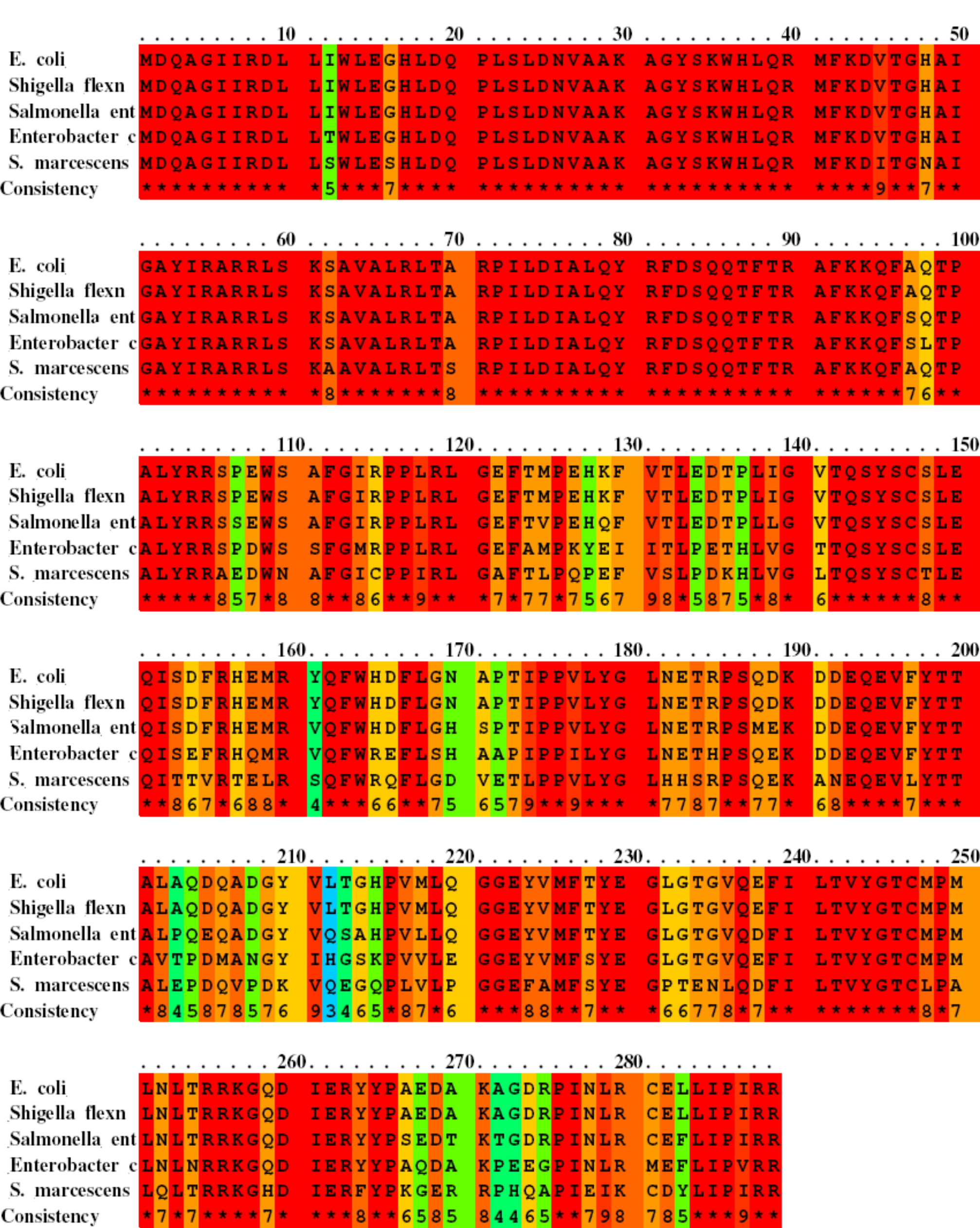


Table. 3.1. Percentage identity of genes flanking *E. coli rob* and *S. marcescens rob*3. The position of each gene was obtained from the Ecogene website. Genes listed from top to bottom of table reflect proximity to down stream or up stream of *E. coli rob*, as indicated. The ORF numbers in *S. marcescens* genome were used according to Artemis program. *S. marcescens* genome sequence was obtained from Sanger website. *S. marcescens* genome was compared to targeted *E. coli* genes using BLAST2 and percentage of identity are indicated in corresponding genes. According to Artemis program, miscellaneous features are the sequences which are yet to be identified. Each miscellaneous feature has been surrounded by two ORF as indicated.

Down stream of *E. coli rob*[a]	Percentage Identity[b]	*S. marcescens* ORF No.[c]	Misc. Feature[d]		Up stream of *E. coli rob*[a]	Percentage Identity[b]	*S. marcescens* ORF No.[c]	Misc. Feature[d]
ytjC	77%	4758	-	***rob*** (4759)[c]	*creA*	84%	4761	-
yjjX	54%	-	4757-4758		*creB*	-	-	-
trpR	62%	4756	-		*creC*	-	-	-
slt	63%	4755	-		*creD*	-	-	-
yjjK	91%	-	4751-4752		*arcA*	93%	4762	-
nadR	83%	4747	-		*yjjY*	48%	-	4762-4763
radA	89%	4746	-		*yjtD*	64%	4763	-
serB	74%	4745	-		*thrA*	27%	4750	-

a: from Ecogene website, **b**: determined using BLAST2, **c**: ORF No. according to Artemis program, **d**: unidentified sequences surrounded by two known ORFs.

similar to the *E. coli rob* than *rob*2. Putative translation products of *rob*1 and *rob*3 possess similar C-terminal domains (CTD), a ligand-binding domain, and two N-terminal (NTD) DNA-binding domains to that of *E. coli rob*. *rob*3 had higher identity at the DNA (71%) and the amino acid level (70%) to the *E. coli rob* than *rob*1. Moreover, high identity was also found with genes on the upstream and down stream of *rob*3 when compared to those of *rob* from *E. coli*, whilst no such identities were found for *rob*1 and *rob*2 (data not shown).

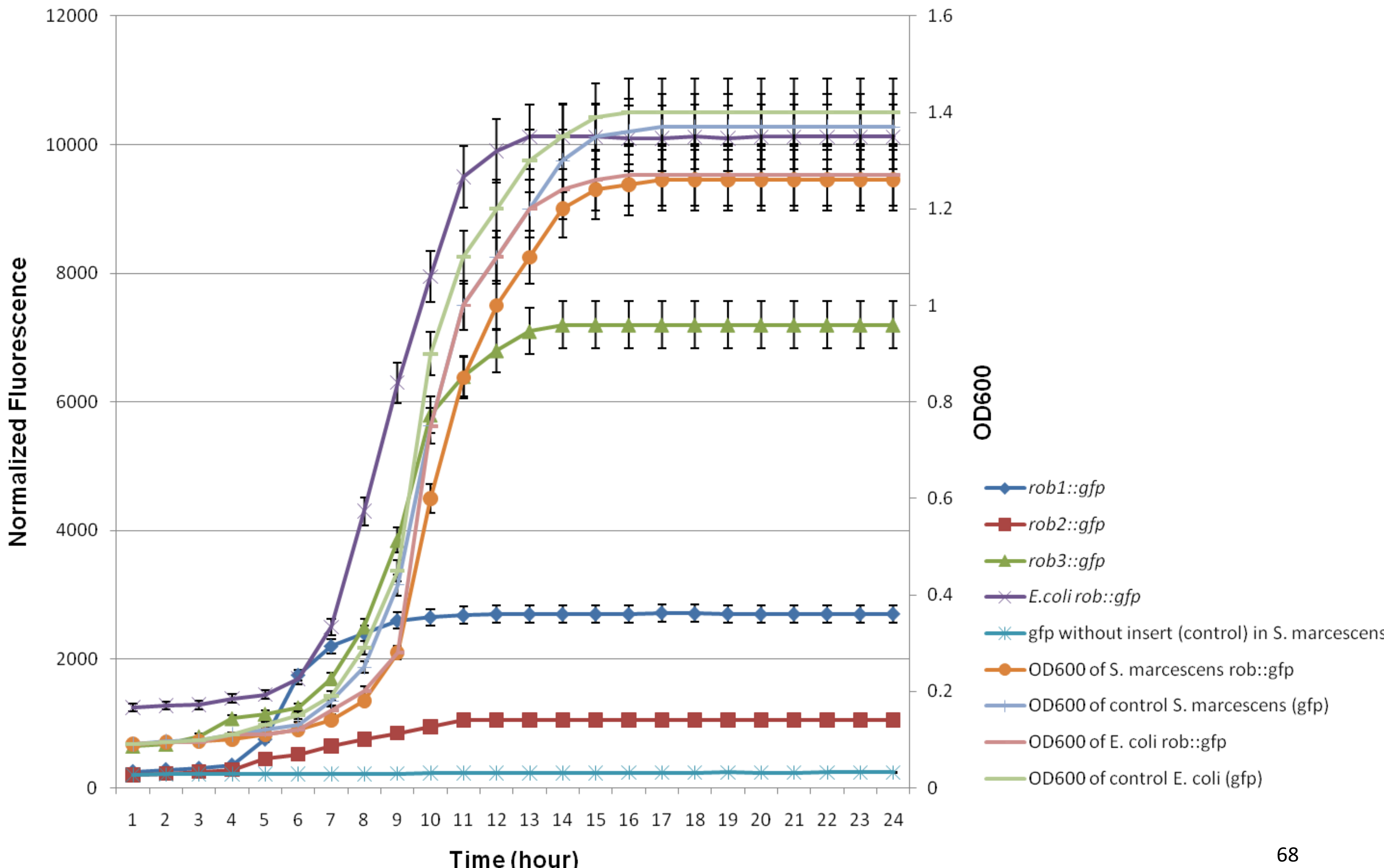
3.2. Promoter evaluation

To measure promoter activities of the *rob* candidates, transcriptional fusions were carried out using a GFP promoterless reporter system. These experiments were designed to try to understand whether the expression of *S. marcescens rob* has adapted more to human physiological temperature (37°C) or a lower temperature (30 °C).

The recombinant plasmids harboring the promoter regions of *rob*1, *rob*2, and *rob*3 were separately transformed into *S. marcescens* UOC-67. The recombinant plasmid containing the positive control, the promoter region of *E. coli rob*, was transformed into wild-type *E. coli* K-12 (GC4468). Fluorescence activities of the *S. marcescens rob* candidates were measured at 30°C and 37°C (Fig. 3.4 and 3.5). The growth curves of *S. marcescens rob* candidates were assayed at the same time points and are also displayed in Fig. 3.4. and 3.5.

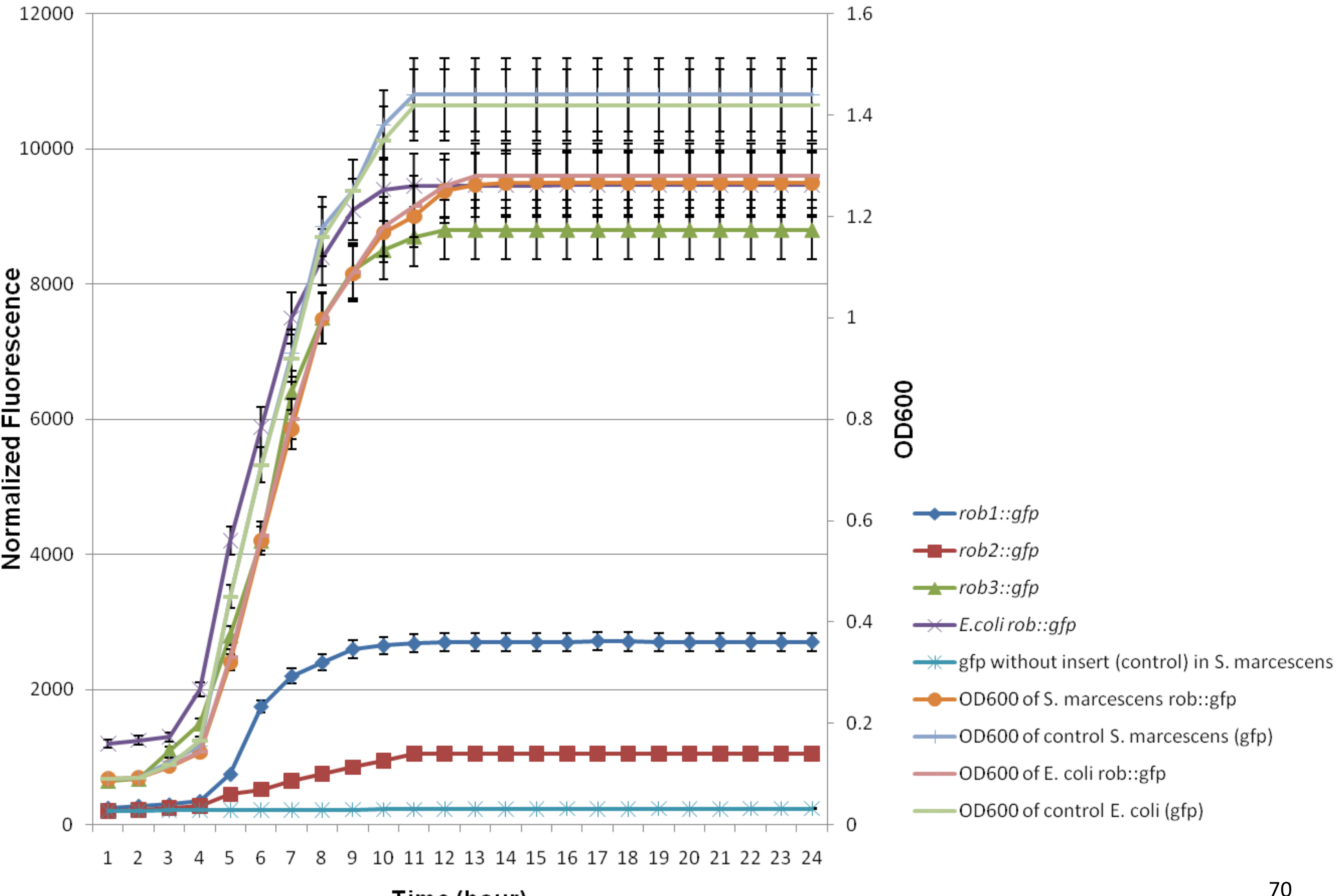
The *rob*3 promoter was found to have the highest promoter activity of all three candidates for both temperatures. Moreover, *rob*3 showed higher activity at 37ºC than

Fig. 3.4. Promoter activity of *S. marcescens rob*1, *rob*2, *rob*3 and *E. coli rob* at 30°C. *rob*1, *rob*2 and *rob*3 promoters were transformed into *S. marcescens* UOC-67 and the positive control *E. coli rob* was transformed into wild-type *E. coli* K-12 (GC4468). Results were drawn by Microsoft Office Excel 2007. Results are depicted as follows: *rob*1::*gfp* ( ), *rob*2::*gfp* ( ), *rob*3::*gfp* ( ) all transformed into *S. marcescens* UOC-67; *E. coli rob* ( ) transformed in wild-type *E. coli* K-12 (GC4468), *gfp* without insert ( ) transformed into both *S. marcescens* and wild-type *E. coli* K-12 (GC4468); growth curve of *S. marcescens* transformed with *rob::gfp* ( ) (as all *rob* candidates showed similar growth curves, just one growth curve is presented), growth curve of *gfp* without insert ( ) tranformed into *S. marcescens*, growth curve of *E. coli rob*::*gfp* ( ) and growth curve of *gfp* without insert ( ) transformed into *E. coli* K-12 (GC4468). All trials were performed in triplicate.



Normalized Fluorescence
OD600
Time (hour)
12000
10000
8000
6000
4000
2000
0
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
rob1::gfp
rob2::gfp
rob3::gfp
E.coli rob::gfp
gfp without insert (control) in S. marcescens
OD600 of S. marcescens rob::gfp
OD600 of control S. marcescens (gfp)
OD600 of E. coli rob::gfp
OD600 of control E. coli (gfp)

Fig. 3.5. Promoter activity of *S. marcescens rob*1, *rob*2, *rob*3 and *E. coli rob* at 37 °C. *rob*1, *rob*2 and *rob*3 were transformed in *S. marcescens* and *E. coli rob* was transformed in wild-type *E. coli* K-12 (GC4468). Results were drawn by Microsoft Office Excel 2007. Results are depicted as follows: *rob*1*::gfp* (), *rob*2*::gfp* (), *rob*3*::gfp* () transformed all in *S. marcescens; E. coli rob* () transformed in wild-type *E. coli* K-12 (GC4468), *gfp* without insert () transformed in both *S. marcescens* and wild-type *E. coli* K-12 (GC4468); growth curve of *S. marcescens* with *rob::gfp* () (as all *rob* candidates had similar growth curves, just one growth curve presented), growth curve of *gfp* without insert ( ) transformed into *S. marcescens*, growth curve of *gfp* without insert () transformed into *E. coli* K-12 (GC4468) and growth curve of *E. coli rob::gfp* (). All trials were performed in triplicate.


12000
10000
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2000
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Normalized Fluorescence
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD600
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Time (hour)
rob1::gfp
rob2::gfp
rob3::gfp
E.coli rob::gfp
gfp without insert (control) in S. marcescens
OD600 of S. marcescens rob::gfp
OD600 of control S. marcescens (gfp)
OD600 of E. coli rob::gfp
OD600 of control E. coli (gfp)

30°C, while *rob*1 and *rob*2 exhibited the similar activities at 37°C and 30°C. The positive control, *E. coli rob,* had higher activity at 30ºC than 37ºC which is consistent with other studies (Hartog et al. 2008; Seoane and Levy 1995). If we consider that *rob*3 is the true homolog of *rob* produced by other members of the *Enterobacteriaceae* family, results for *rob*3 are consistent with those found with *Salmonella enterica* serovar Typhimurium (*Salmonella* Typhimurium) (Hartog et al. 2008) which has higher promoter activity at 37°C than 30°C, while *rob*3 differs from *E. coli* which has higher promoter activity at 30 ° C (Hartog et al. 2008; Seoane and Levy 1995). Although, both *E. coli* and *Salmonella* Typhimurium are enteric human pathogens surprisingly only *rob* from *Salmonella* typhimurium demonstrated a higher expression at 37 ° C suggesting adaptation to the physiologic temperature of the human body. It is not understood that why *E. coli rob* has a lower promoter activity at 37°C.

Begic and Worobec (2006) reported that *S. marcescens ompF* expression was higher at 28 ° C than 37 ° C, in addition, S*. marcescens micF* expression was lower at 28 ° C than 37 ° C which correlates to the finding that expression of *rob* increases at 37 ° C . It is known that any change in *rob* expression can consequently result in alteration in *micF* expression, which is one of the *mar*/*sox*/*rob* regulons, and thereby changes in *ompF* expression also occur (Schmidt et al. 1995). Based on this conclusion and according to our results for *rob* promoter evaluation, increasing the temperature from 30°C to 37°C results in an increase in *rob* expression which should lead to an elevation in *micF* expression and thereby reduction in OmpF production. Reduction of

OmpF production (Harder et al. 1981) and over-expression of the efflux pump/s are considered the main reasons for conferring bacterial resistance to multiple antibiotics (Delcour 2009).

In addition, similar to *S.* Typhimurium and *E. coli*, we found that promoter activity of *rob*3 of *S. marcescens* is higher at both temperatures once stationary phase was reached. The higher activity of *rob* in this phase could be partially attributed to dependency of *rob* expression on the sigma subunit of RNA polymerase (δ^s), encoded by *rpoS* gene. In *E. coli*, deletion of *rpoS* resulted in a decrease in *rob* expression (Hengge-Aronis 1993). Stationary phase conditions can be correlated to an infection condition in the human body when bacteria face non-ideal conditions. Under such conditions, environmental signals trigger the cell to express more *rob* which in turn controls the expression of a variety of systems to combat these extreme conditions, such as to pump out toxic metabolites and antibiotics, and confer the MDR phenotype to survive (Hartog et al. 2008).

Pathogenicity is a multifactorial and sophisticated process. Bacterial pathogens possess powerful defense mechanisms to express divergent virulence factors in response to different environmental signals such as pH, osmolarity and temperature (Hromockyj et al. 1992). Among these various external stimuli, temperature plays an important role (Konkel and Tilly 2000). Various pathogens showed this effect when grown at 37°C (Hromockyj et al. 1992) such as *Yersinia pestis* (Fukui et al. 1960; Bolin et al. 1982), *Bordetella* spp. (Weiss and Falkow 1984), *Salmonella* spp. (Jones and

Richardson 1981), *Shigella* spp. (Maurelli et al. 1984), and enterohemorrhagic *E. coli* (Weinstein et al. 1988). For instance, in the case of *Shigella flexneri*, an enteric bacterium and causative agent of bacillary diarrhea, external temperature is considered a primary signal to trigger the expression of their virulence genes at 37 °C while gene repression occurs at 30°C (Maurelli 1989; Maurelli et al. 1984; Rogers 1999). Findings showed that this is mainly due to the positive regulator *virB* which regulates genes involved in invasion, including *ipa*, *mxi* and *spa* (Tobe et al. 1991). It was shown that suppression of *virB* transcription at 30°C occurred by changing superhelicity around the promoter region (Tobe et al. 1991).

Upon entrance into the human body, bacteria face an elevated temperature. The advantage of being able to grow in a wide range of temperatures has made opportunistic *S. marcescens* more competitive with other human pathogens (Chaloupka 1985). When facing an elevation in temperature, an increase in the expression of certain genes, such as *rob*, which controls the expression of genes related to outer membrane protein production like *micF* and RND efflux pumps like *sdeXY* and *sdeAB*, suggests that this bacterium has evolved to potentially confer its pathogenicity under this condition (37 °C).

3.3. Induction of promoters of *rob* candidates by 2,2'-dipyridyl at 37 °C

2,2'-dipyridyl is a hydrophobic compound which is a strong iron chelator used to lower the iron quantity of media (Tsolis et al. 1995). Rosner et al. (2002) examined a number of unrelated compounds to see if they were able to induce transcription of *E. coli rob*, disregarding their presence or absence in bacterium's natural environment

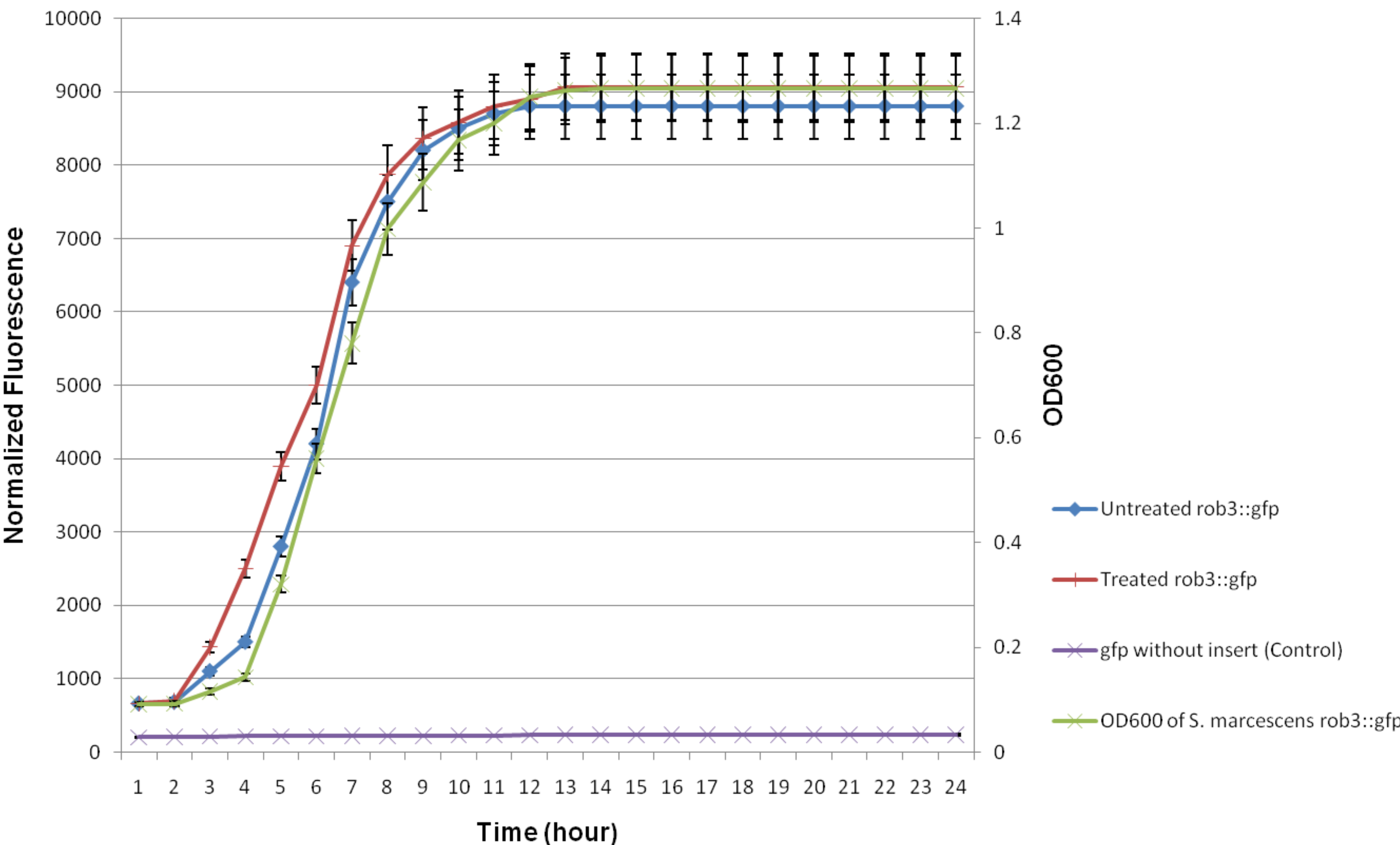
(Rosner et al. 2002). They found that 2,2'-dipyridyl can induce *E. coli rob* transcription and activate pre-existing intracellular Rob molecules, although, 2,2'-dipyridyl does not naturally exist in the bacterium's niches (Rosner et al. 2002).

To investigate if 2,2'-dipyridyl is similarly able to induce *rob* transcription in *S. marcescens*, promoters of the three *rob* candidates were examined using transcriptional fusion constructs transformed into *S. marcescens* UOC-67 and corresponding *rob* null mutant strains. *rob*1 and *rob*2 promoters were not induced upon exposure to 2,2'-dipyridyl in either wild-type *S. marcescens* UOC-67 or their corresponding *rob* mutant strains (data not shown), while the *rob*3 promoter activity was observed to be slightly increased in the early and mid-log phase by 1.4 and 1.1 fold, respectively. Similar values were obtained when the *rob*3 promoter was transformed either into *S. marcescens* UOC-67 or the *rob*3 null mutant SMRD3. The *rob*3 promoter did not show any induction in the stationary phase upon exposure to 2,2'-dipyridyl (Fig 3.6).

Our results are consistent with those found for *E. coli* (Rosner et al. 2002). In *E. coli*, introduction of 2,2'-dipyridyl resulted in the increase of *rob* expression by 1.7 and 1.1-fold in mid-log, and log-phase, respectively. Similar to *S. marcescens*, no elevation in *rob* transcription was reported in stationary phase (Rosner et al. 2002). The authors give no explanation for why expression of *E. coli rob* is affected differently in different growth phases. Moreover, no mechanism has been found to explain how 2,2'-dipyridyl induces *rob* transcription.

Generally speaking, many regulators do repress their own synthesis to keep their

Fig. 3.6. Induction of the *S. marcescens rob*3 promoter by 2,2'-dipyridyl at 37°C. Results were drawn by Microsoft Office Excel 2007. Results are depicted as follows: Untreated *rob*3::*gfp* (—◆—), treated *rob*3::*gfp* (—+—), *gfp* without insert as control (—✕—) and growth curve of *S. marcescens* with *rob*3::*gfp* (—✕—) all recombinant plasmids were transformed into *S. marcescens* UOC-67. As the treated and untreated controls showed the same values, only one curve is shown. All trials were performed in triplicate.


Normalized Fluorescence
10000
9000
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7000
6000
5000
4000
3000
2000
1000
0
1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD600
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Time (hour)
Untreated rob3::gfp
Treated rob3::gfp
gfp without insert (Control)
OD600 of S. marcescens rob3::gfp

intracellular quantity constant (Van Houdt et al. 2007; Bell and Jackson 2001) but this doesn't seem to be the case for either *E. coli rob* or *S. marcescens rob*3, as we found no change in *rob*3 transcription upon transformation into either wild-type *S. marcescens* and *rob*3 null mutant SMRD3. In *E. coli*, 2,2'-dipyridyl, as a Rob activator, binds to the CTD of Rob, stimulates release of pre-existing Rob molecules from intracellular foci and promotes the change of Rob from the inactive or low-active to the high-active state (Rosner et al. 2002). It is suggested that the N-terminal domain (NTD) of Rob from *E. coli* has a different conformation in normal conditions (low-active form) but upon addition of 2,2'-dipyridyl, which binds to the CTD, an alteration in the amide and side chain regions of CTD occurs, resulting in the interaction of NTD with C-terminal domain (CTD). The outcome of this event is converting the low-active form to the high-active form. These changes were not observed in NTD-truncated Rob, which is an important domain for *rob* DNA-binding (Rosner et al. 2002).

In addition, it seems that 2,2'-dipyridyl plays a paradoxical role here. As iron is a necessary element for S*. marcescens* to cause infections (Zimmermann et al. 1989), lowering the iron content by a compound like 2,2'-dipyridyl in the environment decreases the chance of infection by this bacterium. Meanwhile, 2,2'-dipyridyl is able to induce transcription of *rob* which can help the bacterium to establish an infection by expressing less OmpF and over-expressing efflux pumps to pump out toxic compounds. In another study, exposure of *Pseudomonas aeruginosa* to an iron-depleted medium using 2,2'-dipyridyl led to over-expression of the efflux pump MexAB-OprM (Poole et al. 1993). As subsequent experiments in our studies, including tolerance to n-hexane,

ethidium bromide efflux and resistance to certain antimicrobials, showed the over-expression of efflux pumps mediated by over-expression of Rob in *S. marcescens*, we see a similarity to what was reported for *P. aeruginosa*.

3.4. Induction of *rob* promoters by sodium salicylate (SSA) and sodium decanoate (SDE) at 30°C and 37°C

Salicylate is the main metabolite of aspirin in the human body. Salicylate, as a weak acid, is able to elevate the membrane charge and affect membrane permeability (Repaske and Adler 1981; Snyder et al. 1981). Sodium salicylate is not naturally present in the bacterial habitat but it can be found in the human body during the medical therapy with aspirin. Previous studies indicated that the transcription of *E. coli marA* and *Salmonella* Typhimurium *marA* and *rob* transcription are affected by the presence of salicylate (Alekshun and Levy 1999; Hartog et al. 2008). In *E. coli*, no study has been carried out to investigate the effect of salicylate on *rob* transcription.

Sodium decanoate (sodium caprate), a medium-fatty acid chain (ten-carbon-atom), is present in the human intestinal tract in high concentration (10mg/ml) (Batta et al. 2002). Sodium decanoate is naturally found in milk fat and is often used as a food additive (Jensen et al. 1991). Previously, sodium decanoate was examined for the potential to activate intracellular Rob molecules in *E. coli* (Rosenberg et al. 2003) and for its effect on *Salmonella* Typhimurium *rob* transcription (Hartog et al. 2008).

In our study, sodium salicylate and sodium decanoate were examined to see if these compounds affect the transcription of *S. marcescens rob*. Promoters of *rob*1, *rob*2 and *rob*3 were cloned into pGlow-TOPO and then recombinant plasmids were

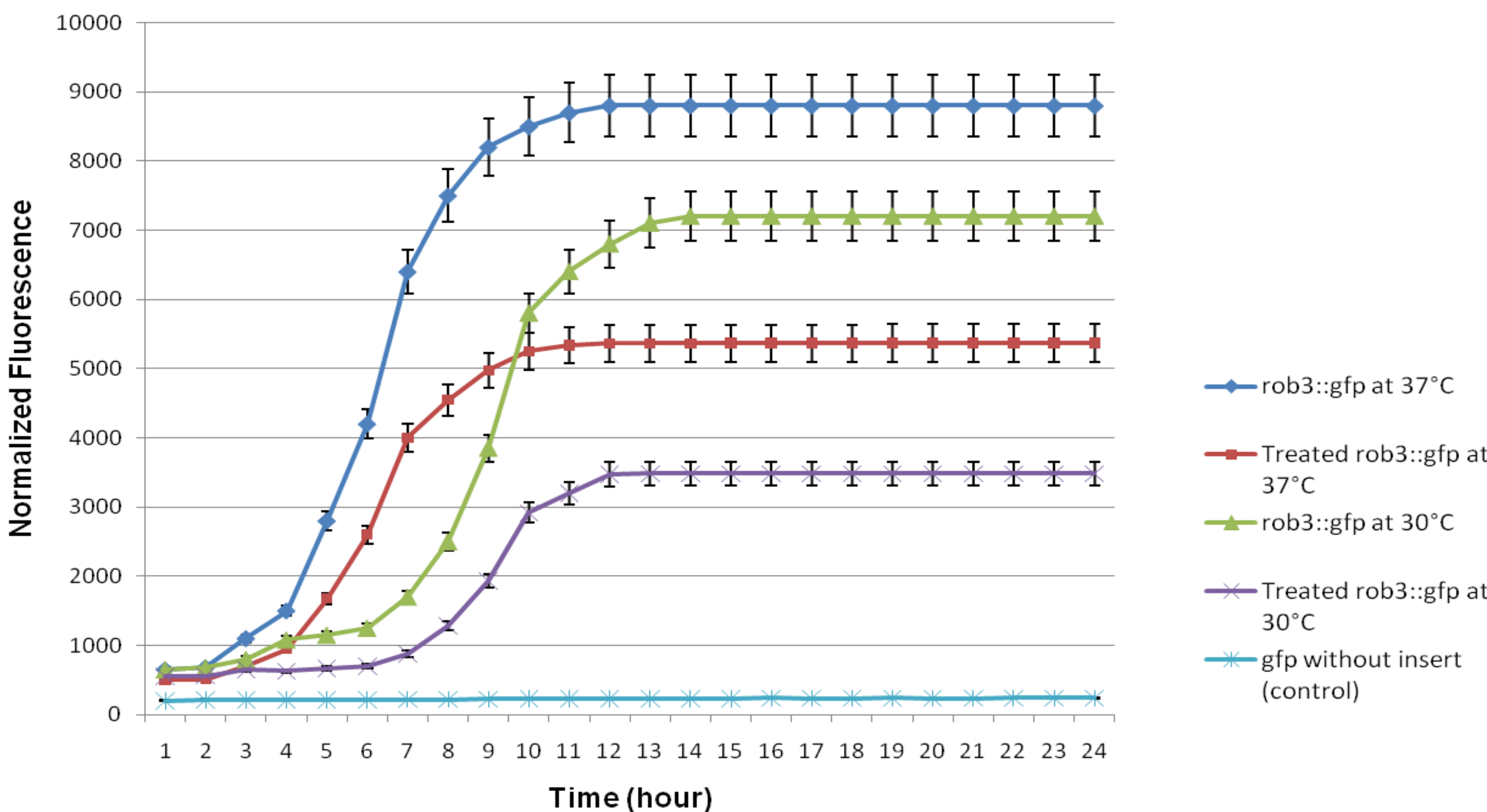
transformed into *S. marcescens* UOC-67. Sodium salicylate and sodium decanoate were added to the growth media of resulting strains according to previously described methods (See Methods and Materials).

The promoters of *rob*1 *and rob*2 did not show any change in their GFP fluorescence activities at 30°C and 37°C upon exposure to either sodium salicylate or sodium decanoate (data not shown), whereas, the promoter of *rob*3 exhibited a reduction in the GFP fluorescence activity, to a differing extent, at both temperatures in the presence of sodium salicylate (Fig 3.7) and sodium decanoate (Fig. 3.8). In stationary and mid-log phases, the promoter of *rob*3 showed a 1.6 fold decrease in activity upon exposure to sodium salicylate at 37 ° C and by 2 fold at 30 ° C. In the case of sodium decanoate, in mid-log and stationary phase, transcription was decreased by 3.3 fold at 37 ° C, while, 8 and 9.5 fold decreases were observed at 30 ° C.

In *E. coli*, salicylate is known as a *marA* inducer but no direct interaction with Rob protein or transcription of *rob* has been reported (Alekshun and Levy 1999). In both *E. coli* and *Salmonella* Typhimurium, *marA* expression is suppressed by its repressor, MarR. Salicylate, as a *marA* inducer, binds to MarR and derepresses *marA* transcription (Alekshun and Levy 1999; Hartog et al. 2008; Sulavik et al. 1997). Using this mechanism, salicylate can induce the transcription of the *marRAB* operon (Fralick 1996). No similar study has been performed on *sdeR*, the *marA* homolog in *S. marcescens*.

Recently, it was found in *E. coli* that *marA* represses *rob* transcription via steric hindrance (McMurry and Levy 2010). As mentioned, upon exposure of *E. coli* to

Fig. 3.7. Induction of the *S. marcescens rob*3 promoter by sodium salicylate at 30°C and 37°C. Results were drawn by Microsoft Office Excel 2007. Results are depicted as follows: Untreated *rob*3*::gfp* at 37ºC (), treated *rob*3*::gfp* (), untreated *rob*3*::gfp* at 30ºC (), treated *rob*3*::gfp* at 30ºC (), *gfp* without insert as control () all transformed into *S. marcescens* UOC-67. All trials were performed in triplicate.



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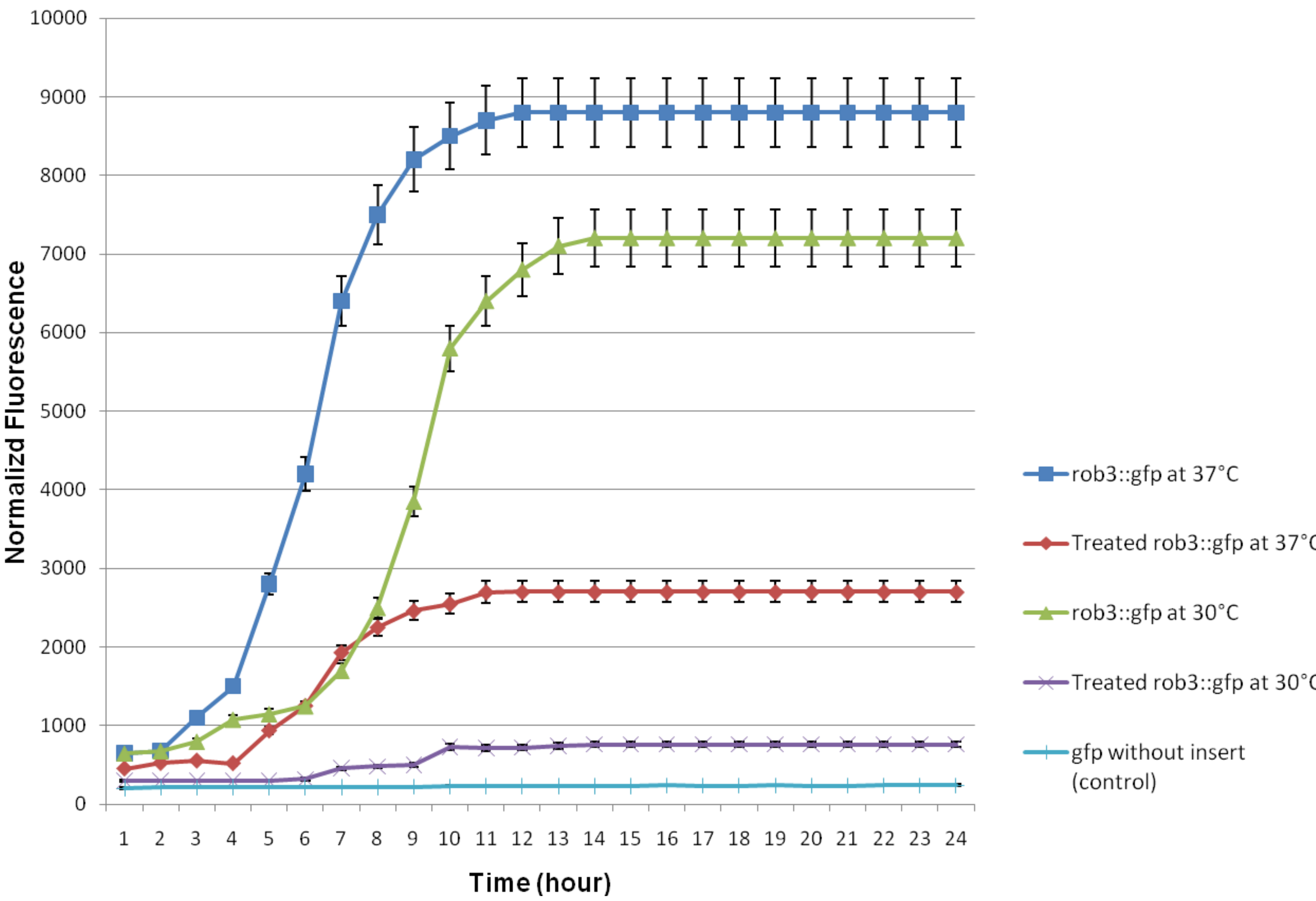
Normalized Fluorescence
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Time (hour)
rob3::gfp at 37°C
Treated rob3::gfp at 37°C
rob3::gfp at 30°C
Treated rob3::gfp at 30°C
gfp without insert (control)

Fig. 3.8. Induction of the *S. marcescens rob*3 promoter by sodium decanoate at 30 °C and 37 °C. Results were drawn by Microsoft Office Excel 2007. Results are depicted as follows: Untreated *rob*3*::gfp* at 37ºC (■), treated *rob*3*::gfp* (♦), untreated *rob*3*::gfp* at 30ºC (▲), treated *rob*3*::gfp* at 30ºC (✕), *gfp* without insert as control (+) all transformed into *S. marcescens* UOC-67. All trials were performed in triplicate.


10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Normalizd Fluorescence
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Time (hour)
rob3::gfp at 37°C
Treated rob3::gfp at 37°C
rob3::gfp at 30°C
Treated rob3::gfp at 30°C
gfp without insert
(control)

salicylate the expression of *marA* is mediated by MarR. MarA can bind to a specific binding site (*mar/sox/rob* box) within the *rob* promoter preventing RNA polymerase (RNAP) from gaining access (Schneiders and Levy 2006; McMurry and Levy 2010) resulting in a decrease in *rob* transcription. We suggest that there is a similar mechanism for the reduction in *S. marcescens rob* transcription upon exposure to salicylate.

Previously, it was found that exposure to salicylate resulted in an increase in *micF* expression and a decrease in *ompF* expression in *S. marcescens* (Begic and Worobec 2006). However, in this study, we report that *rob* expression decreases upon exposure to salicylate and based on what is known about the function of Rob in *E. coli*, one could predict that a decrease in Rob would result in a decrease in *micF* expression, and this in turn would result in an increase in *ompF* expression. RT-PCR experiments in this study confirmed that a reduction in *rob* expression resulted in a decrease in *micF* transcription and an increase in *ompF* transcription (see Section 3.9).

We found that sodium decanoate had a stronger effect on *rob* transcription than sodium salicylate, most notably at 30 °C. Similar results in *Salmonella* Typhimurium were obtained but no clear reason was presented (Hartog et al. 2008). No similar study has been carried out in *E. coli*. Spectroscopic methods indicated that sodium decanoate directly binds to pre-existing Rob via CTD (Rosner et al. 2002; Rosenberg et al. 2003). In this process, intracellular Rob, found in intracellular clusters, is dispersed upon addition of sodium decanoate and inactive or low-active form of Rob molecules are converted to high-active molecules (Rosner et al. 2002; Rosenberg et al. 2003). Our

findings with 2,2'-dipyridyl induction ruled out the repression of *rob* transcription by pre-existing intracellular activated Rob protein, as no difference was found in fluorescence activity when *rob*3 promoter was transformed into either wild-type *S. marcescens* UOC-67 or *rob*3 null mutant SMRD3.

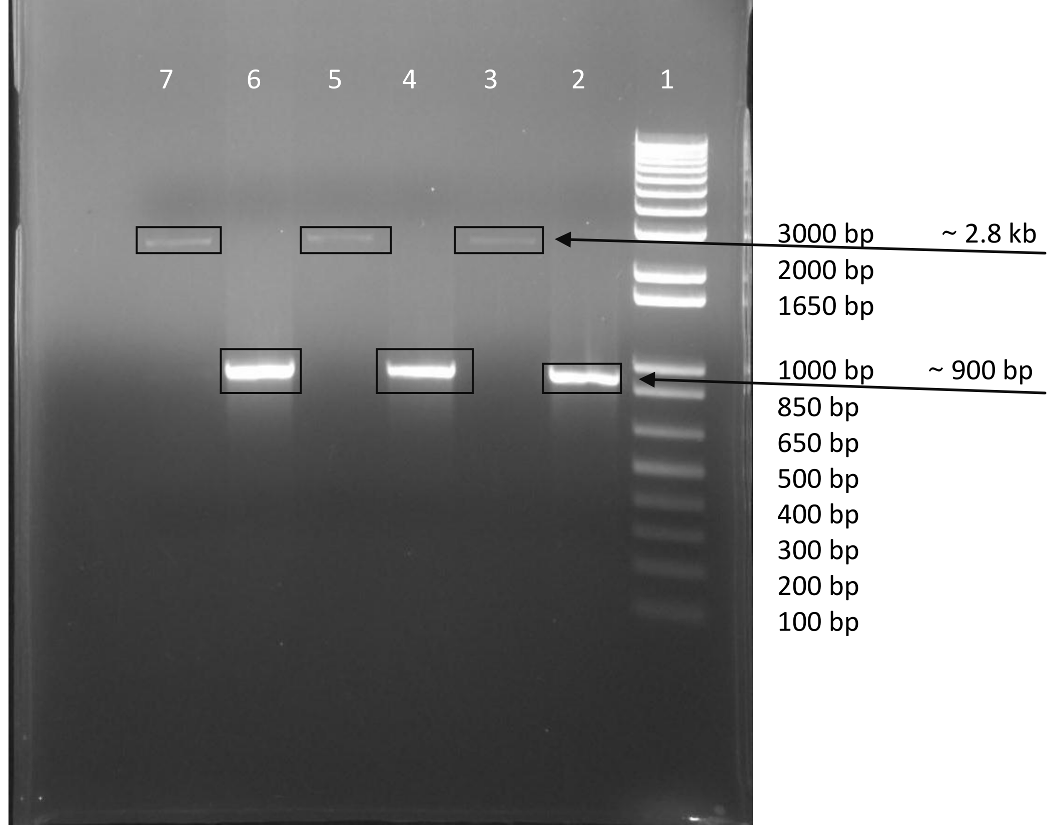
Unlike salicylate, sodium decanoate doesn't affect Mar activity (Rosenberg et al. 2003). Another possibility, such as different penetration of sodium decanoate versus salicylate across membranes, could be considered for lowering *rob* transcription in the presence of sodium decanoate at 30 °C and 37 °C. Sodium decanoate is an amphiphilic compound, having both hydrophilic and hydrophobic properties. Being amphiphilic enables sodium decanoate to easily cross biological membranes (Caboi et al. 2001). Sodium salicylate is not an amphiphilic compound. Giving this difference in permeability, more sodium decanoate than sodium salicylate can accumulate inside the cell and potentially having an impact on *rob* transcription.

3.5. Construction of *rob* knock-out mutant strains

*S. marcescens rob*1, *rob*2 and *rob*3 knock out strains were constructed using the TargeTron Knockout System. *rob*1, *rob*2 and *rob*3 all were ~ 0.9 kb in size. Upon insertion of intron RNA into chromosomal DNA of *S. marcescens* UOC-67, the *td* group intron was removed and the kanamycin marker became activated. These cells were able to grow on LB kanamycin plates and were screened using colony PCR (Lu 2003). The total size of intron RNA, which contains the kanamycin marker, is 1.9 kb. Therefore, the total size of all knock-outs were the same (~2.8 kb). To confirm the size of resulting knock-outs, the same primers used for cloning the *rob* gene were used

Fig. 3.9. PCR products from wild type *S. marcescens* and *rob*1, *rob*2 and *rob*3 knock-out strains SMRD1, SMRD2 and SMRD3, respectively, using TargeTron Gene Knockout System. Lane 1, 1 Kb Ladder; Lane 2, *rob*1 ~900 bp product; Lane 3, SMRD1 *rob*1 disrupted by intron RNA with total size of 2.8 kb; Lane 4, *rob*2 ~900 bp product; Lane 5, SMRD2 *rob*2 disrupted by intron RNA with total size of size 2.8 kb; Lane 6, *rob*3 900 bp product; Lane 7, SMRD3 *rob*3 disrupted by intron RNA with size of 2.8 kb. Bands marked and shown by arrows.

Fig. 3.9.



(Table 2.3). As shown in Fig 3.9, the expected 2.8 kb PCR fragments were obtained after disruption of *rob* by intron RNA containing the Kanamycin marker. Knock-out constructs were confirmed by sequencing.

3.6. Minimum Inhibitory Concentrations (MICs)

As previously reported, over-expression of Rob in *E. coli* resulted in conferring the MDR phenotype to multiple antibiotics, predominantly due to over-expression of the RND efflux pump AcrAB (Jair et al. 1995; Alekshun and Levy 1999) and reduction in OmpF synthesis due to over-expression of *micF* (Bennik et al. 2000; Hirai et al. 1986b; Aoyama et al. 1987; Cohen et al. 1989; McMurry et al. 1994). In our study, we assessed the effect of Rob over-expression in *S. marcescens* on resistance to a variety of antibiotics. MIC experiments were performed using wild type *S. marcescens* UOC-67, *E. coli rob* null mutant RA4468, *rob* deficient *S. marcescens* strains SMRD1, SMRD2, SMRD3, and *E. coli* RA4468, SMRD1, SMRD2, SMRD3 complemented with *rob*. To over-express the different *rob* candidates, *rob*1, *rob*2 and *rob*3 from wild-type *S. marcescens* genome were amplified and then cloned into pUS19, a high copy number plasmid. The resulting plasmids, called pUSRob1, pUSRob2 and pUSRob3, were transformed into *rob* deficient *S. marcescens* SMRD1, SMRD2, SMRD3, and *rob* deficient *E. coli* RA4468. MIC values for resistance to ciprofloxacin, ofloxacin, norfloxacin, nalidixic acid, tetracycline, and chloramphenicol are ≥4, ≥8, ≥16, ≥32, ≥ 16 and ≥ 32 μg/ml, respectively (CLSI 2007).

Results of the MICs are summarized in Table 3.2. We found deletion of *rob*3 led to increase sensitivity to norfloxacin, ofloxacin, ciprofloxacin, tetracycline, chloramphenicol,

Table 3.2. Minimum Inhibitory Concentration (MIC) (µg/ml) for wild-type, *rob*-deficient and *rob* over-expressing strains.

Antimicrobial agent	**MIC (µg/ml)**		**MIC (µg/ml)**		**MIC (µg/ml)**	
	***E. coli* RA4468 /pUS19 (control)**	***E. coli* RA4468 /pUSRob3**	***S. marcescens* SMRD3 /pUS19 (control)**	***S. marcescens* SMRD3 /pUSRob3**	***S. marcescens* UOC-67**	***S. marcescens* UOC-67/pUS19**
Ciprofloxacin	0.0078	0.0625	0.004	0.032	0.032	0.032
Ofloxacin	0.0625	0.25	0.125	0.5	0.5	0.5
Norfloxacin	0.0625	0.25	0.0312	0.125	0.125	0.125
Chloramphenicol	2	10	5	10	10	10
Tetracycline	1.25	5	8	16	16	16
Nalidixic acid	2	4	1.25	5	5	5

n=3

and nalidixic acid. Our MIC results are similar to those for *E. coli* and *E. cloacae* (Nakajima et al. 1995; Ariza et al. 1995; Lee et al. 1996).

The over-expression of *rob*1 (pUSRob1) and *rob*2 (pUSRob2) in S*. marcescens rob* deficient SMRD1 and SMRD2 and *rob* mutant *E. coli* RA4468 did not show any change in MIC values for all tested antibiotics. These results are not shown. Over-expression of *rob*3 (pUSRob3) in both *E. coli* RA4468 and *S. marcescens* SMRD3 resulted in reduced susectibility to fluoroquinolones (norfloxacin, ofloxacin and ciprofloxacin), tetracycline, chloramphenicol, and nalidixic acid (Table 3.2). No change in MIC values compared to the wild-type *S. marcescens* UOC-67 was found when cephalothin, cefoperazone, cefotaxime, cefuroxime, gentamycin, streptomycin, amikacin, carbenicillin, and novobiocin were used for all tested strains (data not shown).

These results indicate that *S. marcescens rob*3 functions similarly to *E. coli rob* in terms of reducing the susceptibility to certain antibiotics in over-expressing strains. As already mentioned, in *E. coli* the MDR phenotype, mediated by *rob*, is due to the over-expression of the antibiotic efflux pump AcrAB, and decrease in OmpF production (Jair et al. 1996b; Bennik et al. 2000). In *S. marcescens*, two MDR efflux pumps, SdeAB and SdeXY, which have the similar substrates to *E. coli* AcrAB, contribute to confer resistance to most antibiotics. It was previously found that *sdeAB* null mutant strains resulted in decreased MIC to norfloxacin, chloramphenicol and tetracycline (Begic and Worobec 2008; Maseda et al. 2009). In addition, it was previously reported that the

detection of *sdeXY* increases sensitivity to tetracycline and norfloxacin (Chen et al. 2003). Moreover, similar to *E. coli*, a decrease in *S. marcescens* OmpF synthesis, mediated by *micF* expression as a *rob* regulon, could be involved in conferring antibiotic resistance in *S. marcescens*.

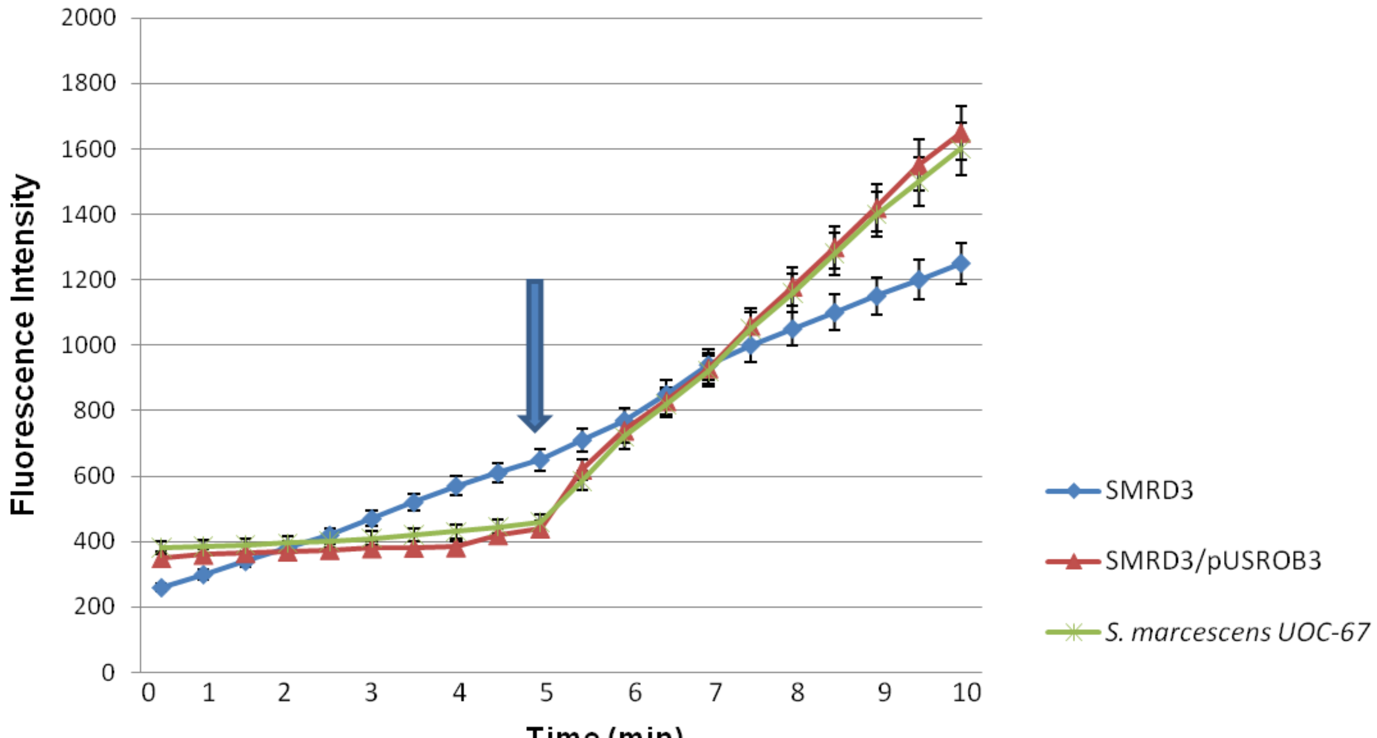
3.7. Ethidium Bromide (EtBr) accumulation assay

Ethidium bromide is considered as a substrate for various efflux pumps (Paulsen et al. 1996). To investigate the involvement of *rob* in controlling expression of efflux pumps, ethidium bromide accumulation assays were performed.

As shown in Fig. 3.10, wild-type *S. marcescens* UOC-67 maintained the constant intracellular amount of ethidium bromide initially, but upon introduction of CCCP, a proton motive force inhibitor, an elevation was observed in the accumulation of ethidium bromide. CCCP is known to disrupt RND pump function by interfering with pump energization (Kaback et al. 1974).

Among the three *rob* knock-outs SMRD1, SMRD2 and their corresponding over-expression strains showed similar accumulation results as wild-type *S. marcescens* UOC-67 (data not shown), while SMRD3 demonstrated a different pattern. SMRD3 showed a steady elevation in ethidium bromide accumulation and addition of CCCP did not cause any significant increase in ethidium bromide accumulation. Complementing SMRD3 with pUSROB3 resulted a similar increase upon exposure to CCCP as wild-type *S. marcescens* UOC-67. These results indicate that *S. marcescens rob* is involved

Fig. 3.10. Ethidium bromide accumulation by wild-type *S. marcescens*, SMRD3 and SMRD3 complemented with *S. marcescens rob*3 (SMRD3/pUSROB3). Results were drawn by Microsoft Office Excel 2007. Results are depicted as follows: SMRD3 (—◆—), wild-type *S. marcescens* UOC-67 (—✳—) and SMRD3/pUSROB3 (—▲—). Arrow shows when CCCP was added. Experiments were carried out in triplicate.



Fluorescence Intensity
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0
1
2
3
4
5
6
7
8
9
10
Time (min)
SMRD3
SMRD3/pUSROB3
S. marcescens UOC-67

in the regulation of efflux pump/s which participate/s in extruding ethidium bromide. These findings substantiate our hypothesis that Rob is a transcriptional activator of RND efflux pump genes, in this case *sdeXY* and *sdeAB*.

3.8. n-hexane Tolerance Test

Tolerance to organic solvents, such as n-hexane, is an indication of the presence of an efflux pump mechanism (Ma et al. 1993; Fralick 1996; Ramos et al. 2002). The n-hexane tolerance test was carried out for *E. coli rob* deficient RA4468 with and without *S. marcescens rob*1*, rob*2 *and rob*3 (*E. coli* RA4468/pUSRob1, RA4468/pUSRob2, RA4468/pUSRob3), and *S. marcescens rob* knock-out strains SMRD1, SMRD2 and SMRD3 with and without their corresponding expression vector pUSRob1, pUSRob2 and pUSRob3, wild-type *S. marcescens* UOC-67 with and without pUS19 and *E. coli rob* deficient RA4468.

Only three strains, *E. coli* RA4468/pUSRob3, wild-type *S. marcescens*/pUSRob3 and SMRD3/pUSRob3, demonstrated tolerance to n-hexane while no other tested strains showed any sign of growth in the presence of n-hexane (Fig. 3.11). Data for these latter strains are not shown.

In the present study, findings showed that expression of *rob* resulted in tolerance to n-hexane. *S. marcescens* type strain UOC-67, *E. coli rob* deficient RA4468, and *rob* deficient *S. marcescens* SMRD3 were sensitive to n-hexane, while upon complementation of these strains with *S. marcescens rob*3, they became resistant to

Fig. 3.11. n-hexane tolerance assay. A) Representative photograph of growth in the presence of n-hexane for *rob* deficient *E. coli* RA4468, SMRD3/pUSRob3 and *S. marcescens* UOC-67 with pUSRob3. B) Representative photograph of no growth in presence of n-hexane for the rest of the tested strains such as wild-type *S. marcescens* UOC-67, *E. coli* RA4468.

Fig. 3.11.

n-hexane. Similarly in *E. coli*, *rob* inactivation resulted in decreasing its sensitivity to n-hexane but over-expression of Rob from a high copy number plasmid increased the tolerance to organic solvent due to over-expression of AcrAB-TolC (Nakajima et al. 1995; White et al. 1997).

In *E. coli*, all three transcriptional activators, *marA*, *soxS*, and *rob* are able to confer resistance to n-hexane as mediated by the efflux pump AcrAB. Studies showed that mutations in *rob* and/or *acrAB* could increase tolerance to organic solvents in *E. coli* (Ariza et al. 1995; Ma et al. 1995; White et al. 1997). Indeed, Kumar and Worobec (2005) also reported SdeAB is involved in tolerance of *S. marcescens* to n-hexane.

3.9. RT-PCR results

To measure the transcription of genes which may be under control of *S. marcescens* Rob, we performed RT-PCR for the *E. coli acrAB*, *tolC* and *micF* homologues *sdeAB*, *sdeXY*, *hasF* and *micF*, respectively. In addition, we examined *ompC* and *ompF* which may be indirectly affected by Rob through *micF* expression.

RT-PCR was carried out for wild-type *S. marcescens* UOC-67 and *S. marcescens rob* knock-out strains SMRD1, SMRD2 and SMRD3. SMRD1 and SMRD2 did not show any changes in the expression of any tested genes (data not shown), while the *rob*3 deficient strain SMRD3 did have changes in the expression of the targeted genes. As shown in Table 3.3, there was a slight decrease in expression of *micF*, *ompC*, *sdeXY* , *sdeAB* and *hasF* and a sizeable increase in *ompF* expression (4.5

Table 3.3. RT-PCR of wild-type *S. marcescens* UOC-67 and *rob3* knock-out strain (SMRD3) for the evaluation of the expression of various genes.

Genes / Strain	*micF*	*ompF*	*ompC*	*sdeXY*	*sdeAB*	*hasF*
wild-type *S. marcescens* UOC-67	1	1	1	1	1	1
S. marcescens SMRD3	0.25±0.04	4.5±0.5	0.22±0.08	0.26±0.04	0.56±0.08	0.65±0.04

n=3

fold) for SMRD3 when compared to *S. marcescens* UOC-67.

Our RT-PCR results for *micF* were consistent with what was found with an *E. coli rob* mutant, although, a different technique, Northern blotting, was used in that study (Bennik et al. 2000). As *micF* is one of the *mar/sox/rob* regulons and *micF* regulates the *ompF* expression, any alteration in *micF* transcription will affect OmpF production. In addition, various environmental signals can affect the ratio between OmpF and OmpC (Delihas and Forst 2001) due to compensatory mechanisms by which the cell tries to maintain the relative copies of total OmpC and OmpF constant (Lugtenberg et al. 1976). Similar to this study, we found an increase in *ompF* expression in S. *marcescens* SMRD3, while a decline in *ompC* transcription was observed.

Moreover, our RT-PCR results showed that transcription of genes encoding MDR efflux pumps, *sdeXY* and *sdeAB*, and their probable common outer membrane portal, *hasF* were decreased in the *S. marcescens rob*3 mutant. In *S. marcescens*, it is suggested that both SdeXY and SdeAB are similar to *E. coli* AcrAB in the context of substrate, specificity, while structurally SdeXY has a higher identity with *E. coli* AcrAB (Kumar and Schweizer 2005: Chen et al. 2003). AcrAB is considered to be the major efflux pump found in members of the Enterobacteriaceae family (Fralick 1996; Oethinger et al. 2000; Okusu et al. 1996; Fabrega et al. 2010) and TolC is an important residue to form tripartite MDR efflux pumps such as AcrAB, AcrEF, EmrAB, MacAB (Rosner and Martin 2009; Piddock 2006; Aono et al. 1998; Koronakis et al. 2004). TolC

synthesis is regulated by transcriptional activators, MarA, Rob and Sox.

In *S. marcescens*, SdeAB and SdeXY are two major RND efflux pumps, having divergent substrate specificities (Chen et al. 2003; Kumar and Worobec 2005; Kumar and Schweizer 2005) while *S. marcescens* HasF possesses 98% identity with *E. coli* TolC at the amino acid level and has a similar function (Maseda et al. 2009). HasF is thought to function as the outer membrane portal of SdeAB and SdeXY (Maseda et al. 2009; Begic and Worobec 2008; Hornsey et al. 2010). Our study showed that a decrease in Rob results in the decrease of these two pumps and their common outer membrane component HasF.

CHAPTER 4: Conclusion and Future Studies

The overall goal of this research was to identify and characterize a Rob-like transcriptional activator in *S. marcescens*. My hypothesis was *S. marcescens* produces a *rob*-like transcriptional activator that contributes to antibiotic resistance by controlling the expression of the SdeAB-TolC pump and OmpF and OmpC porins. This hypothesis was substantiated and the overall goal was achieved by completing the following objectives:

Objective 1. Identify and clone a *rob*-like gene from *Serratia marcescens*. Three different candidates for the *E. coli rob* homologue in *S. marcescens* were identified, of these *rob*3 showed the highest identity with that of *E. coli*. In addition, subsequent experiments demonstrated that *S. marcescens rob*3 has similar properties to *E. coli, Salmonella* Typhimurium and *Enterobacter cloacae rob*.

Objective 2. Evaluate *rob* gene promoter function by transcriptional fusion using a GFP reporter system. Promoter activity assessment using the pGlow-TOPO reporter plasmid, showed that all three candidates had promoter activity, with *rob*3 having the highest activity at both 30°C and 37°C temperatures. Moreover, *rob*3 had higher promoter activity at 37°C than 30°C. This differed from what was found for *E. coli rob* but was similar to what was found in *S.* Typhimurium.

Induction of *rob* transcription by 2,2'-dipryridyl was found to be similar to that of *E.coli*. In this process, conversion of inactive or low-active Rob to high-active form of

Rob is likely. As we found similar levels for induction by 2,2'-dipyridyl, for both wild-type *S. marcescens* and *rob*3 knock-out *S. marcescens*, suggesting that unlike most other bacterial regulator proteins which negatively modulate their over-expression, the active form of Rob is not able to repress transcription of *rob*.

We showed that sodium decanoate and sodium salicylate can negatively effect the transcription of *rob* at 30°C and 37°C. This reduction was observed more potently when *rob* was exposed to sodium decanoate at 30°C. In *E. coli*, it was recently found that salicylate is able to trigger MarA synthesis and consequently, MarA binding to the *rob* promoter prevents the accession of RNA polymerase to the *rob* promoter by a steric hindrance mechanism. A similar mechanism in *S. marcescens* is likely.

Although, it was shown in *E. coli* that either dipyridyl and sodium decanoate can directly bind to pre-existing low-active state of intracellular Rob and change conformation to make it active, these compounds can also affect transcription of *rob*. How this occurs is not well understood. It is likely that these two compounds alter the structure of Rob in different ways, allowing for differential binding by 2,2'-dipryridyl to the *rob* promoter resulting in transcription activation.

Objective 3. Create a *rob* gene knock-out strain of *Serratia marcescens*. Using the TargeTron Gene Knockout System, we created *rob* null mutants for each *rob* candidate in *S. marcescens*. Mutants were confirmed by PCR and DNA sequencing and used in subsequent experiments.

Objective 4. Compare Minimum Inhibitory Concentration (MIC) for diverse antibiotics for wild type and knock-out strains of *Serratia marcescens.* Over-expression of *rob*3 (pUSRob3) in both *rob*-inactivated *E. coli* and *rob* null mutant of *S. marcescens* (SMRD3) resulted in reduced susceptility to multiple antibiotics that are considered substrates for the efflux pumps, SdeXY and SdeAB. These results suggest that *sdeAB* and *sdeXY* are regulated by Rob. Moreover, as found in *E. coli*, the other factor contributing to the antibiotic resistance was the over-expression of OmpF, resulting in limited permeability to various antibiotics.

Objective 5. Study the effect of the *rob* knock-out on efflux pump expression. RT-PCR studies using wild-type *S. marcescens* and *rob*3 null mutant strains showed a decrease in *sdeAB*, *sdeXY* and *hasF* expression. As SdeXY and SdeAB are two important efflux pumps in *S. marcescens* and HasF is likely their common portal, RT-PCR results suggest that Rob is involved in regulation of the main efflux pumps in *S. marcescens* as a transcriptional activator. This was confirmed by ethidium bromide accumulation assays and organic solvent tolerance experiments.

Objective 6. Study the effect of the *rob* knock-out on outer membrane protein expression. RT-PCR results showed that expression of *ompC* is decreased and concomitantly *ompF* is increased in *rob* null mutant strains of *S. marcescens* indicating that *rob* is involved in the expression of these porins, likely via *micF* which is also decreased in the null mutant strains. *micF* is a repressor of *ompF*.

Future studies:

To date, two transcriptional activators, SdeR and Rob, have been identified in *S. marcescens*. To investigate the effect of these transcriptional activators on the expression of genes comparable to those found in *E. coli mar*/*sox*/*rob* regulons, construction of *rob* and *sdeR* double mutant strains is necessary. Using these double mutant strains, examination of the antibiotic susceptibility for divergent antibiotics and RT-PCR of *sdeAB*, *sdeXY*, *ompF* and *micF* should be performed.

I also suggest that the role of *S. marcescens rob* in resistance in clinical isolates should be examined by assessing *rob* promoter function and expression (RT-PCR) of the key resistance genes (i.e. *mar*/*sox*/*rob* regulon).

Appendix

*rob*1 sequence from Sanger website and ORF Finder and Rob1 sequence from Expasy.

```
  1 atgcaccagcaacaggtgattgaacagctgctggcctggatcgag
    M  H  Q  Q  Q  V  I  E  Q  L  L  A  W  I  E
 46 cagagcctggatcagccgctgacgctggacgacattgccgccaag
    Q  S  L  D  Q  P  L  T  L  D  D  I  A  A  K
 91 tccggctactccaagtggcatttgcagcggatattcaagcagcat
    S  G  Y  S  K  W  H  L  Q  R  I  F  K  Q  H
136 accggccatattctcggcacttacgcgcgccgcagaaggctgacc
    T  G  H  I  L  G  T  Y  A  R  R  R  R  L  T
181 gccgccgcgcgcgaactgcgcctgaccggcaccagcgtggcctgc
    A  A  A  R  E  L  R  L  T  G  T  S  V  A  C
226 atcgccgatacttaccagttcgattcacagcagaccttcacccgc
    I  A  D  T  Y  Q  F  D  S  Q  Q  T  F  T  R
271 tgcttccgcaagcaattcggtttgccgccggccagctatcgccgc
    C  F  R  K  Q  F  G  L  P  P  A  S  Y  R  R
316 agccaggattggtcgagctatggcctgcagccgccgctgcggctg
    S  Q  D  W  S  S  Y  G  L  Q  P  P  L  R  L
361 accgaagcgccgttgccgcaggccgacatcgtgacgctgcccgcc
    T  E  A  P  L  P  Q  A  D  I  V  T  L  P  A
406 atgcagctggtgggcaacacccagcgccgcagcttcacgctgggg
    M  Q  L  V  G  N  T  Q  R  R  S  F  T  L  G
451 cagttggcggactccaagtgcgagctgcgccgccatgcctggcgg
    Q  L  A  D  S  K  C  E  L  R  R  H  A  W  R
496 caactgctgcggccgcaggcgctgccggaggtggtgtacggcctc
    Q  L  L  R  P  Q  A  L  P  E  V  V  Y  G  L
541 accagcctggaggtcgacaggcaacgccggggctgcccgcgtatg
    T  S  L  E  V  D  R  Q  R  R  G  C  P  R  M
586 gcgtataccgccgctttgccggacgaaggggcgatgggggagcgg
    A  Y  T  A  A  L  P  D  E  G  A  M  G  E  R
631 gtgactatcgaacagggggagtatgcccgtttcacctatcagggg
    V  T  I  E  Q  G  E  Y  A  R  F  T  Y  Q  G
676 caggcggaagggttacaaaactttattgtccgattgtatgacact
    Q  A  E  G  L  Q  N  F  I  V  R  L  Y  D  T
721 gccatgccgcagatgaacgccatccgccggccggggcaggatatc
    A  M  P  Q  M  N  A  I  R  R  P  G  Q  D  I
766 gagcgtttctacccggcgcaggagggctgttgcccgctcggcggc
    E  R  F  Y  P  A  Q  E  G  C  C  P  L  G  G
811 gcggcgatccgctgtgaatatctgatccctattcggcgggtggaa
    A  A  I  R  C  E  Y  L  I  P  I  R  R  V  E
856 gcgttggccgccgccagttag 876
    A  L  A  A  A  S  *
```

*rob*2 sequence from Sanger website and ORF Finder and Rob2 sequence from Expasy.

```
  1 atgacaaatgaagacatttttttattgaagagctaattgagtgg
    M  T  N  E  D  I  F  F  I  E  E  L  I  E  W
 46 gtggagatacatctggagaaacggccaaacctggatgaagtcgcg
    V  E  I  H  L  E  K  R  P  N  L  D  E  V  A
 91 cgtatttcgggctattccaagtggcatctgcagcgtaaattcaag
    R  I  S  G  Y  S  K  W  H  L  Q  R  K  F  K
136 cgtattaccggcattcaactcgccacctatatccgttcgcgcatc
    R  I  T  G  I  Q  L  A  T  Y  I  R  S  R  I
181 ctgacgcgcgccgcggtggcgctgcgcattactcgccgctccatc
    L  T  R  A  A  V  A  L  R  I  T  R  R  S  I
226 atcgacatttccgatgagctgggcttcgattcacagcagaccttc
    I  D  I  S  D  E  L  G  F  D  S  Q  Q  T  F
271 acccgcatgttcaagcagcgcttcggcaccacgcccaatcgctac
    T  R  M  F  K  Q  R  F  G  T  T  P  N  R  Y
316 cgctcgatggcgcactgggacgtgaaaaacctgatgccgcgcttt
    R  S  M  A  H  W  D  V  K  N  L  M  P  R  F
361 aacttcgaagccagctacggtgccggttattatccggaagtgaag
    N  F  E  A  S  Y  G  A  G  Y  Y  P  E  V  K
406 cggttgacgctgcccgacatgcagttggtcggcttcacgcgccgg
    R  L  T  L  P  D  M  Q  L  V  G  F  T  R  R
451 ttggatttcgcctccgaacaggagctggaatattcttcctgcatg
    L  D  F  A  S  E  Q  E  L  E  Y  S  S  C  M
496 gcgatgaaggacgagattttcaacgacttcttcaaggggttgcac
    A  M  K  D  E  I  F  N  D  F  F  K  G  L  H
541 gttgactgtcggcgaatttacagcatttattctcctcatgccggg
    V  D  C  R  R  I  Y  S  I  Y  S  P  H  A  G
586 gagggcgacgagctctcgtccacgttggtgatggcggtcgatcct
    E  G  D  E  L  S  S  T  L  V  M  A  V  D  P
631 gaacacaaaaaggatattctttccaaccatcagatcgacaccttc
    E  H  K  K  D  I  L  S  N  H  Q  I  D  T  F
676 catctgccgagccgcgagtttatctccatcaaccataagggcacg
    H  L  P  S  R  E  F  I  S  I  N  H  K  G  T
721 gcgaaagagtgtctgcagtttttcggctacctgatgtcgcatgtg
    A  K  E  C  L  Q  F  F  G  Y  L  M  S  H  V
766 atgccggggctgaaggatgaggtgcgcggcagtatggaaatggag
    M  P  G  L  K  D  E  V  R  G  S  M  E  M  E
811 atcattcaaaccaaagagtggaaccccgagtccaaactgcgccag
    I  I  Q  T  K  E  W  N  P  E  S  K  L  R  Q
856 attgaagtggattacacctacctgatttctatcgattaa 894
    I  E  V  D  Y  T  Y  L  I  S  I  D  *
```

*rob*3 sequence from Sanger website and ORF Finder and Rob3 sequence from Expasy.

```
870 atggatcaagccggtatcattcgtgatctgcttagctggctggaa
    M  D  Q  A  G  I  I  R  D  L  L  S  W  L  E
825 agccatttggaccaacccttgtcgctggataacgtggcggccaag
    S  H  L  D  Q  P  L  S  L  D  N  V  A  A  K
780 gccggctactccaaatggcatctgcaacggatgttcaaagatatt
    A  G  Y  S  K  W  H  L  Q  R  M  F  K  D  I
735 accggtaatgccatcggtgcttacatccgggcaaggagactgtcc
    T  G  N  A  I  G  A  Y  I  R  A  R  R  L  S
690 aaagccgccgtcgcgctgcgcctgaccagccggccgattttggat
    K  A  A  V  A  L  R  L  T  S  R  P  I  L  D
645 atcgccctgcaatatcgtttcgactcgcagcagaccttcacccgc
    I  A  L  Q  Y  R  F  D  S  Q  Q  T  F  T  R
600 gccttcaaaaaacagtttgcgcaaacgccggcgctgtaccgccgc
    A  F  K  K  Q  F  A  Q  T  P  A  L  Y  R  R
555 gccgaggactggaatgcgttcggcatctgtccgccgatccgtctg
    A  E  D  W  N  A  F  G  I  C  P  P  I  R  L
510 ggggccttcactctgccgcagccggaattcgtctcgctgccagac
    G  A  F  T  L  P  Q  P  E  F  V  S  L  P  D
465 aaacacctggtcggcctgacccaaagctactcctgtacgctggaa
    K  H  L  V  G  L  T  Q  S  Y  S  C  T  L  E
420 cagatcaccaccgtgcgcactgaactgcgctcgcagttctggcgt
    Q  I  T  T  V  R  T  E  L  R  S  Q  F  W  R
375 cagttcctcggtgacgtcgaaaccctgccgccggtgctgtacggg
    Q  F  L  G  D  V  E  T  L  P  P  V  L  Y  G
330 ctgcaccactcgcgcccaagccaggagaaggcgaacgaacaggaa
    L  H  H  S  R  P  S  Q  E  K  A  N  E  Q  E
285 gtgctgtataccacggcgctggagccggatcaggtgcctgacaag
    V  L  Y  T  T  A  L  E  P  D  Q  V  P  D  K
240 gtacaggaaggccagccgctggtgttgccgggcggtgagtttgcg
    V  Q  E  G  Q  P  L  V  L  P  G  G  E  F  A
195 atgttcagctacgaagggccaaccgagaacctgcaagactttatt
    M  F  S  Y  E  G  P  T  E  N  L  Q  D  F  I
150 ctgacggtgtacggcacctgcctgccggcgctccagctgacgcgc
    L  T  V  Y  G  T  C  L  P  A  L  Q  L  T  R
105 cgcaaagggcacgacatcgaacgcttctacccgaaaggcgagcgc
    R  K  G  H  D  I  E  R  F  Y  P  K  G  E  R
 60 cgtccgcatcaggcgcctatcgagatcaagtgcgattacctgatc
    R  P  H  Q  A  P  I  E  I  K  C  D  Y  L  I
 15 ccgattcggcgttaa 1
    P  I  R  R  *
```

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