resistance of serratia marcescens from food industry to quaternary ammonium compounds

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RESISTANCE OF SERRATIA MARCESCENS FROM FOOD INDUSTRY TO QUATERNARY AMMONIUM COMPOUNDS

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Abbreviations

ATCC	American Type Culture collection
BAC	Benzalkonium Chloride
CPC	CetylPyridimine Chloride
DNA	Deoxyribonucleic Acid
MIC	Minimum Inhibitory Concentration
PCR	Polymerase Chain Reaction
PPM	Part perMilliion
QAC	Quaternary Ammonium compound
RND	Resistance Nodualtion cell Divison superfamily
RPM	Revolution per minute
RT	Reverse Transcription
RT PCR	Real time Polymerase Chain Reaction
TSB	Trypticase Soya Broth
TSA	Trypticase Soya Agar
TAE	Tris Acetate EDTA
O.D	Optical Density

ABSTRACT

In earlier days, antibiotic resistance was more concern than disinfectant resistance. During the last 20 years there has also been a focus on disinfectant resistance because of fear that wide spread used of disinfectant in any of the settings hospital, food industry may overall contributes for resistance to antibiotics. The aim of the study is to elucidate the mechanism of resistance to quaternary ammonium compounds, a group of disinfectants, among *Serratiamarcescens* from the food industry. In this study we determined the minimum inhibitory concentration (MIC) and bactericidal value for 13 *Serratiamarcescens* strains isolated from the food industry and two laboratory strains of *Serratiamarcescens* with two quaternary ammonium compounds Benzalkonium chloride (BAC) and CetylPyridimine Chloride (CPC). Strains from food industry were resistant having less than 3 $\log_{10}$reduction on the Bactericidal test whereaslaboratory strains has more than 5 $\log_{10}$reduction. One of the laboratory strains was sensitive to CPC in the MIC test, while the other was sensitive to BAC. The laboratory strains were adapted to higher concentration as footbath strain by serially subculturing them in increasing concentration up to 200 ppm.

*Serratiamarcescens*has been reported to gain resistance to QACs by expression of sdeAB efflux pumps, due to mutation in *sdeS,* a gene encoding the repressor protein sdeS. In our study we sequenced *sdeS* and found that there were 11 nucleotide substitutions on the *sdeS* gene between sensitive and adapted/resistant strains. These substitutions refer to two amino acid substitutions in the putative sdeS proteins common in all the strains (adapted and resistant) at position 157 and 159 from threonine to alanine and aspartic acid to glycine respectively. Besides this there was insertion of glutamine in position 153 in all the adapted strains and two footbath strains. Since the amino acid substitutions were found in all strains, this indicates the*sdeS* may be involve in resistance, however further studies is needed in order to prove this.

Biocide resistance confers a biological cost.We observe that BAC adapted strains exhibited a fitness cost, having reduced growth rate; there was

significant difference on the growth rate ($p<0.05$). Strains lost its acquired adaptation, in our study also from 17 up to 85 percentages of adapted strains lose its acquired resistance after ten cultivation in media without quaternary ammonium compounds.

PCR revealed that all tested strains contained the gene for efflux pumps (sdeAB, sdeXY, sdeCDE).

In conclusion the work in thesis showed that *Serratiamarcescens*from the food industry have common mutation in the *sdeS* gene. Further studies are necessary in order to elucidate the resistance mechanism. Resistance to QAC is not a stable trait. The presence of resistant strains in the food industry, indicate that the use of QAC in the food industry selects for QAC resistance *Serratiamarcescens.*

1. INTRODUCTION

1.1 Bacterial resistance to disinfectants

In earlier days, antibiotic resistance was more concern than disinfectant resistance. During the last 20 years there has also been a focus on disinfectant resistance because it's found that wide spread used of disinfectant in any of the settings hospital, food industry may overall contributes for resistance to antibiotics(A. Russell, 2003).Biocides used as part of a thorough combined cleaning and disinfection programs, are an essential weapon in the food industry hygiene to control pathogenic and spoilage microorganisms (Holah, Taylor, Dawson, & Hall, 2002). The industry also recognizes the microorganisms that adhered to surfaces are more resistant to disinfectants than those in suspension (Foschino, Nervegna, Motta, & Galli, 1998; Frank & Koffi, 1990; Gibson, Elton, Peters, & Holah, 1995; Ridgway & Olson, 1982)

The term resistance is a relative term in case of disinfectants. Resistant strains are those strains that are either not killed or not inhibited by the concentration that usually kill/inhibit the most of the strains of that organism(A. Russell, 2010). In this study strains are termed as resistant if it's have less then 5log reduction with recommended use in concentration of disinfectants.

Bacterial resistance can broadly classify in to two types:

1.1.1Intrinsic resistance

Innate resistance is due to the innate natural characteristic properties of the organism as phenotypic, physiological or biochemical properties or seen in bacterial spores, biofilms. Chromosomally transcribed efflux pumps and membrane permeability barrier are phenotypic properties conferring intrinsic resistance to QACs. Intrinsic resistance due to outer membrane in gram negative bacteria that acts as protective barrier, they are generally less susceptible to antiseptic and disinfectants than non-sporulation, non-mycobacterial gram positive bacteria(A. Russell, 1997; A. Russell & Gould, 1988). The outer membrane of the Gram-negative bacteria acts as a barrier that limits the entry of many chemically unrelated types of antibacterial agents.An efflux pump pumping out a variety of agents mostly (lipophilic or amphipathic molecules) is also responsible for intrinsic resistance of Gram-negative bacteria (Beumer et al., 2000).

In case of *Serratiamarcesces*SdeAB,SdeXY,SdeCDEefflux system acts as transporters for a whole range of biocide and antibiotics (Chapman, 2003).

Intrinsic resistance besides efflux pump,impaired uptake some microorganism shows intrinsic resistance through inactivation of biocides. Inactivation of biocides like phenol and some aldehydes by pseudomonas species(Chapman, 2003). Inactivation of QACs have been seen but that cannot be considered as resistance mechanism because the compound is only inactivated below the concentration used in practice.(Fraise, Maillard, & Sattar, 2012).

Intrinsic resistance also may be due to physiological adaptation of microorganism that increases resistance to biocides due to change in environmental conditions. The physiological adaptation might be due to the change in the outer cell layer structure, which prevents access to its site of action. Biofilm formation of microorganismis also associated with the microorganism in the solid surface accounts for the intrinsic resistance in microorganism(Gilbert, Collier, & Brown, 1990).

1.1.2 Acquired resistance

Resistance due to relative changes in disinfectant tolerance upon sub lethal exposure or due to the result of genetic changes either by mutation or acquisition of plasmid and transposons as seen in Methicillin resistance *Staphylococcusaureus* (MRSA) strains, (A. Russell, 1995; Tezel & Pavlostathis, 2011). Acquired resistanceis often unstable, and they may lose their resistant properties if grown in a biocide free environment for a long time (Fitzgerald et al., 1992).

1.1.2.1Acquired resistance due to plasmid mediated mechanism

Although there is increased resistance of bacteria having plasmid to biocides but the exact physiological basis for increased resistance is unknown(Beumer et al., 2000). There is also no specific evidence of direct involvement of plasmid on bacterial resistance to plasmid. First example of plasmid involvement was seen in increased resistance to metal such as silver and mercury(A. Russell, 1997).In case of *Staphylococcus aureus* plasmid mediated biocide resistance is explained on the basis of MIC value. Reduced susceptibility to QACs and BAC is mediated by structurally similar group of plasmid encoding *qac* genes(Mitchell, Brown, & Skurray, 1998; Tennent, Lyon, Gillespie, May, & Skurray, 1985).

1.1.2.2Acquired resistance due to mutation

Mutation on the specific target site of the biocide also involves in the resistance due to biocides but it is not a common phenomenon as in case of antibiotics (Beumer et al., 2000). In *E.coli*triclosan acts on enoylreductase and the mutants lacking this enzyme shows increased resistance towards triclosan(McMurry, Oethinger, & Levy, 1998).

1.2 Cationic and amphoteric tensides as disinfectants

Quaternary ammonium compounds (QACs) cationic surfactants, are important antiseptic and disinfectants in various industry and health care centers(Frier, 1971)because they have several advantages over other commonly used disinfectants, such as no corrosiveness, high surface activity and relatively low toxicity(Langsrud and Sundheim, 1997). Although QACs were described in 1916 but was commercially used after around 20 years(A. Russell, 2002).

QACs are organic compounds having a central nitrogen atom attached covalently to four functional groups (R4N+). Among the four functional groups at least one group (R) is a long-chain alkyl and the other is either benzyl or a methyl group (Cross and Singer, 1994). Dialkonium and Benzalkonium chloride (BAC) are most commonly used QACs(Tiedink 2001). The biocidal activity of QACs is dependent on the chain length of alkyl groups. QAC with hydrophobic tail (C16) affects the outer membrane of gram negative bacteria more effectively then shorter-chain compounds, it might be due to strong interaction between the fatty acid portion of the lipid and long alkyl chain. Monoalkyl QACs binds to the membrane surface by ionic and hydrophobic interactions with the cationic head group facing outwards and the hydrophobic tails inserted into the lipid bi-layer,causing rearrangement of the membrane and subsequent leakage of intracellular constituents (Ioannou et al.,2007).

QACs target mostly at the cytoplasmic membrane of the bacteria(Ioannou, Hanlon, & Denyer, 2007; A. D. Russell & Block, 2001)and membrane bound enzymes. QACs attack on cytoplasmic membrane causes leakage of intracellular material and initiation of autolysis so there is entire loss of structural organization and integrity. QAC at higher concentrations also causes coagulation and precipitation of cytoplasmic constituents. Mechanism of interaction is electrostatic ionic interaction with phospholipid(Denyer, 1995).

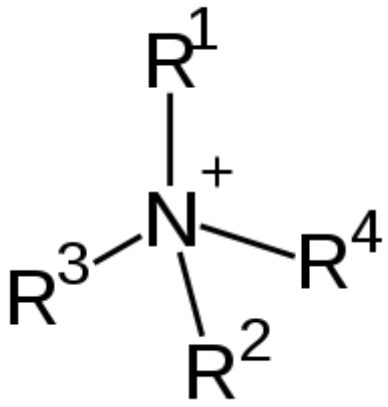

Fig1: Quaternary ammonium cation. The R groups may be the same or different alkyl or aryl groups. Also, the R groups may be connected.

1.3Serratiamarcescens

*Serrratiamarcescens*is a gram negative, oxidase negative bacillus and a member of Enterobacteriaceae family. The name *S. marcescens* was assigned by the Bizio in 1823 and was defined by Grimont and Grimont(Hejazi & Falkiner, 1997) .

Serratiamarcescens is responsible for nosocomial infection, its first infection was reported in 1951 at Stanford university Hospital in 1951 (Papapanagiotou & Aligizakis, 1959). In the food industry, *Serratiamarcescnes* is a concern because of their growing ability under extreme conditions. It is widely found in food particularly in starchy variants and its ability to utilize the wide range of nutrients including in antiseptics(Nakashima, Highsmith, & Martone, 1987),disinfectants(Parment, Rönnerstam, & Walder, 1986; Sautter, Mattman, & Legaspi, 1984).

1.4 Efflux pumps and regulation of efflux in *Serratiamarcescens*

Serratiamarcescens encodes at least three RND-type efflux pumps, SdeAB, SdeCDE and SdeXY, which play a significant role in the resistance to antibiotics and biocides (Maseda, Hashida, Konaka, Shirai, & Kourai, 2009). The SdeAB pump transports ciprofloxacin, norfloxacin, ofloxacin, chloramphenicol and surfactants; SdeCDE for novobiocin; and SdeXY for erythromycin, tetracycline, norfloxacin, ampicillin and biocides(Chen, Kuroda, Huda, Mizushima, & Tsuchiya, 2003). The wild-type *S.*

*marcescens*only expresses SdeAB and SdeCDE(Begic & Worobec, 2008). The wild type *S. marcescens* produced undetectable level of sdeB protein but when it is exposed to gradually increasing concentration of biocide, it gains resistance and shows increased levels of sdeAB protein(Maseda et al., 2009). In sdeAB operon, expression of the sdeAB operon is regulated by the repressor,*sdeS* gene located on the downstream of the sdeAB operon(Maseda, Hashida, Shirai, Omasa, & Nakae, 2011). *sdeS* gene encodes a sdeSrepressor protein that binds to the promoter operator region of the sdeAB operon and blocks the transcription, so any non-sense mutation on *sdeS*gene may result into the expression of the sdeAB operon(Maseda et al., 2011).

1.5 Cross Resistance

From the different studies carried out some studies shows that there is an association between the increased biocides resistant and antibiotic resistance and vice versa while some investigation shows that there is no such association.(Beumer et al., 2000)

Resistance to one disinfectant might cause increased resistance to other antimicrobial agents. Cross resistance between two or more antibacterial agents may occur due to following reasons:

(A) Their similarity of mechanism of action (B) Similarity in mechanism of resistance (C) Have the similar pathway to reach the target (Jones, Herd, & Christie, 1989; Sundheim, Langsrud, Heir, & Holck, 1998). Cross resistance of CPC resistant *Serratiamarcescens* to flouroquinolonedue to the involvement of sdeAB pump is one of the example of cross resistance of biocides and antibiotics(Maseda et al., 2011).

There are also the cases where the antibiotic resistance strain does not show any resistant to biocides.Antibiotic resistance Enterobacteriace strains were not resistant to amphoteric Tego disinfectants(Stecchini, Manzano, & Sarais, 1992).Clinical and environmental isolates including *Pseudomonas aeuroginosa*, *Klebsiella*ps, *E.coli* were not resistant to bactericidal activity of disinfectants like QACs, povidine iodine, chloroxylenol(Payne, Babb, & Bradley, 1999) .

In Gram-negative bacteria chromosomally mediated efflux pumps are intrinsic resistance of bacteria to both biocides and antibiotics so plays an important role in multiresistance. Environmental stimuli or phenotypic adaptation increases the

expression of these pump which in turn increases the expression of these pump and increases resistance(Beumer et al., 2000).

1.6 costs of fitness and stability of resistance

The association of antimicrobial resistance mechanism and fitness cost is observed as decreased bacterial growth rate. The rate of development and stability of resistance are dependent on the magnitude of the cost (Andersson & Hughes, 2010). The biological cost of resistance can predict the risk of resistance development because if resistance severely reduces the fitness, even though mutation rates are high number of resistant mutation will be outcompeted by wild type strains(Andersson, 2003; Andersson & Hughes, 2010)

2. MATERIAL AND METHODS

2.1 Primers

Primers used in this study and their sources are listed in table 1

Table1. Primers used in this study this study

Primer Name	Target	Sequence (5'-3')	Source
sdeB1 LC F	sdeB	AGATGGCCGATAAGCTGTTG	(Hornsey et al., 2010)
sdeB1 LC R		CAGCGTCCAGCTTTCATACA	(Hornsey et al., 2010)
sdeD LC F	sdeD	AGCTTCATTCATCCGGTCAC	(Hornsey et al., 2010)
sdeD LC R		CATGATGGCGTTCTTCTTCA	(Hornsey et al., 2010)
sdeY LC F	sdeY	TCCATCAACGAAGTGGTGAA	(Hornsey et al., 2010)
sdeY LC R		GTTTATCGAGAAGCCGAACG	(Hornsey et al., 2010)
SmarpoB F	rpoB	CTAACGAGTATGGCTTCCTG	(Hornsey et al., 2010)
SmarpoB R		CTTCTTCATCCAGGTTGGAG	(Hornsey et al., 2010)
SdeS3Hin	SdeS	CAGAAGCTTGTGCGATTAAG CGGTGTGTTCG	(Maseda et al., 2011)
SdeS4Eco		CAGGAATTCGACGACGCCG GTGTTCTACGTCAC	(Maseda et al., 2011)
sdeS_seq	*sdeS*	CGGAATTCTTGGCAAACGGC GATCGGGT	(Maseda et al., 2011)
sdeS_seq		GTGCGATTAAGCGGTGTGTT CG	(Maseda et al., 2011)
sdeB2 F	*sdeB*	CAGCGTCCAGCTTTCATACA	(Dalvi & Worobec, 2012)
SdeB2 R		TACGGTGGTGTTTACGACGA	(Dalvi & Worobec, 2012)
RplU F	*rplU*	GCTTGGAAAAGCTGGACATC	(Dalvi & Worobec, 2012)
Rpl U R		TACGGTGGTGTTTACGACGA	(Dalvi & Worobec, 2012)

2.2 Bacterial strains and growth conditions

The *Serratiamarcescens* strains used in this study are described in table 2. Seven of these strains had previously been isolated from Norwegian dairies from disinfection footbaths containing the tenside TEGO103G or TP-99, an alkylaminoacetate. The strains were previously shown to be resistant to the quaternary ammonium compound (QAC), benzalkoniumchloride (BAC) (Langsrud, 2003). The strains MF2325, MF2326

and MF3324 were previously isolated from a floor of the department of a poultry processing plant. The flooring material contained low concentrations of triclosan, but the floor had extremely low antibacterial activity. The poultry processing plant alternated between QAC and hypochlorite for disinfection (Møretrø,2006). MF3613 was previously isolated from meat saw in an abattoir. The abattoir was daily disinfected with TP-99 (Heir, unpublished).

Table 2.Bacterial strain used in this study

Mf number*	Strain name	Origin	Refrences
2336	N9	Footbath Tego 103G	Langsrudet.al.,2003
3309	B25	Footbath TP99	Langsrusd et.a.,l2003
3306	O6	Footbath Tego 103G	Langsrusd et.a.,l2003
3308	B19	Footbath Tego 103G/TP99	Langsrusd et.al.,2003
3299	N6	Footbath Tego 103G	Langsrusd et.al.,2003
3297	No17	Footbath TP99	Langsrusd et.al.,2003
3300	N7	Footbath Tego 103G	Langsrusd et.a.,l2003
2325	13R	Floor with triclosan Poultry processing plant	Møretrø, 2006
2326	13H	Floor with triclosan poultry processing plant	Møretrø, 2006
3224	32B	Floor with triclosanPoultryprocessing plant	Møretrø ,2006
3613	6.2	Meatsaw, abattoir	Heier,unpublished
ATCC 13880	MF 2602		American Type culture collection
ATCC 14041	MF 2006		American Type culture collection

*MF numbers refer to Nofima's strain collection

2.3Preparation of stock culture

Bacterial glycerol stock was prepared and stored at -80 °C for long time storage. Under sterile condition, a single colony was transferred from TSA plate to TSB and incubated at 30°C at

200 RPM for 24 hours. Then One ml of overnight bacterial culture was mixed with 300µl of 87% (v/v) glycerol and was frozen immediately at -80°C for future uses.

2.4 Identification of the strains (API)

For the identification of the organism as *Serratiamarcescens*Rapid 20E API system (BioMerieuxSA,Marcy 1'Etoile, France) was used. Protocol in the kit was followed. Apilabplusversion 3.2.2(BioMerieux SA) software was used for the analysis.

2.5 Minimal Inhibitory Concentration (MIC)

The MIC value of two different quaternary ammonium compounds (QACs) Benzalkoniumchloride(BAC) and Cetylpyridimine chloride(CPC) was determined for all the wild type *Serratiamarcescens*from the food industry and laboratory strains. Concentrations of disinfectant starting form 0 up to 200 ppm were made on the interval of 25 (0,25,50 … 200 ppm) for both disinfectants in 3 ml TSB tubes. Overnight culture was prepared by transferring a single pure colony from TSA plate into 5ml TSB and incubated at 30°C for 18-20 hours with continuous shaking at 200 rpm. For every strain 50µl of the overnight culture was inoculate into each tube with different disinfectant concentration and were incubated at 30°C for 18-20 hours with continuous shaking at 200 rpm. A tube with 200 ppm of disinfectants were also incubated for 18-20 hours at 30°C, to check if incubation of disinfectants for long time might give false result.

The lowest concentration of disinfectantsthat prevented growth was noted as MIC value. MIC determinants were repeated twice. We only tested BAC and CPC up to 200ppm, because above that concentration BAC/CPC was precipitated in the media.

2.6 Adaptation to antimicrobial compounds

Strain ATCC 14041(sensitive to BAC) MF 3299, MF 3300(tolerant to BAC) and ATCC 13880(sensitive to CPC) were serially adapted up to 200 ppm on respective disinfectants. Strains ATCC 14041, MF 3299, MF3300 were serially sub cultured on a higher concentration of BAC and ATCC 13880 on CPC starting from their respective MIC value. Every day 50µl from the TSB tube with highest concentration of disinfectants permitting the growth, was transferred to 3 ml TSB tubes with increasing concentration of disinfectant, and incubated at 30°C with continuous shaking at 200 rpm

for 24 hours. Growth was observed by direct visualization. The process was repeated every day until growth was observed up to 200 ppm. Adaptation experiment was performed twice.

2.7 Bactericidal test

The disinfectant bactericidal effect was tested using modified Council of Europe suspension test (1987) for testing antimicrobial activity of disinfectant (S Langsrud & Sundheim, 1998)

For the preparation of bacterial test suspension, overnight culture of each strain was prepared by inoculating single colonies from the their respective TSA plate to individual 4.5 ml TSB tubes, and incubated at 30°C for 18-20 hours with continuous shaking at 200 rpm. Then 50 µl of the overnight culture was transferred to the 4.5 µl TSB tubes, and incubated at 30°C for 18-20 hours with continuous shaking at 200 rpm. So that cell density was expected to be approximately 10^9 cells ml^{-1}. Overnight bacterial culture was diluted to a concentration 10^8 cells ml^{-1} in peptone water.

For exposure of disinfectant One ml of peptone water diluted cell was transferred to nine ml of distilled water containing 200 ppm BAC. Control sample was prepeared by transferring 1ml of peptone diluted sample to nine ml distilled water without BAC, and were exposed for five minutes.

For neutralization of disinfectant 0.5 ml from both tubes were transferred to 4.5 ml Dey/Engley neutralizing broth (DE, Difco, Detroit USA). The final cell concentration was about 10^5 Cells ml^{-1}. The serial dilution on peptone water (Up to 10^{-4} dilution was performed from each Neutralization broth and plated on TSA plate usingwhitely spiral automatic plater, (Don whitley scientific limited, UK). The plates were incubated at 30°C for 2-3 days and colony count was performed using Protocol 2 (synbiosis, UK).

2.8 Isolation of DNA from bacterial cells

For isolation of DNA, each strain single colony was dissolved in 50 µl of TE buffer in a 96 well microtitre plate.The plate was heated for 10 min at 90°C, and was centrifuged for 3 min at 5000 rpm, Then 30 µl of supernatant was transferred to the new plate. Plates were stored at 4° C for further work.

2.9 Expression of pumps genes

For the Screening of the three pump gene *sdeB, sdeD, sdeY,* one house keepinggene*rpoB*and repressor gene *sdeS* simple PCR was performed as per following steps

2.9.1Polymerase chain reaction (PCR)

Polymerase Chain Reaction (PCR) invented KaryMulisin 1983, has become the most useful technique in molecular biology for the amplification of the specific region of DNA(Wolcott. 1992). In PCR for amplification of DNA fragments, Template DNA is mixed with specific primers, nucleotides(dNTPs),Buffer and thermo stable DNA polymerase. In this study PCR was used for the amplification of specific region of RND pump encoding genes responsible for resistance of *Serratiamarcescens*to antibacterial compounds.

PCR reaction and mixture for *sdeS*gene are shown below and in table 3, and primer used in this study are show in (Table 1).Gene Amp9700 (Applied biosystem, UK) was used for PCR reactions. PCR was performed for all the 13 strains from food industry, two laboratory strains and four adapted strains.

The following reagents were added to 25 µl PCR tubes for PCR of *sdeS*

19.5 µl of dH20

2.5 µl of hot start buffer

0.5 µl of dNTPs

0.5 µl of forward primer

0.5 µl of reverse primer

0.5 µl hot start polymerase

1 µl template DNA (DNA isolated from *Serrratiamarcescens*

Table 3.PCR optimized for Hot start polymerase

Functions	**Temperature(^{0}C)**	**Time**	**Cycles**
Initial denaturation	94	10 min	1
Denaturation	94	30 seconds	30
Annealing	60	30 seconds	30
Extension	72	30 seconds	30
Final extension	72	7 minutes	1
Storage	4	Hold	

PCR reaction mixture and condition for the screening of pump genes (*sdeB, sdeD,sdeY),* and housekeeping gene (*rpoB*)was carried out according to the condition shown below(Table 4) and the primer used is shown in (Table 1).

The following reagents were added to 25 µl PCR tubes

18.5 µl of dH20

2.5 µl buffer

1 µl of dNTPs

0.5 µl forward primer

0.5 µl reverse primer

1 µl polymerase

1 µl template DNA (DNA isolated from *Serrratiamarcescens*

Table4. PCR condition for pump gene (*sdeB,sdeY,sdeD*and*rpoB)*

Functions	**Temperature(^{o}C)**	**Time**	**Cycles**
Initial denaturation	94	4 min	1
Denaturation	94	30 Seconds	30
Annealing	60	30 Seconds	30
Extensions	72	45 Seconds	30
Final extension	72	7 minutes	1
Storage	4	Hold	

2.9.2Agarose Gel electrophoresis

Agarose gel electrophoresis is a technique of separation of DNA molecules varying in size (Watson 2008). The agarose gel consists of microscopic pores that act as a molecular sieve, which separates molecules based upon charge, size and shape. DNA is negatively charged molecule at neutral pH so it migrates towards anode in an electrical field. In agarose microscopic pores, larger molecules have more difficulty in mobility compare to the smaller DNA molecules. So smaller DNA molecules migrates faster in the gel. UV fluorescence intercalating molecules like Ethidium bromide and Gel red are used for the visualization

In this study, we used 1% agarose gel and stained with gel red for visualization of the amplified product. Amplified products were identified on the basis of their respective position compared to that of standard used. We used standard VI as the marker DNA.

Materials

Agarose

Tris-acetate(TAE) buffer 50 x

Gel red 10 mg/ml (aqueous)

DNA standard (Standard VI)

Loading buffer (Methyl Orange)

Procedure:

One percent agarose gel was made (4 gm of agarose was weighed and mixed with 400 ml TAE buffer).Agarosemixture was heated in microwave until it completely dissolves. The agar was cooled till it reaches around 40°C, and then poured into 60 ml agarosetray for solidification (ca. 20 minutes). A well comb was placed in the tray.The solidified gel was transferred to the electrophoresis chamber filled with 1X TAE buffer. Methyl orange was used as loading buffer .DNA sample was prepared by mixing with 10X methyl orange in the ratio 1:1 and applied in the wells of the gel. Standard VI was used as marker DNA. Gel was run at 80V for around 60 minutes. Visualization of the gel was done on gel doc ez Image analyzer (BioradInc, uk)

2.9.3 DNA sequencing

To find out the mutation of the *sdeS* gene on the resistance and adapted strains sequencing of *sdeS* gene was performed. ABI prism Big Dye Terminator cycle sequencing kit version 3.1 (Applied biosystem) was used for sequencing. Template DNA for sequencing was made by Exosap treatment to the earlier PCR product. Presequencing reaction was performed to remove the left-over of dNTP and primer .5 µl of the PCR product and 2 µl of the exosap was mixed and run in PCR for 37°C for 30 minutes and 80°C or 15 minutes and stored at 4° C.For DNA sequencing the following mixture was used .PCR reaction was performed as described in table no. 5

Reagents:1.5 µl big dye buffer(Applied biosystem)

1.0 µl big dye v 1.1(Appliedbiosystem)

1 µl primer

1 µl template DNA

8 µl dH2O

Table 5. PCR condition for DNA sequencing of *sdeS* gene

Temperature(^{o}C)	**Time**	**Cycles**
96	15 seconds	25
60	4 minutes	25
4		Hold

After PCR, in a new eppendrof tube 10 µl of the PCR product was mixed with 10 µl of x-terminator (Applied biosystem) and 45 µl of the SAM solution (Applied biosystem).The mixture was put on shaker for 30 minutes at 1500 RPM. Then it was centrifuged at 2500 rpm for 2 minutes and was sequenced in a genetic analyzer3130XL(Applied biosystem).

2.9.4Expression of *sdeB* gene

Expression of *sdeB* gene was performed, to find out difference in the level of expression of this gene between sensitive adapted and resistant strains.

2.9.4.1 RNA isolation

Total RNA was isolated from the strains (ATCC 13880, ATCC 14041, ATCC 13880 adpI, ATCC 14041 adpI).For the gene expression analysis, RNA should be extracted from the log phase of bacterial growth. So to calculate the log phase of bacterial growth, inoculum size of 10^{6}cells ml^{-1} was made by 10^{-3} dilution of the overnight culture on TSB, then 5ml of it was transferred to 45 ml of TSB and then incubated at 30°C at 200 rpm. In the interval of every 20 minutes, O.D was measured and was also plated on TSA. Then TSA plates were incubated at 30°C for 24 hours, colony count was made on protocol2 (symbiosis, UK). The O.D. vs. time vs. cell count graph was plotted to calculate the log phase of bacterial growth (See Appendix fig.A, fig.B and fig. C). Then 3ml of the bacterial cells from the log phase were mixed with 6ml of RNA protect bacteria reagent (Qiagen) in 15 ml tubes. Bacterial pellets were extracted as per the manufacturer's instructions and stored at -80°C for further work.

Total RNA was extracted using RNeasy protect bacteria Mini prep kit(QiagenInc) further purification of RNA was done by using the RNase-free DNase set (QiagenInc)as instructed by the manufacturer.

The purity and concentration of extracted RNA was analysed using NanoDrop-1000 spectrophotometer (Nanodroptechnologies) and Agilent 2100 bioanalyzer (Agilent technologies)

The RNA was transferred to new eppendrof tubes and stored at -80°C for further work.

2.9.4.2 Real- Time polymerase chain reaction

Real time polymerase chain reaction is mostly used for qunatifying nucleic acids and genotyping. In this study, we use real time- SYBR green PCR to quantify the expression of *sdeB* gene quantitative real time PCR was carried out for two sensitive strainATCC 13880, ATCC 14041, and two adapted strains ATCC 13880 adpI, ATCC 14041 adpI.

In this experiment,we used two different primers to check the expression of *sdeB*gene.

Two different primers were used to check the *sdeB* gene expression because with the first primer set described below as set (A) the RTPCR product was different between adapted and sensitive stains (observed from the dissociation curve). The two primer set used were as follows:

(A) First set of *sdeB* primer set Listed as sdeB1 and *smarpoB* in table 1 and

(B)Second set of *SdeB* primer set listed as *SdeB2* and *RpIU* as in table 1.

The synthesis of cDNA from isolated RNA for ATCC13880, ATCC14041, ATCC13880 ADPI, ATCC 14041 ADPI, was performed using superscript II Reverse transcription kit (Qiagen) according to the manufacturer instruction. Three reaction containing reverse transcriptase enzyme (RT positive) and two reactions without reverse transcriptase enzyme (No RT) were made for each RNA sample. For both set of primer same cDNA synthesis process was followed.

A) Using the cDNA synthesized for above mention strains, experiment for RNA expression of *sdeB*was performed, by using thefirst set of primer (A) listed in table 1 as *SdeB1* and housekeeping gene *rpoB for* qualitative control. Thermo Scientific Maxima SYBR green/ROX qPCR master Mix (2X) (Thermo scientific) was used for all real time PCR according to the instruction of the manufacturer. 25µl of PCR master mix for RTPCR contains 12.5µl of Maxima SYBR green/ROX qPCR master Mix (2X), 0.7µM of forward primer and 0.7µM of reverse primer, 1 µl cDNA and 10µl of nuclease free water.

qRTPCRreaction was performed in the Abi prism 7900HT sequence detection system (Applied biosystem). The cycling condition for PCR was 95°C for 10 minute as initial incubation, and then 40 cycles of 15 seconds at 95°C and 30 seconds of 60°C. Triplicates of each sample were run including two sets of negative RT control. For each run, nuclease free water was added instead of cDNA to make a no template control. Quantification of the gene of interest can be calculated by ΔΔCt methods. To calculate the change in gene expression, the cycle threshold (C_T) for the housekeeping gene (*rpoB*) is subtracted from the cycle threshold of the gene of interest (*sdeB*). The resulting value, ΔC_T, is similarly calculated for the calibrator strain (ATCC 13880 and ATCC 14041). Subtracting the ΔC_T of the calibrator strain from the ΔC_T of each of the experimental strains resulted in the $\Delta\Delta C_T$ value: that is ΔC_T of a stain $_{is}$ calculated as $C_{T(sdeB)} - C_{T\ (rpoB)}$ and$\Delta\Delta C_T$ is calculated as $\Delta C_{T(\text{adapted strains})} - \Delta C_{T(\text{calibrator strain(s)})}$. The formula ($2^{-\Delta\Delta C_T}$) can then be applied to calculate the fold change in gene expression relative to the calibrator strains.

(B) As we did not get the same amplified product for sensitive and adapted strains we tried with new primer set listed as *sdeB2* and *rpIU* as the housekeeping gene. The qRTPCR mixture concentration and condition were same as for the first set of primer.

With the second set of primer, melting curve looked fine but there was high signal from the negative RT control so we extracted RNA form ATCC 13880 and ATCC 13880 adpI following the same process as mentioned above for RNA isolation (see 2.9.4.1) tried the one step RTPCR . We used the EXPRESS one- step SYBR Green ER universal kit(Invitrogen life technologies Inc.,). Following reaction mixture was made for one step PCR

EXPRESS SYBR Green ER qRTPCRsupermix with premixed ROX: 10µl;10µmforward Primer(200nm Final(F): 0.4µl;10µm reverse primer (200nm final):0.4µl;EXPRESS superscript Mix for one step SYBR Green ER :0.5µl;Template RNA :5µl; distilled water:3.7 µl to make a total reaction volume of 20 µl. The triplicates of reaction were set up on 96 well reaction plates(Applied biosystem). To test for genomic DNA contamination of the RNA sample and No template controls, appropriate no-RT and were run in the plate.

2.10 Antibiotic sensitivity test

Antibiotic sensitivity test was performed by two different methods

2.10.1 Broth dilution method

MIC value of norfloxacin of sensitive, resistant and adapted strains was tested.Stock solution of norfloxacin 100ppm was made in acetone. Overnight culture of ATCC 13880, ATCC 13880 adp I, ATCC 13880adp II and MF2336 was prepared by incubation at 30^{o}C for 18 hours. Norfloxacin solution was diluted in a stepwise manner starting from 0.1 µg/ml, 0.2 µg/ml, 0.3µg/ml up to 2µg/ml in 4.5 ml TSBand 50µl of the overnight culture of each strain was transferred to all the above mention dilution of norfloxacin. The highest concentration that allows the growth was noted as MIC value of that strain for norfloxacin.

2.10.2 Agar disc test

Antibiotic susceptibility test was performed with norfloxacin and ofloxacin disc (Oxoid). A single colony from TSA plate was transferred to 3mlTSB and incubated at 37°C for 3 hour.O.D. at 600nm was measured in a spectrophotometer. O.D. of all the strains was adjusted to 0.125 with TSB that is equivalent to 0.5 MC Farlandstandards. From TSB culture was swabbed in a plate with sterile cotton swab and antibiotic disc was placed in the plate and incubated at 30°Cfor 24 hour and size of zone of inhibition was measured by scale (Kwok, So, Wang, & Ge, 2005).

2.11 Fitness cost of resistant strains

The cost of resistance due to adaptation was calculated by the direct comparison of growthof biocide resistant strain against the sensitive parental strains at 30°C. Equal densities of the biocide sensitive and biocide resistant strains was prepared by making O.Dvalue of 0.6 at 600 nm measurement for all Overnight culture of strains (ATCC 14041,2206adpI,ATCC 14041adpII,MF ATCC 13880,ATCC 13880adpI,ATCC 13880adpII) was measured by ultrospec 3000 spectrophotometer (pharmacia biotech). Then inoculum was prepared by diluting the 15µl of OD adjusted culture in a respective eppendrof tube with 1485 µl of TSB. From the 1500µl of each sample, 250µl was transferred to the three different wells of the bioscreen plate and last 5 wells were filled with the TSB without any strains as control .The $OD_{600}$was measured for every 20 min

for 48 hours at 30°C using a bioscreen C unit (Labsystem).Annovatukeys test was applied for statistical analysis.

2.12 Stability of QAC adapted strains

To check the stability of the adapted strains, First cconformation of resistance characteristics of adapted strain ATCC 14041adpI, ATCC 14041 adpII and MF ATCC 13880 adpI, ATCC 13880 adpII was performed, by inoculating the overnight culture of strains at 30°C in 200 ppm of BAC and CPC respectively.

Percentage stability of the resistance in adapted strain was calculated as the difference in the bacterial count before and after tenth transfer in a biocide free medium.

Overnight culture of bacterial strain was made by inoculating single colony from TSA plate to the 5ml TSB tubes and incubating at 30° C for 24 hours at 200 rpm. Overnight culture of strains 14041,14041 AI, 14041AII, 13880, 13880AI,13880 AII were serially diluted in a TSB up to 10^{-7} dilution .Then 14041,14041AI, 14041 AII dilution were plate in a three different TSA plate with no BAC, with 100ppm BAC and 200ppm BAC , similarly 13880 ,13880 AI, 13880 AII in a plate without CPC,with 100ppm CPC and 200 ppm CPC by using WSAP(Whitley spiral automatic plater), and incubated at 30° C for 24 hours and colony count was performed using protocol(symbiosis).

50µl of the overnight cultures were continuously transferred to the new 5 ml TSB tubes. On 10^{th} transfer both the 13880 AI,13880 AII were plated in a two different TSA plate with 100 and 200ppm CPC and 14041AI,14041 AII on 100 and 200 ppm BACcontaining TSA plates and colony count was performed using protocol(symbiosis).

3. RESULTS

3.1 Susceptibility of wild type strains toBenzalkonium chloride (BAC) and cetylpyridimine chloride(CPC)

The susceptibility of thirteen strains of *S. marcescens* against the two QACs , BAC and CPC was tested. BAC was tested in a bactericidal suspension test and in a MIC test, while CPC was tested only in the MIC test.

In the bactericidal test with 200 ppm BAC the eleven food industry strains were resistant with a reduction of 0.8-2.7 log (Table 6). The two ATCC strains were completely sensitive with >5 log reduction.

MIC values of the two commercial disinfectants BAC and CPC on bacteria were investigated (Table 6). Out of the 13 different isolates,10 strains were found to have MIC values for BAC greater than 200 ppm and were considered to be resistant (BACr). Growth was not tested at higher concentrations due to precipitation in the media. Two food related strains were found to have intermediate sensitivity to BAC (BAC1), having MIC value of 110 ppm and the ATCC11041strain was sensitive to BAC(BAC^s) having MIC value of 30ppm.Similarly twelve strains were resistant to CPC (CPC^r), they were found to have MIC greater than 200ppm, one strain ATCC 13880 was sensitive to CPC having MIC value of 30 ppm. Results were confirmed by repeated analysis all the strains.

Table 6. MIC of BAC and CPC and bactericidal effect of BAC for reference strains

STRAIN	MIC BAC(ppm)	MIC CPC(ppm)	log reduction on BAC
ATCC 14041	30	>200	>5.55#
ATCC 13880	>200	30	>5.15
2336	>200	>200	0.97± 0.13*
3309	>200	>200	0.76 ± 0.47
3306	>200	>200	2.47 ± 0.47
3308	>200	>200	2.70 ± 0.52
3299	110	>200	1.25 ± 0.33
3297	>200	>200	1.94 ± 0.03
3300	110	>200	1.82 ± 0.01
2325	>200	>200	1.74± 0.05
2326	>200	>200	0.83 ± 0.03
3613	>200	>200	1.78 ± 0.20
3224	>200	>200	1.93 ± 0.24

* refers mean and Standard Error (S.E.) of two replicate experiments

#indicates that no surviving bacteria was observed. The reduction is greater than the detection limit. The data for the replicate with highest detection limit is presented.

3.2 Adaptation to benzalkonium chloride

Strains with MIC of BAC or CPC <200 ppm were adapted by exposing them to gradually increased concentrations. The BAC sensitive strain ATCC 14041 developed increased resistance to BAC on regular repeated exposure to BAC.Fig.2 shows the adaptation rate of ATCC 14041 strains on BAC. ATCC 14041 strains become equally resistant as the strains from food industry (growth at 200 ppm) up on 17th transfer in first adaptation and same concentration adaptation was seen on replicate experiment on 16th transfer. In both experiments color of the colony changed from red to yellow.

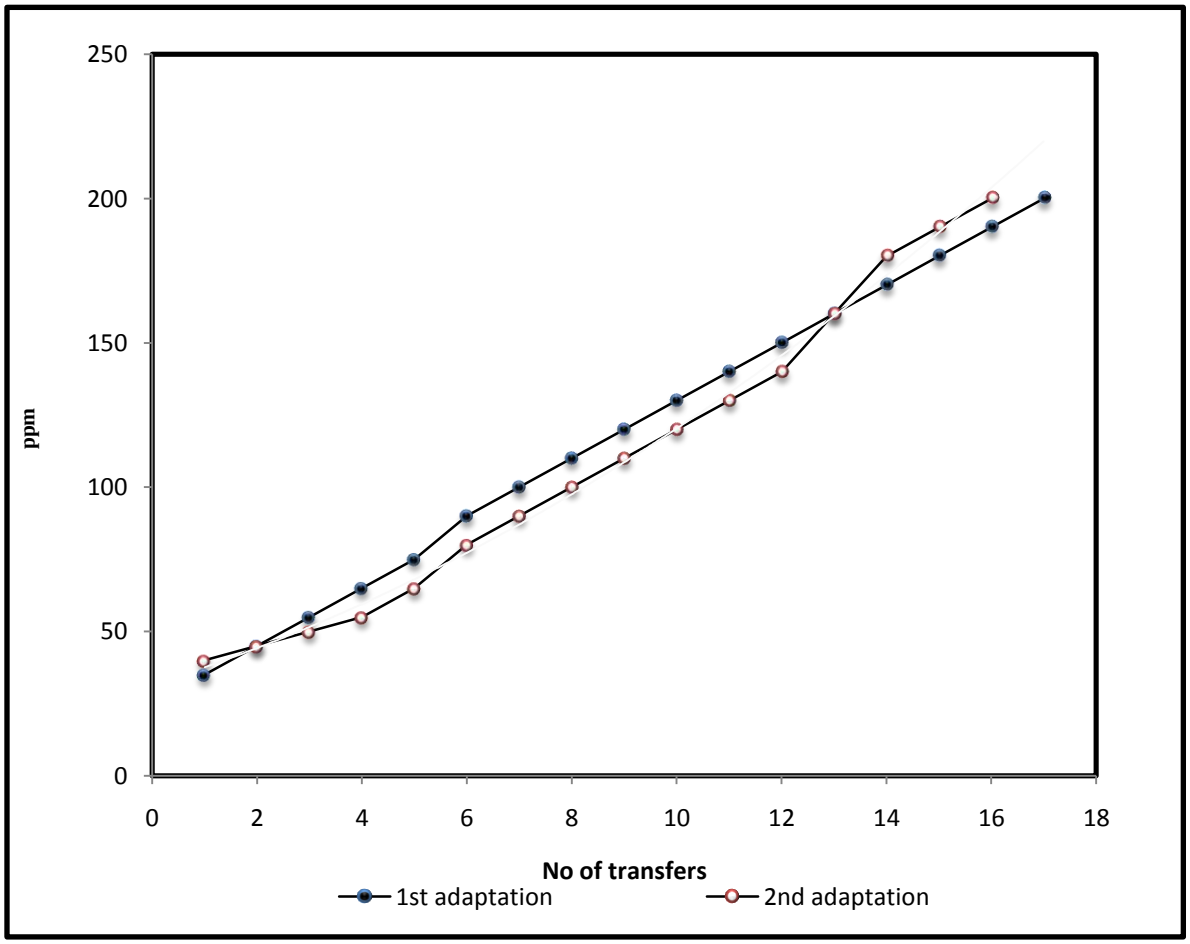

Figure2. BAC adaptation of *Serratiamarcescens* ATCC 14041 maximum growth concentration plotted against time

3.3 Adaptation to CPC

CPC sensitive strain ATCC 13880 developsincreased resistance to CPC on regular repeated exposure to CPC. MF ATCC 13880 strains become equally resistant as the strain from the food industry up on 15th transfer on first adaptation and 14th transfer on second adaptation(Figure 3). Adaptation was performed twice. Mixed color(red and yellow) was seen in sensitive strains. Adapted culture was yellow in color.

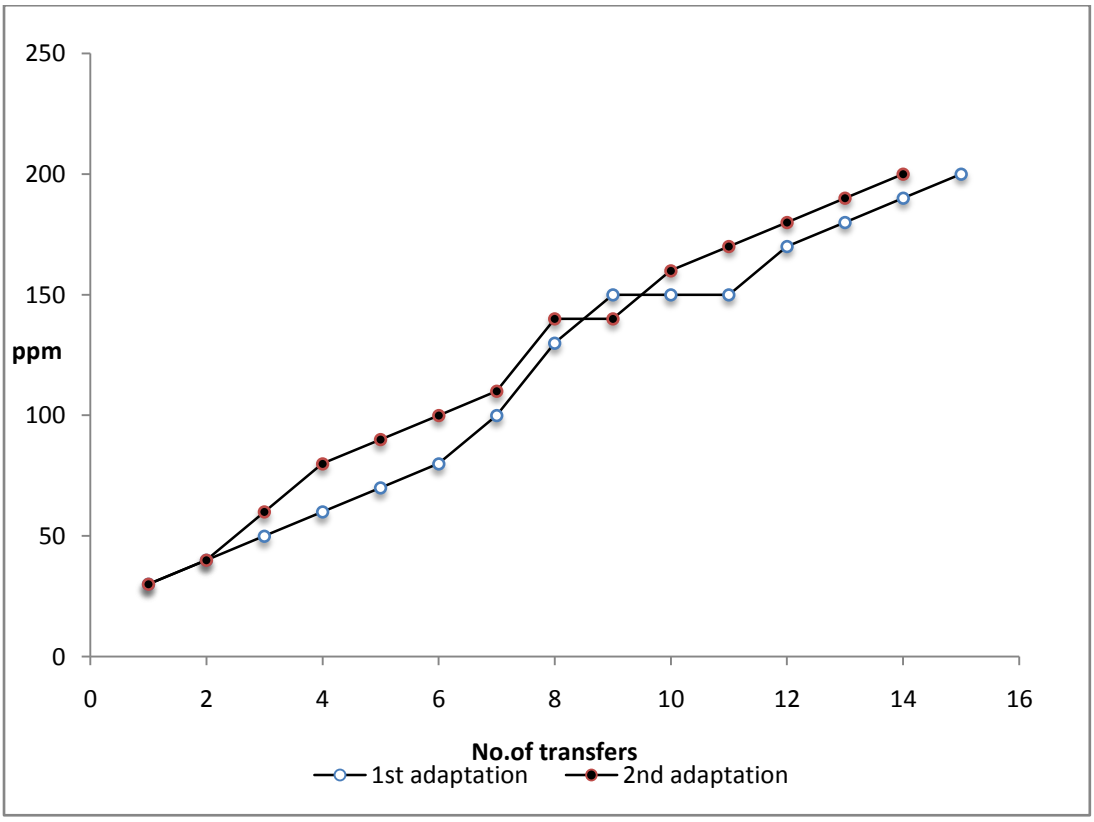

Figure 3.CPC adaptation of ATCC 13880 Maximum growth concentration plotted against time

3.4. Adaptation of 3300 strain to BAC

MF 3300 strains was adapted from 110PPM to 200 PPM up on repeated exposure to higher concentration of BAC .(figure 4).MF 3300 requires 4 transfer in 1st adaptation and five transfer in second adaptation(figure 4).Adaptation was performed twice.

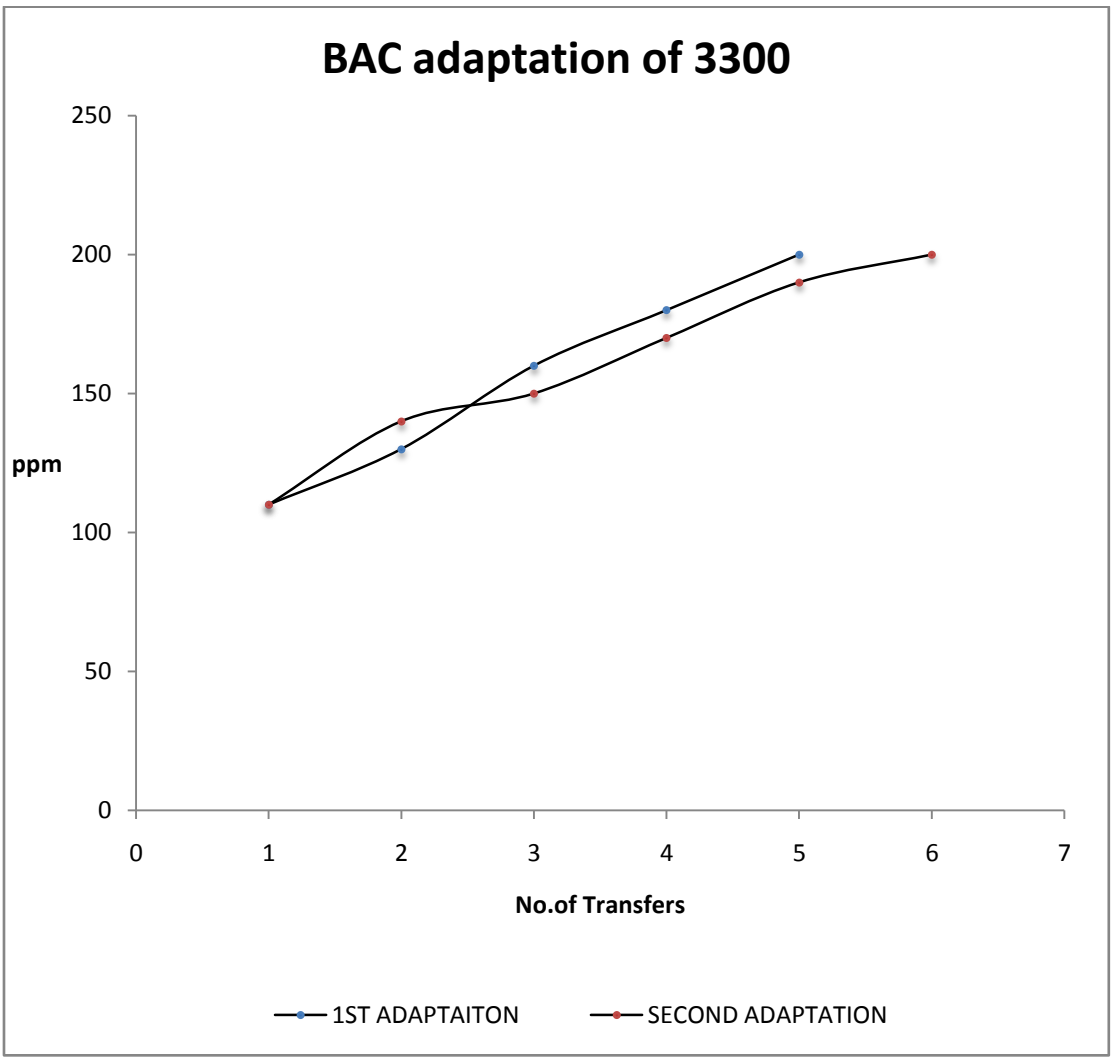

Figure 4. BAC adaptation of 3300 Maximum growth concentrations plotted against time

3.5 Adaptation of 3299 strain to Benzalkonium chloride

MF 3299 strain was adapted from 110PPM to 200 PPM up on repeated exposure to higher concentration. MF 3299 adaptation requires seventransfers in first adaptation and six transfers in second adaptation (fig.5). Adaptation was performed twice.

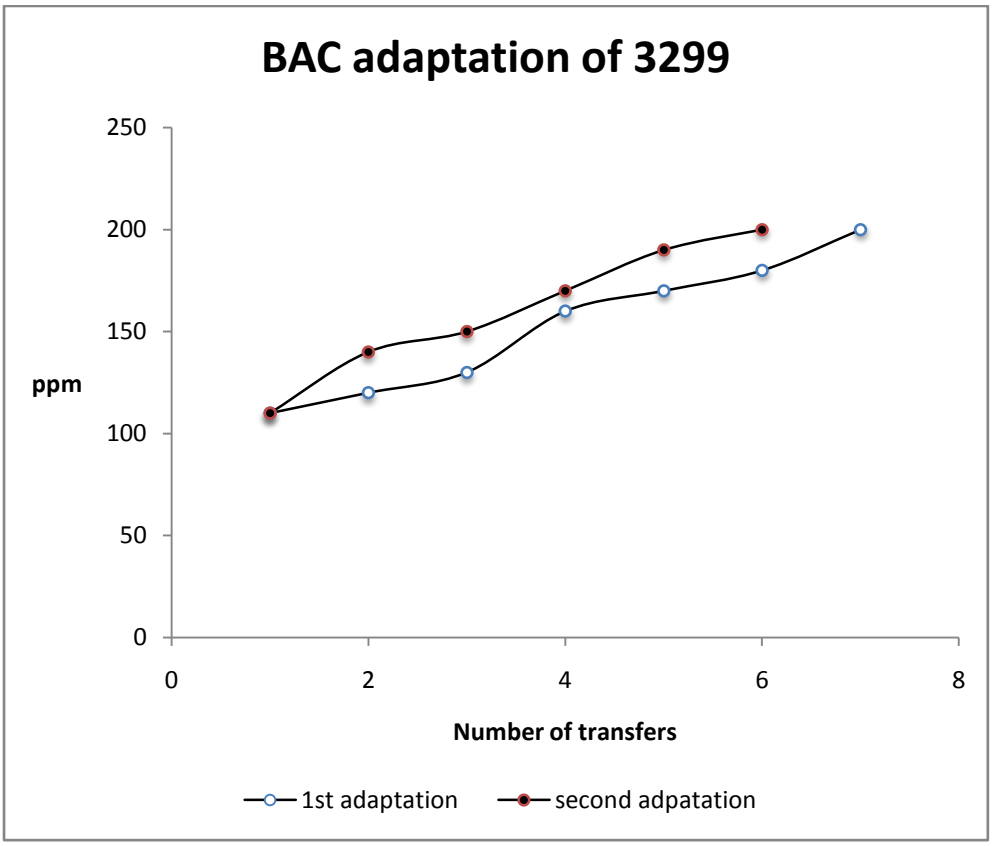

Figure5. BAC adaptation of 3299 maximum growth concentration plotted against time

3.6 Characterization of adapted strains

The adapted strain ATCC 13880 adpI, ATCC 13880adpII, ATCC 14041adpI and ATCC 14041adpII were reduced by less than 2.5 log $_{10}$ reduction when exposed to 200 ppm of BAC for 5 minutes (Figure 6).

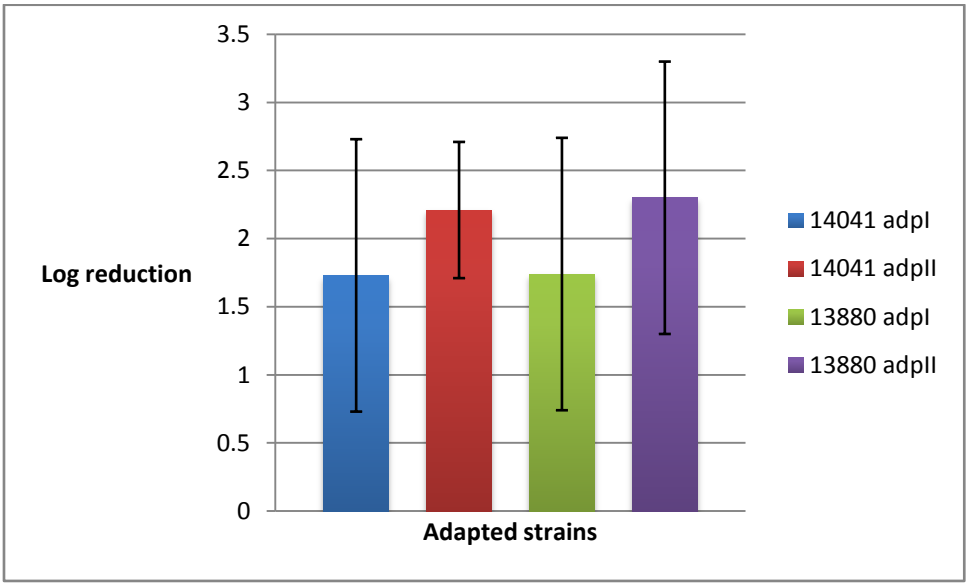

Figure 6. Bactericidal effect of 200 ppm BAC for 5 min on *Serratiamarcescens* ATCC 13880 and ATCC 14041 adapted strains. Mean of two replicates with standard errors are presented.

3.7 Prevalence of the *sdeB,sdeD,sdeY,*and*rpoB* gene

Screening of the *sdeB,sdeD, sdeY and rpoB* gene was done by simple PCR. The primers used are listed in (Table 3). The PCR products were identified as respective gene positive from their sizes on agarose gel stained with gel red. The PCR product for *sdeB,sdeD, sdeY* and housekeeping gene *rpoB*was detected in all the strains.

3.8 Agarose gel electrophoresis for *sdeB* new, *rplY*

Previous *sdeB* primer did not work correctly so we screen thewith new *sdeB2* primer and *rplY* as housekeeping gene. Simple PCR was performed for the screening of *sdeB* gene. PCR product was then identified on the basis of their sizes on agarose gels (Fig.7 and fig 8) and again the screening of *sdeS* gene was done on the same mannerFig .9).

The results from *sdeS*PCR product were sequenced to identify the any amino acid changes and there was 11 differentnitrogen bases substitution among the strains.

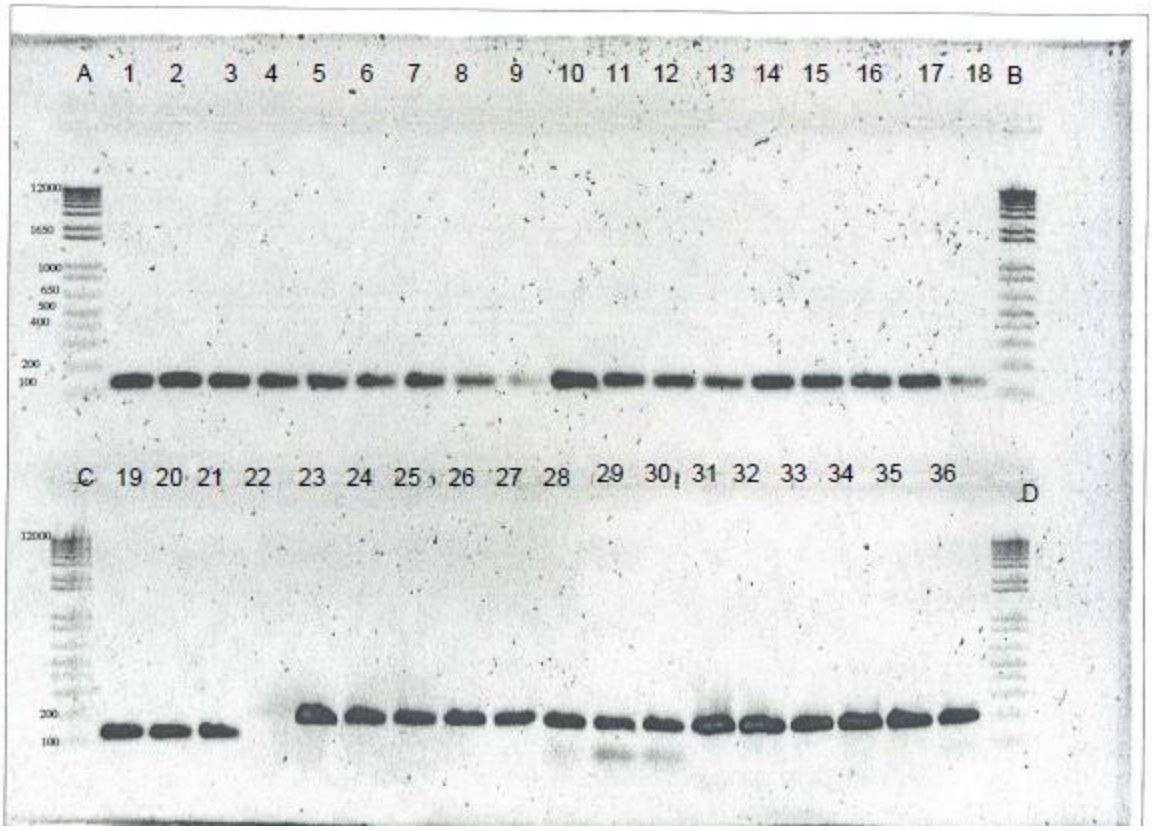

Figure 7.SdeB (Lane A= 1 kb plus DNA ladder. Lane 1=MF 2336.Lane 2=ATCC 13880.Lane 3=ATCC 13880 AdpI.Lane 4=ATCC 13880 adpII.Lane 5=3297. Lane 6=3613. Lane 7 =3224. Lane 8=MF ATCC 14041, Lane 9=MF ATCC 14041 adpI.Lane 10=ATCC 14041 adpIi. Lane11= 3299 adpII, lane 12=3299 AdpI. Lane 13 =3299 adpII.Lane 14 =3300. Lane 15 =3300 adpI,Lane 16 =3300 adpII,Lane 17=2325, Lane 18=2326. Lane 19= 3306. Lane 20 =3308. Lane 21 =3309.Lane 22=Negative control.)

rplY (Lane 23 =2336. Lane 24=ATCC 13880.Lane 25=ATCC 13880 AdpI. Lane 26=ATCC 13880 AdpII. Lane 27=3297. Lane 28 =3613.Lane 29 =3224. Lane 30 =ATCC 14041. Lane 31=ATCC 14041 Adp I. Lane 32 =ATCC 14041 AdpII. Lane 33=3299. Lane 34 =3299 Adp I. Lane 35 =3299 Adp II. Lane 36=3300

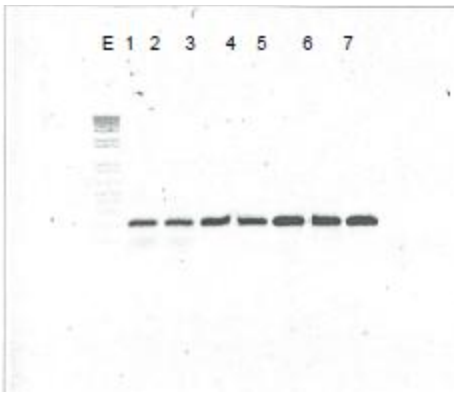

Figure 8. Lane E=1 kb plus DNA ladder.RPI Y Lane 1=3300 AdpI.Lane 2=3300 AdpII. Lane 3=2325.Lane 4=2326. Lane 5=3306. Lane 6=3308. Lane 7=3309. Lan8 =Negative control

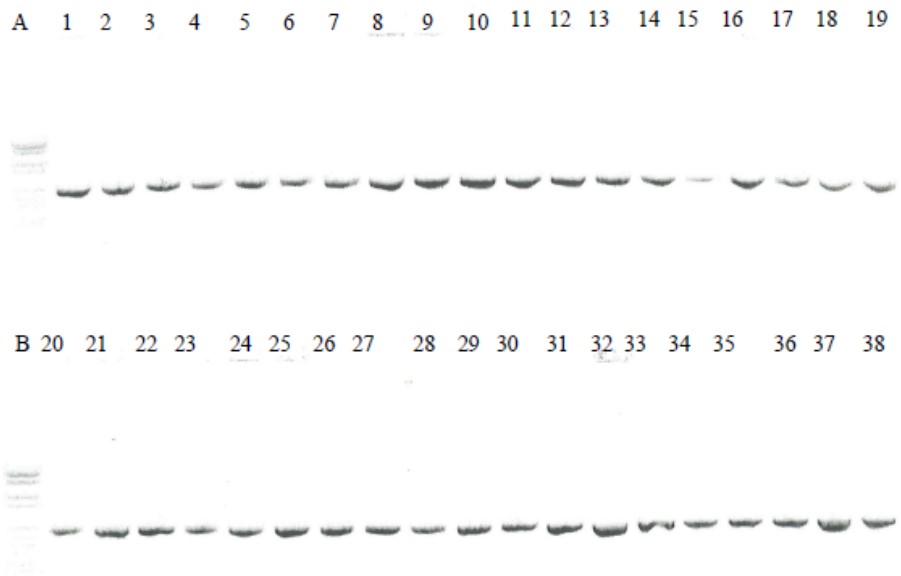

Figure 9.SdeS(Lane A= Std VI DNA ladder. Lane 1=MF 2336.Lane 2=ATCC 13880.Lane 3=ATCC 13880 AdpI.Lane 4=ATCC 13880 adpII.Lane 5=3297. Lane 6=3613. Lane 7 =3224. Lane 8=MF ATCC 14041, Lane 9=MF ATCC 14041 adpI.Lane 10=ATCC 14041 adpIi. Lane11= 3299 adpII, lane 12=3299 AdpI. Lane 13 =3299 adpII.Lane 14 =3300. Lane 15 =3300 adpI,Lane 16 =3300 adpII,Lane 17=2325, Lane 18=2326. Lane 19= 3306. Lane 20 =3308. Lane 21 =3309.lane 22= 3300 AdpI. Lane 23 =2336. Lane 24=ATCC 13880.Lane 25=ATCC 13880 AdpI. Lane 26=ATCC 13880 AdpII. Lane 27=3297. Lane 28 =3613.Lane 29 =3224. Lane 30 =ATCC 14041. Lane 31=ATCC 14041 Adp I. Lane 32 =ATCC 14041 AdpII. Lane 33=3299. Lane 34 =3299 Adp I. Lane 35 =3299 Adp II. Lane 36=3300

3.9Antibiotic susceptibility test

MIC value of norfloxacin for four strains MF ATCC 13880,ATCC 13880 adpI,ATCC 13880 adpII and 2336 is shown in table Table 7. The MIC value of the resistant (MF 2336)and adapted strain ATCC 13880 AI and ATCC 13880 AII shows slightly higher value of MIC then BAC sensitive strain ATCC 13880.

Table 7. MIC of Norfloxacin

Strain	**MIC Norfloxacin (µg/ml)**
ATCC 13880	0.7
ATCC 13880 ADPI	0.9.5 ± 0.5
ATCC 13880 ADPII	0.9 ± 0.5
2336	0.9±0.5

* refers to mean of two samples ± Standard deviation

8 strains MF ATCC 14041,MFATCC 14041 adpI.ATCC 14041 adpII,ATCC 13880,ATCC 13880 adpI,ATCC 13880 adpII, MF2336 and MF 3297 were tested for antimicrobial resistance against ofloxacin and norfloxacin. Both the sensitive and adapted strains show almost similar size of zone of inhibition (table 8).

Table 8.Size of zone of inhibition for Norfloxacin and Ofloxacin

Strain	Zone of inhibition (cm)	
	Ofloxacin*	Norfloxacin*
ATCC 14041	4.7±0.71	3.1±0.14
ATCC 14041 AdpI	3.25±0.18	3.45±0.18
ATCC 14041 AdpII	3.05±0.1	3.45±0.18
ATCC 13880	2.65±0.3	3.35±0.24
ATCC 13880 AdpI	2.2±0.7	3.45±0.14
ATCC 13880 Adp II	2.25±0.1	4±0.18
2336	2.9±0.07	3.35±0.10
3297	2.2±0.07	3.95±0.10

* = Mean of zone of inhibition ± standard error

3.10 Growth rates of wild type and adapted strains

There is significance difference on the growth rate of the sensitive ATCC 14041 strain and ATCC 14041 adpI and ATCC 14041 adp II strain (fig 10) ($p<0.05$), while there was no significant difference on the growth rate between two adapted strains ATCC 14041 AdpI and ATCC 14041 AdPII ($p>0.05$) (appendix E)

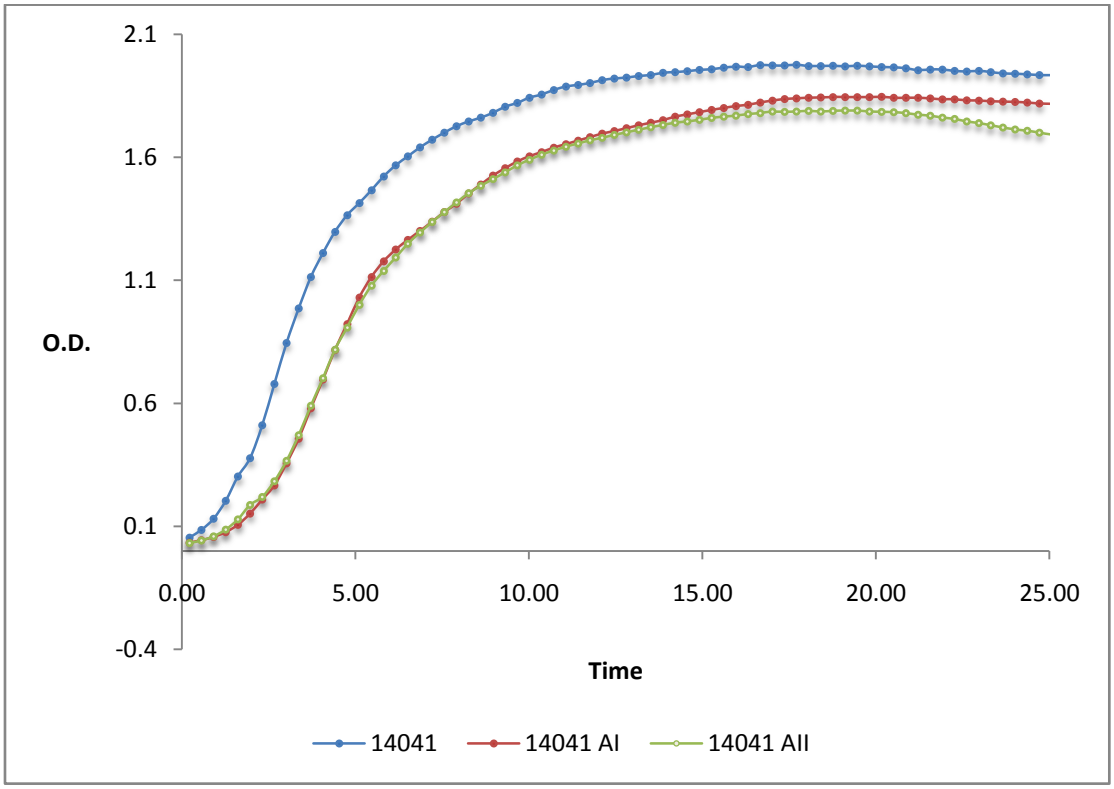

Fig. 10.Fitness costs during growth in a TSB at 30^{o}C of ATCC 14041, ATCC 14041AI, ATCC 14041 AII.

There was no significant difference on the growth rate between CPC sensitive and CPC resistant strains(fig 11)(Appendix f)

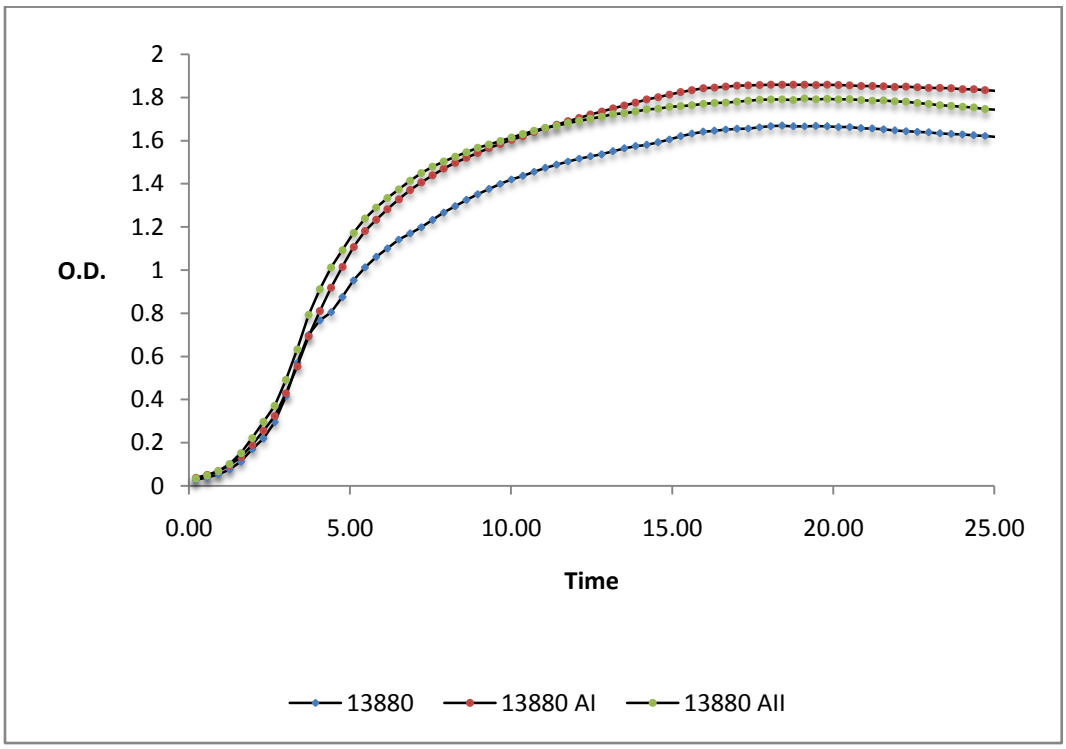

Fig.11Fitness costs during growth in a TSB at 30^{o}C of sensitive mutants of *Serratiamarcescens* MF ATCC 13880, first line adaptation mutants ATCC 13880AI, second line adaptation ATCC 13880.

3.11 Growth rates of sensitive 3300, 3300AI and 3300AII strains and footbath strain 2336

There was no significant differenceon growth between the sensitive strain 3300 strain and its adapted strain 3300AI and 3300 AII ($p > 0.05$).(fig 12)(Appendix G)

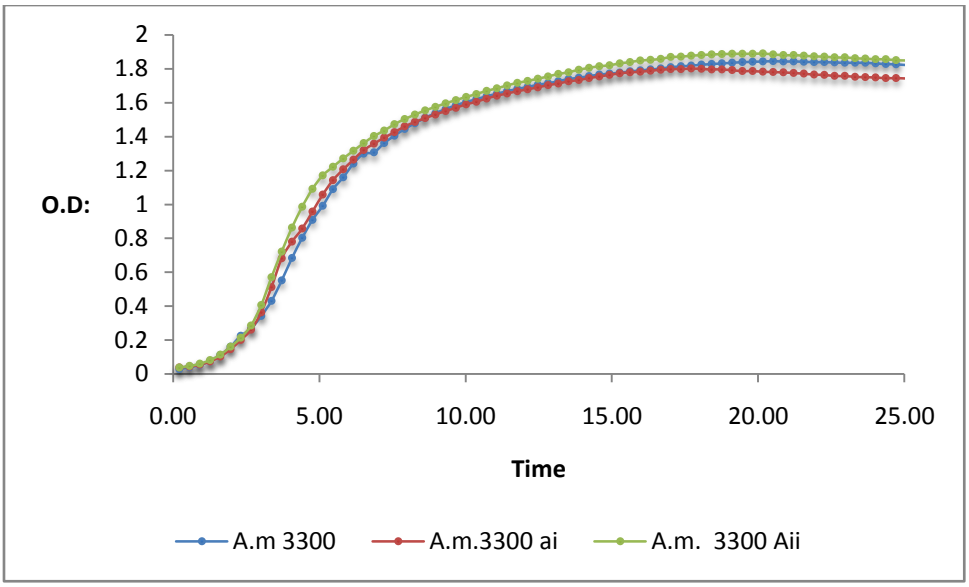

Fig12.Fitness costs during growth in a TSB at 30^{o}C of sensitive mutants of S. marcescen sensitive strains 3300,first line adaptation mutants 3300AI,and second line adaptation 3300 AII.

3.12 stability of adapted strains

Percentage stability of the resistance in adapted strain was calculated as the difference in the bacterial count before and after tenth transfer in a biocide free medium.In stability test 17.13 and 18.24 percentage of 14041 AI and 47.67 and 22.39percentage of 14041AII (figure 13) strains losses it's resistant at 200 and 100 ppm of BAC respectively.

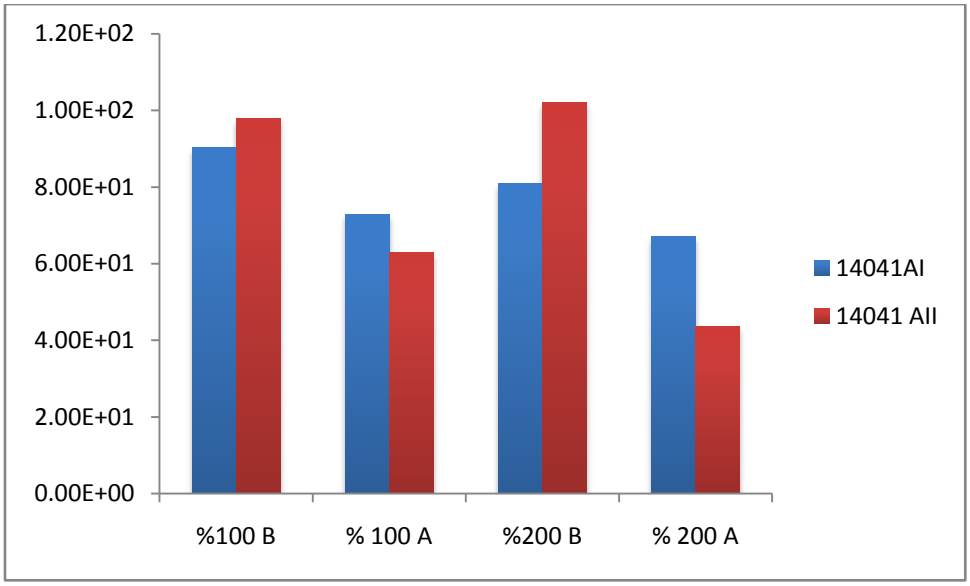

Figure13. Percentage of survival of 14041AI and 14041 AII on 100ppm and 200ppm BC before and after 10th transfer on CPC free environment.

Similarly in case of 13880 Adapted strains28.14 and 22.13 percentage of 13880 AI and 36 and 85.5 percentage of 13880 AII(figure 14) strains loses its resistance ability at 200 and 100 ppm of CPC respectively.

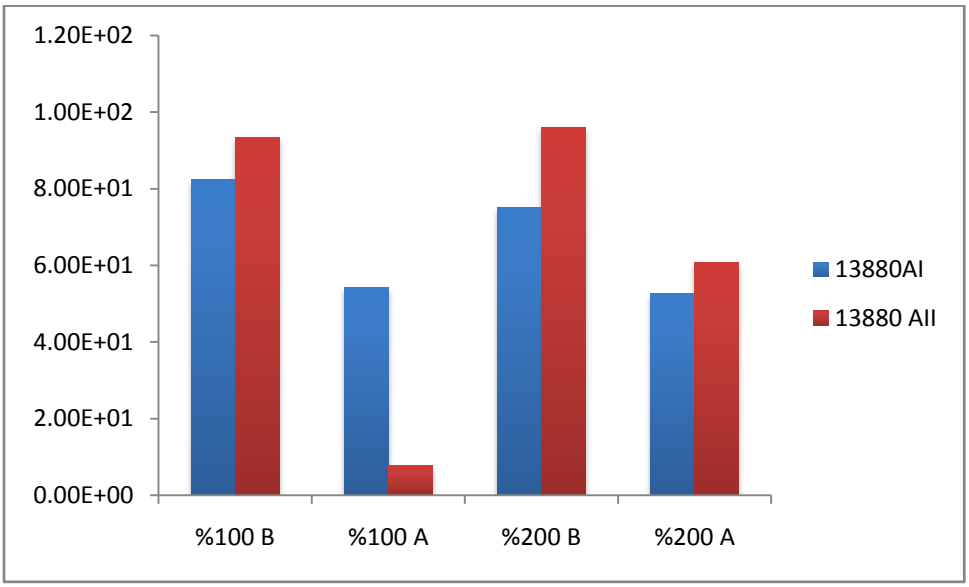

Figure 14.Percentage of survival of 13880AI and 13880 AII on 100ppm and 200ppm CPC before and after 10th transfer on CPC free environment.

In case of 14041 and 13880 strains, there was no growth on 100ppm and 200ppm of BAC and CPC respectively.

3.13 *SdeS* genesequence profile for footbath and adapted strain

On *sdeS*genesequencing of the strains including adapted strains revealed 11 different single point mutation .In all the adapted strains there was same pattern of nitrogen base substitution. There was some difference on nitrogen base substitution on footbath strains which is shown on table 9.

Table 9 .Sequence of *sdeS* gene on resistant and adapted strain

Strain	origin	141 G→C	150 C→T	171 C→T	201 C→T	234 C→T	246 G→A	285 A→G	288 G→A	378 G→C	387 C→T	396 C→T
ATCC 14041		-	-	-	-	-	-	-	-	-	+	-
ATCC 13880		-	-	-	-	-	-	-	-	-	-	-
2336	Footbath tego 103 G	+	+	+	+	+	+	+	+	-	-	-
3306	Footbath tego 103 G	+	+	+	+	+	+	-	-	-	-	-
3299	Footbath tego 103 G	+	+	+	+	+	+	+	+	-	-	-
3300	Footbath tego 103 G	+	+	+	+	+	+	+	+	-	-	-
3308	Footbath tego 103 G/TP99	+	+	+	+	-	+	+	+	-	-	-
2325	Floor with tricolsan	+	+	+	+	-	+	-	-	+	-	+
2326	Floor with tricolsan	+	+	+	+	+	+	+	+	-	-	+
3309	Footbath TP99	+	+	+	+	+	+	+	+	-	-	+
3297	Footbath TP99	+	+	+	+	-	+	-	-	+	-	+
3613	Meatsaw	+	+	+	+	+	+	+	+	-	-	+
3224	Floor with tricolsan	+	+	+	+	-	+	+	+	+	-	+
ATCC 14041 AI		+	+	+	+	-	+	-	-	+	-	+
ATCC 14041 AII		+	+	+	+	-	+	-	-	+	-	+
13880		+	+	+	+	-	+	-	-	+	-	+
13880AI		+	+	+	+	-	+	-	-	+	-	+
13880 AII		+	+	+	+	-	+	-	-	+	-	+
3299I		+	+	+	+	-	+	-	-	+	-	+
3299II		+	+	+	+	-	+	-	-	+	-	+
3300I		+	+	+	+	-	+	-	-	+	-	+
3300 AII		+	+	+	+	-	+	-	-	+	-	+

3.14Amino acid substitution on footbath and adapted strains:

Table 10 shows that there weretwo amino acid substitutions common in all the strains (adapted and resistant) at position 157 and 159 from threonine to alanine and aspartic acid to glycine respectively. Besides this there was insertion of the glutamine in position 153 in all the adapted strains and two footbath strains 3297 and 2325.

Table 10.Amino acid substitution of the strains

Strain	**Origin**	**(-) →Q**	**T→ A**	**D →G**
2336	Footbath Tego 103G	-	+	+
3309	Footbath TP99	-	+	+
3306	Footbath Tego 103G	-	+	+
3308	Footbath Tego 103G/TP99	-	+	+
3299	Footbath Tego 103G	+	+	+
3297	Footbath TP99	-	+	+
3300	Footbath Tego 103G	-	+	+
2325	Floor with triclosan Poultry processing plant	+	+	+
2326	Floor with triclosan poultry processing plant	-	+	+
3224	Floor with triclosanPoultryprocessing plant	-	+	+
3613	Meatsaw, abattoir	-	+	+
ATCC 13880		-	-	-
ATCC 14041		-	-	-
3299 AI		+	+	+
3299 AII		+	+	+
3300AI		+	+	+
3300AII		+	+	+
ATCC 13880 AI		+	+	+
ATCC 13880AII		+	+	+
ATCC 14041AI		+	+	+
ATCC 14041 AII		+	+	+

3.15 Prediction of secondary structure of protein

Secondary structure for SdeSprotein of 159aa chain length can be predicted by the use of software （http://minnou.cchmc.org/). Three different profiles of theSdeSprotein structure predictedby (http://minnou.cchmc.org/） are shown as below. If we compare the secondary structure protein between sensitive strains with adapted and resistant strains,both ATCC strains has helical form from 141 to 149aa. At the same position helical form is changed into coil form in case of resistant and adapted strains.

Profile 1:

This profile of protein structure is shown by two laboratory strain ATCC 13880, ATCC 14041. The position of the change of secondary structure are marked with red square in the figure. In the figure 15 H represents helix, C represent coil and E represent strands.

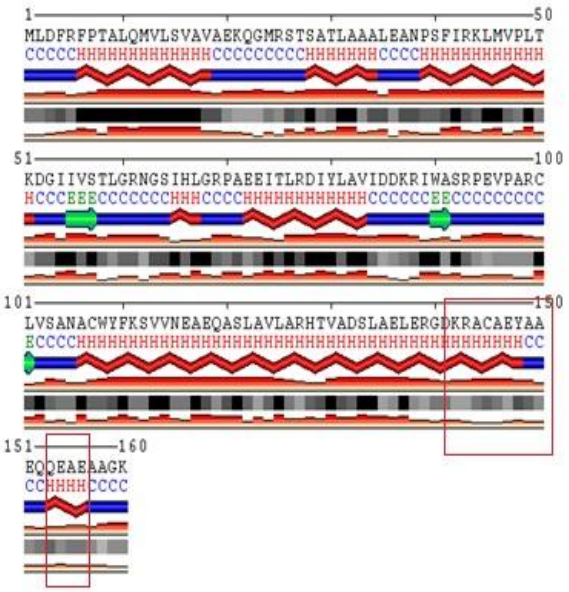

Fig15 . Secondary structure prediction of sdeS protein of two laboratory strains

profile 2:

This secondary structure of sdeS protein is predicted for all the food industry strain except MF 2325 and MF 3297. In the figure 16 H represent helix, C represents coil and E represents for strands.

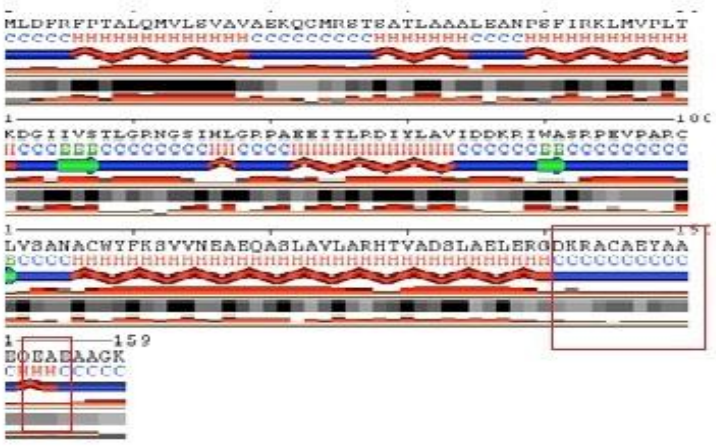

Fig16 . Secondary structure prediction of sdeS protein of food industry strains

profile 3:

Third profile of amino acid is shown by the all the adapted strain and two foot bath strainMF 3297 and MF 2325. In figure 17 H reperesentshelix , C represents coil and E represent strands . Changes in the secondary structure are marked with red square.

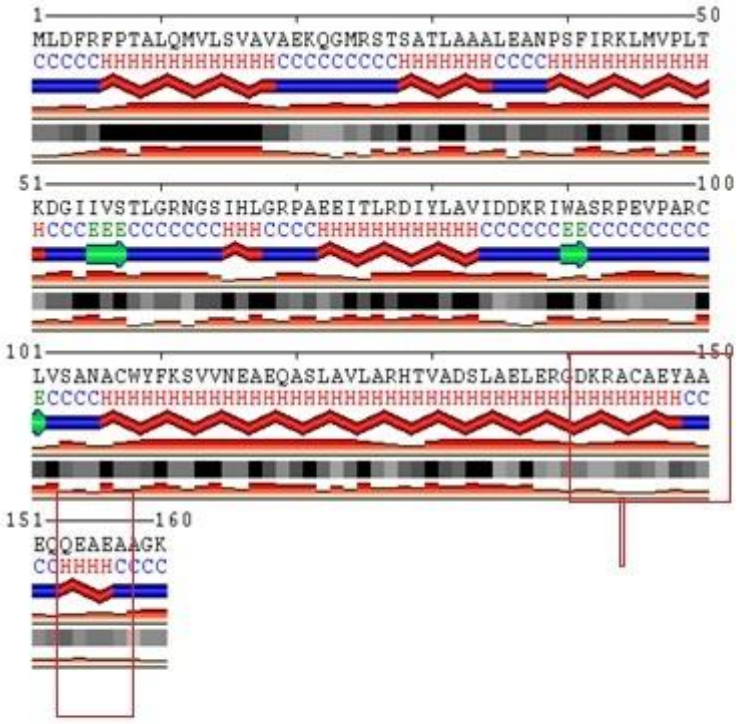

Fig 17 . Secondary structure prediction of sdeS protein of adapted strains

3.16 qRT-PCR

Out of the 13 isolates, ATCC13880,ATCC 14041, ATCC 14041 adpI, ATCC 13880 ADPI were analysed for *sdeB* expression with two different *sdeB*primer sets and *smarpoB* and *rplU*as house keeping genes for respective primer sets.With the first *sdeB1* Primer,there was a shift in the melting curve in the adapted strains compared to the sensitive strains. This indicates that there was the formation of another qRT- PCR product in the adapted strains than in sensitive strains. The peak was not at the very early stage, so it signifies that it´s not the primer dimer formation.

With the second primer sdeB2, expression of *sdeB* gene on same four strains as the first set of primer was performed. The melting curve looks fine, which indicates the formation of same PCR product between adapted and sensitive strains, but the result was not reliable because there was high signal even from the no RT controls. It was almost similar Ct value between with and without reverse transcriptase sample.

Then we came to know that primer was fine, so we tried to remove the cDNA synthesis step by the use of one step RTPCR with same second primer set , but unfortunately again there was high signal from the no RT control. Some examples are ATCC 13880 strain with or without reverse transcriptase has almost equal Ct value approximately 20. For ATCC 14041 with or without reverse transcriptase, Ct value was around 21.

Experiments could not be proceededforward because of time constrains during this study.

4 DISCUSSION

4.1 Determination of resistance to QACs

Frequent use of QACs for better hygiene and sanitary practices in food processing environment and hospitals enhances the level of resistance to QAC and antibiotics (Marrie&Costerton, 1981). In our study all the thirteen*Serratiamarcescens* strains from the food industry shows resistant to BC and CPC. The results indicate that disinfecting footbath containing TEGO103G, TP99, and floordaily treated with triclosan and meatsaw, abbatoir daily treated with QAC can select for the resistant*Serratiamarcescens*. These strains had the ability to multiply up to the recommended use in concentration, but the laboratory strain of *Serratiamarcescens* was relatively sensitive to one of the disinfectant used in this experiment, so it explains that either resistance to these disinfectants is not common properties of this species or laboratory strains lost their resistant traits.

The term resistance is a relative term in case of disinfectants. Resistant strains are those strains that are either not killed or not inhibited by the concentration that usually kill/inhibit the most of the strains of that organism(A. Russell, 2010). In this study,MIC and bactericidal test was used to primarily investigate the resistance. MIC test also shows the possibility of microbial growth adaptation to occur. Although MIC and Bactericidal test measure different properties, both test are important for the use of any disinfectant in the food industry(Sundheim et al., 1998).

As per the common definition of disinfectant, the elimination of hazard of infection or contamination is the main purpose of the disinfectants(Reybrouck, 1998),so the bactericidal effect of a disinfectant is the main purpose in the food industry .To determine the bactericidal effect suspension test was performed on QAC sensitive, resistant and adapted strains. The observation showed that *Serratiamarcescence* that have higher MIC value of QAC, results in high survival in bactericidal test, but it was different in case of ATCC 13880 which showed higher MIC value on BC but was greatly reduced in suspension test by more than than 5 log10 reduction. This is because in bacteriostatic test exposure time of bacteria and disinfectant is longer than in bactericidal test, so neutralization of the disinfectant may occur after specified time of exposure to the bacteria in media. This result is in accordance with the earlier investigation that the correlation between bactericidal and bacteriostatic action has not always been observed (Sundheim et al., 1998).

4.2 Adaptation of sensitive strains

Adaptation curves indicate sensitive *Serratiamarcescens* adapt to higher concentration on gradual exposure to increase concentration of QACs.The tolerant footbath strains MF 3299, MF300 which has high MIC value than ATCC 14041 comparatively acquired resistance in few transfer than ATCC 14041 strains. In case of ATCC 13880 curves indicate that it has to overcome at least one barrier to grow in high concentration of CPC (Fig. 2). Up to 140-150 ppm there was consistent increase in the resistance. The inoculum size was increased to 100µl from 50µl because there was no increase in resistance after that concentration. Similar pattern of adaptation was seen in previous study, adaptation of *E.coli* on BAC (Solveig Langsrud, Sundheim, & Holck, 2004). The colony of a laboratory strain loses its red colour pigmentation during adaptation. The mechanism behind pigmentation and resistant is unknown but it has been reported that most of the strain that causes infection does not produce pigment (Phillips & King, 1977) .These results are in accordance with the previous result reporting that sub lethal exposure to disinfectant might develop resistance(Sidhu, Sørum, &Holck, 2002).This result also highlight on the fact if sub lethal exposure induced adaptation then in the food industry QACs on equipments, floors, surfaces there are QAC left over which might cause microbial growth and adaptation, this could have serious effects on disinfectant procedures and practices used in the food industry. Bactericidal test performed for adapted strains also confirms the adapted strains were resistant to BC.

4.3 Resistance mechanism

Gram-negative bacteria show intrinsic resistance mechanism to QACs either by the expression of the efflux pump or changes in the membrane permeability(Jones et al., 1989). Focus has been put into the efflux pump because of its ability to pump structurally unrelated compounds (Poole, 2005). RND family transporters are most common efflux pumps in gram negative bacteria(Poole, 2000).*Serratiamarcescens* encodes at least three different RND pump SdeAB, SdeCDE and sdeXY(Maseda et al., 2009). In this study, we performed PCR for the primary screening of the Pump gene (*sdeB,sdeD,sdeY*). All the strains showed the presence of pump gene along with the adapted strains. Primary screening of this gene by conventional PCR revealed the presence of thesegene in all the strain.Unfortunately expression of this gene could not be revealed because there was shift in melting curve in adapted strains compared to the sensitive strains which indicates the formation of another qRT-PCR product on the adapted strains. Peak was not the primer dimer because it was not in

the early stage. There was also high signal from no RT control. In this study we were more interested in the sdeAB pump because from the previous finding sdeCDE pump has limited substrate specificity specially novobiocin(SanelaBegic& Elizabeth A Worobec, 2008).SdeXY pump was also the topic of interest because previous finding shows that it pumps for several antimicrobial compounds like erythromycin, norfloxacin,benzalkonium chloride and acriflavine (Chen et al., 2003). We were more interested in *sdeB* because we had mutation on the *sdeS* gene on adapted and resistant strains.We tried with the different primer set with same assay but unfortunately we had the same problem as before. We checked the RNA purity by Nano drop and Bioanalyzer (Agilent 2100)

The *sdeB*gene codes an efflux pump and is one of the key elements in the mechanism of resistance to the biocides (Begic&Worobec, 2008).The *sdeS* gene located on the downstream of the operon regulates the expression of sdeAB,Mutation in the *sdeS* gene causes the high level expression of the sdeAB pump genes resulting the multidrug and biocide resistant Serratia*marcescens*.(Maseda et al., 2011).In this experiment we amplify the short fragments of *sdeB* twice with two different set of primers and whole *sdeS* gene. Sequencing of the *sdeS* gene revealed a mutation on the resistant and adapted strains. There were 11 different base substitutions on *sdeS* nucleotide sequencing which results into the two amino acid substitution in protein sequence with one insertion. This result is contrast with the previous investigation reporting that there was a single point mutation at 269 position of the nucleotide sequence converting 90th position of aminoacid sequence tryptophan to stopcodon(Maseda et al., 2011). In this study the adapted strains and two foot bath strains (MF 3297 and 2325) at 153 position of amino acid has an insertion of extra glutamine.This phenomenon was absent in other foot bath strain. Two amino acid substitution tryptophan to alanine and Aspartic acid to glycine were common in adapted and food industry strains. This suggests that this mutation might have some change in the function of *sdeS*protein that finally effects on sdeAB expression. The amino acid substitution in this study is the conversion of acidic and polar amino acid to non-polar amino acid. Software Prediction of secondary structure of *sdeS* protein shows that helical structure of protein(sensitive strain) was changed to coil form(resistant and adapted strain) from position 141 to 149 in amino acid sequence.

4.4 Cost of fitness and stability of adapted strains

Biocide resistance confers a biological cost.It's generally accepted that the acquisition of antimicrobial resistance produces a global burden on fitness cost, so wild type bacteria are more competitive than their susceptible resistance partners(Andersson& Levin, 1999; Gravesen et al., 2002).

In this study we observed that 17 to 48 percentage of BC adapted ATCC 13880 stains lost its resistance when sub cultured on BC free medium up to 10th culture and there was significant difference on the growth rate between sensitive and adapted strain type($p<0.05$). Biological cost may be due to the overexpression of the efflux pump, which may cause the metabolic imbalances (Sánchez et al., 2002). The fitness cost will be observed as reduced growth rate because mutated genes that cause resistance are also important for other bacterial physiology (Martinez et al., 2009). For example in *E.coli*, mutation in the *rpoB* gene lowers the rate of elongation of transcription(Reynolds, 2000). Mutation in *Salmonellatyphimurium* of *rpsL* gene decreases the growth rate by decreasing the translation elongation rate

In case of strain ATCC14041 there was loss of resistance up to 85 percentage on ten passage on CPC free medium and there was no difference on the growth rate between adapted and sensitive strains ($p>0.05$). From these two cases(ATCC 13880 and ATCC 14041) we can conclude that due to higher instability of a resistant strain in ATCC13880 there was no significant growth rate difference between adapted and non adapted strains. This result is in accordance with (Ender, McCallum, Adhikari, & Berger-Bächi, 2004) where there was an increase in growth rate after removing resistance genes from resistant strains. .Also it seems that actual fitness cost is isolate specific (Gravesen et al., 2002).

The stability test in both the cases during the evolution in the absence of QAC selective pressure, some percentage of strains may results into mutational events favouring towards reversion to lower level resistance phenotype that result in lower fitness cost.This result is in accordance with the previous investigation by (O'Regan et al., 2010)where phenotypically characterised *Salmonella enteritica*in the absence of antibiotic selection pressure acquires reversible mutation making an intermediate level ciprofloxacin resistance phenotype with less significant fitness cost.

4.5 Cross resistance

Cross resistance may results due to the mutation by selective pressure of antimicrobial agents which results into expression of efflux pump or changes in the membrane permeability.(Legakis, Tzouvelekis, Makris, & Kotsifaki, 1989; Poole, Krebes, McNally, & Neshat, 1993)

Biocides resistance *Serratiamarcescen*s shows cross-resistance with antibiotics(Maseda, Hashida, Konaka, Shirai, &Kourai, 2009). In this study there was no increase in NorfloxacinMIC value on increasing the BAC and CPC resistance. There were only 0.2µg/ml differences in the MIC value of Norfloxacin between adapted and wild type strains. This result is in contradiction to the previous investigation by (Maseda et al., 2011) where there was 16fold rise in MIC value of Norfloxacin in resistant strain than in wild type strains.One fo the reason for this may be MIC value of Norfloxacin of Maseda sensitive strain was relatively low (MIC=0.2) as compared to sensitive strains of this study (MIC=0.7). There was no such difference in MIC value between adapted and sensitive strains so there might be involvement of other mechanism besides sdeAB pump but due to the fact of common mutation on *sdeS* gene in adapted and sensitive strains it cannot be fully accepted.

5. Conclusion

From this study, we can conclude that frequency of resistant*Serratiamarcecens* to QACs (Benzalkonium chloride and Cetylpyridimine chloride) is high in food industry. Sensitive *Serratiamarcescens* can be adapted to higher concentration of QACs by sublethal exposure to QACs. Mutation in the *sdeS gene* causes the expression of the SdeAB pump(Maseda et al., 2011).In this study, there was also a mutation on the *sdeS* gene, all the adapted strains have the same amino acid substitution with the insertion of glutamine at 153 position which is in contrast to the most footbath strains they don't have an insertion. Other two amino acid substitution tryptophan to alanine, and aspartic acid to glycine are common in the adapted and food industry strains.Unfortunately we were not able to analyse the expression of *sdeB*gene though we tried with two different assays and two different primer sets**.** Due to time constrains, we could not do further studiesin order to elucidate the resistance mechanism.In this study we did not observe any crossresistance betweenQACs resistance strain and Norfloxacilin**.**Resistance to QAC is not a stable trait.We observed that 17 to 85 percentage of QAC adapted ATCC stains lost its resistance when subcultured on QAC free medium up to 10^{th} culture.

The presence of resistant strains in the food industry, indicate that the use of QAC in the food industry selects for QAC resistance *Serratiamarcescens*.

Biocide resistance confers a biological costwe observe that BC adapted strains exhibited a fitness cost, having reduced growth rate, there was significant difference on the growth rate between adapted and sensitive strains ($p<0.05$).

6.Further work:

We found that there was mutation on the *Sde*S gene. To check whether this mutation causes resistance this mutated gene can be inserted to the biocide sensitive strain for further analysis. RNA expression of the *sdeB*gene can be tried more accurately. Further we can look for RNA expression for SdeXY.

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8. APPENDIX

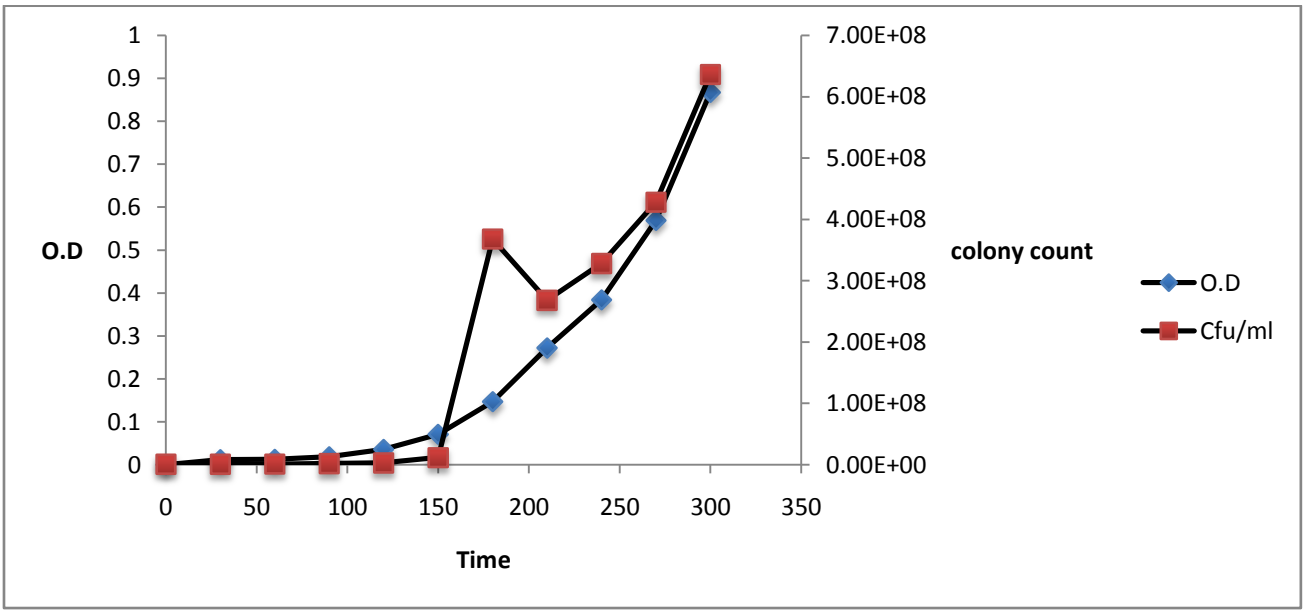

Figure A. O.D vs Colony forming unit count vs time graph for ATCC 14041

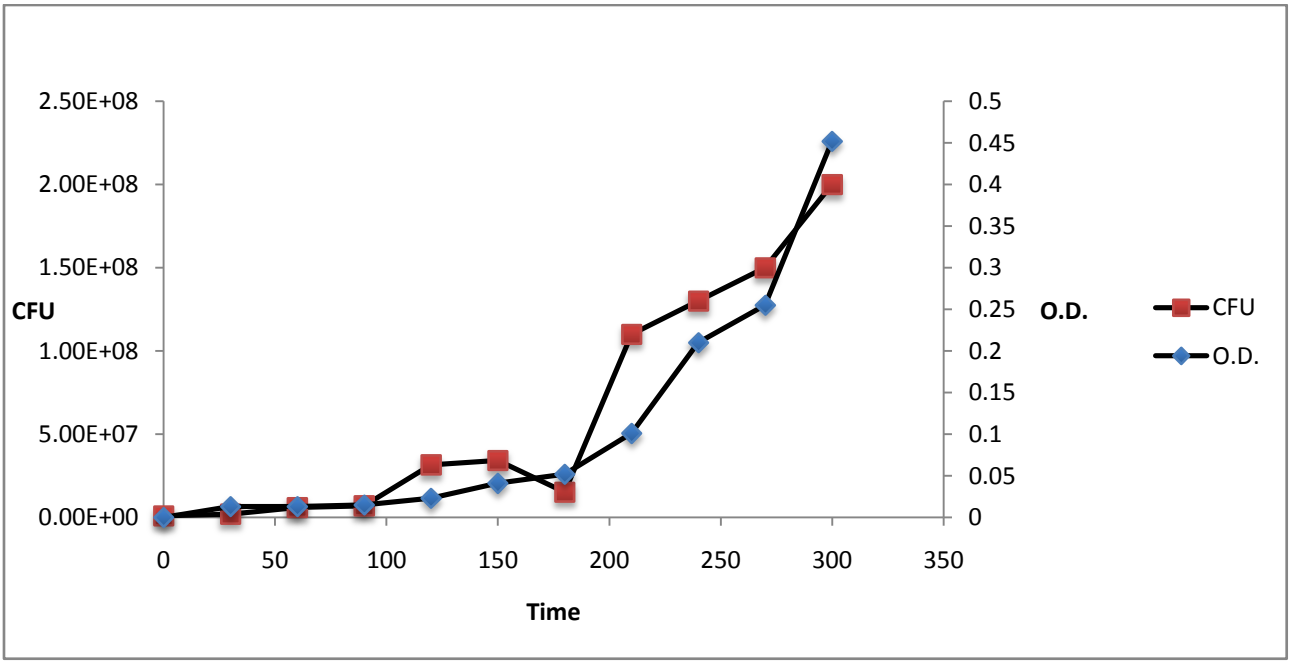

Figure B O.D vs Colony froming unit vs time graph for ATCC 13880

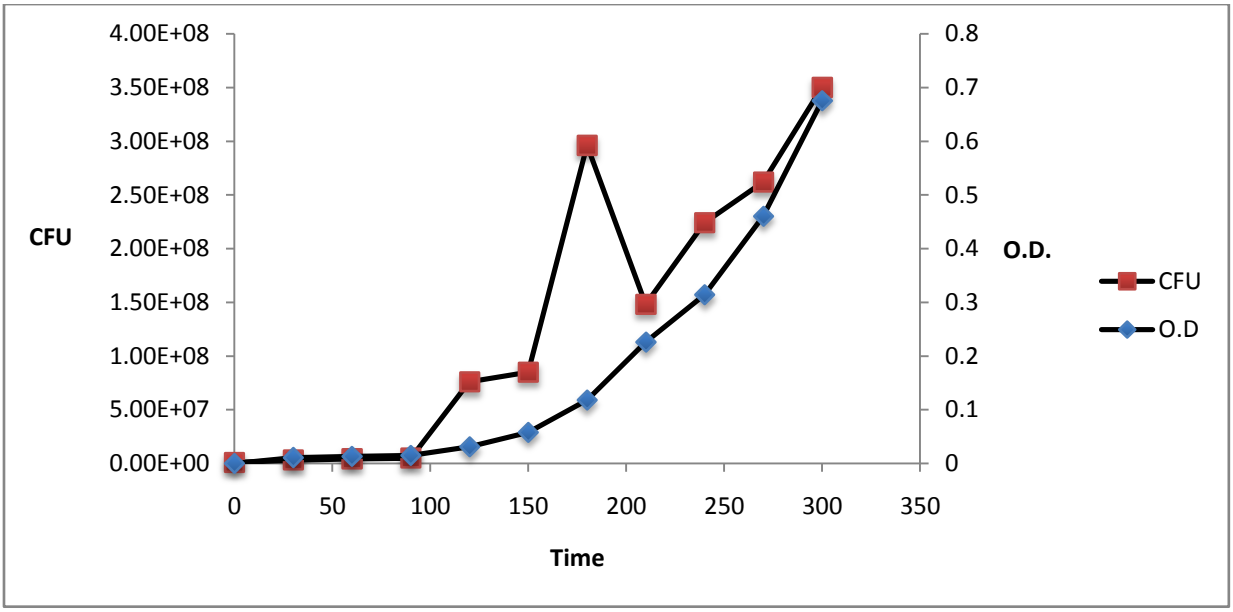

Figure c O.D vs time vs colony count for 14041 strains

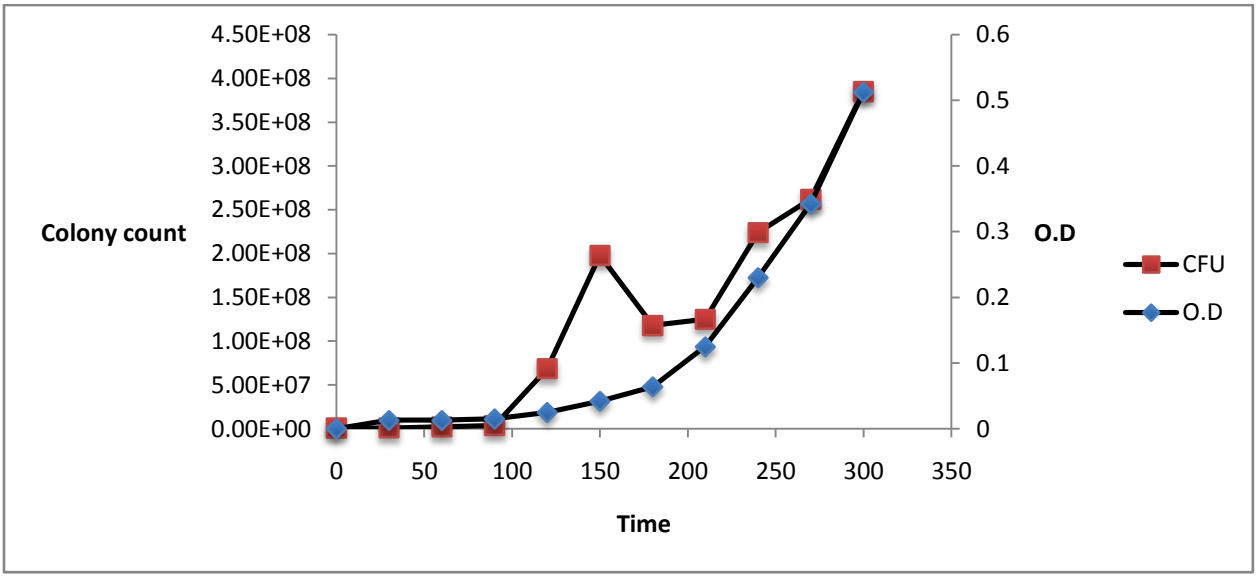

Figure D. O.D vs colony vs time graph for 14041 ADP strains

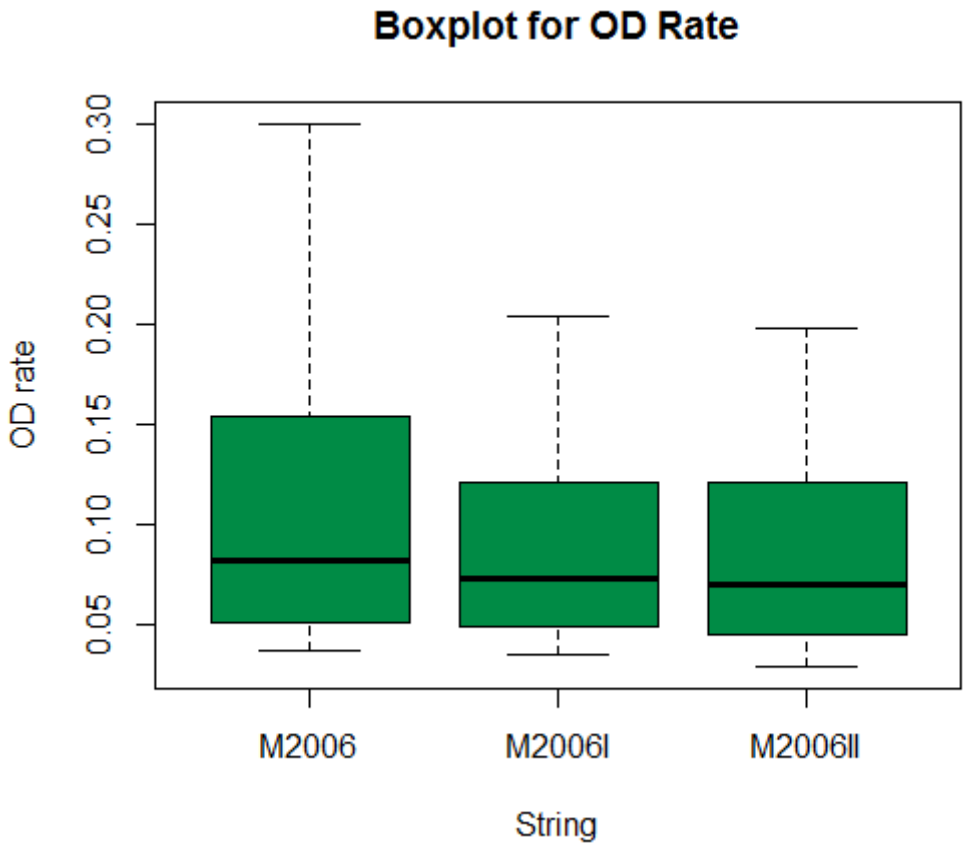

Figure E. Box plot for OD rate for ATCC 14041

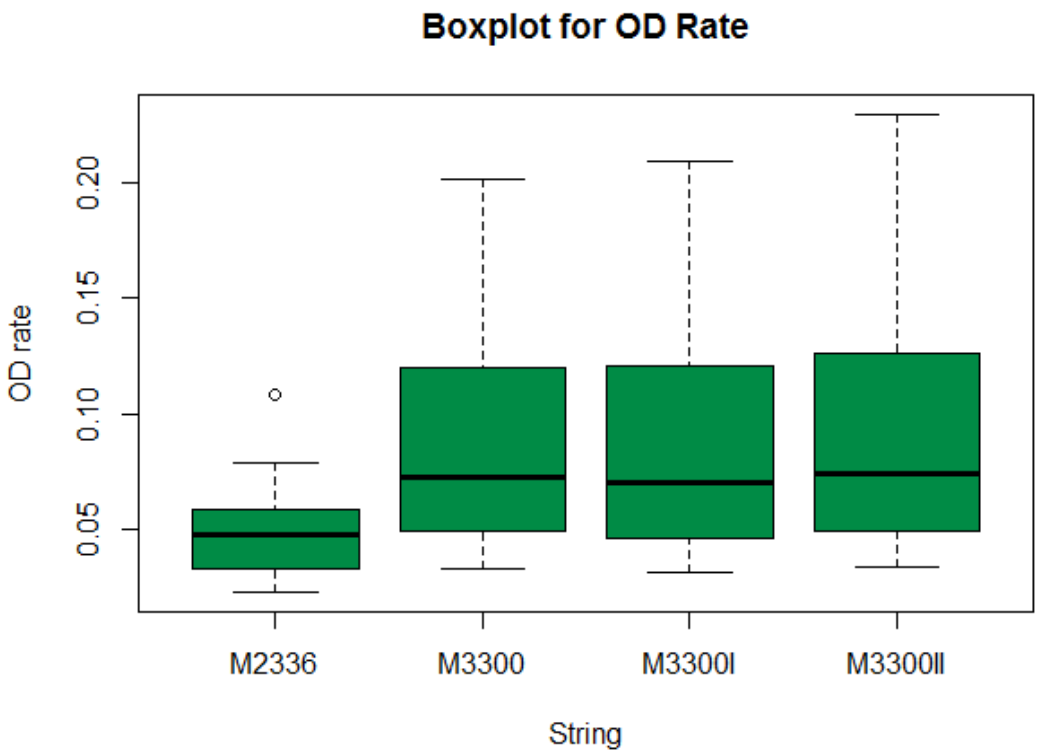

Figure f. Box plot forgrowth rate for MF 3300 and adapted strains

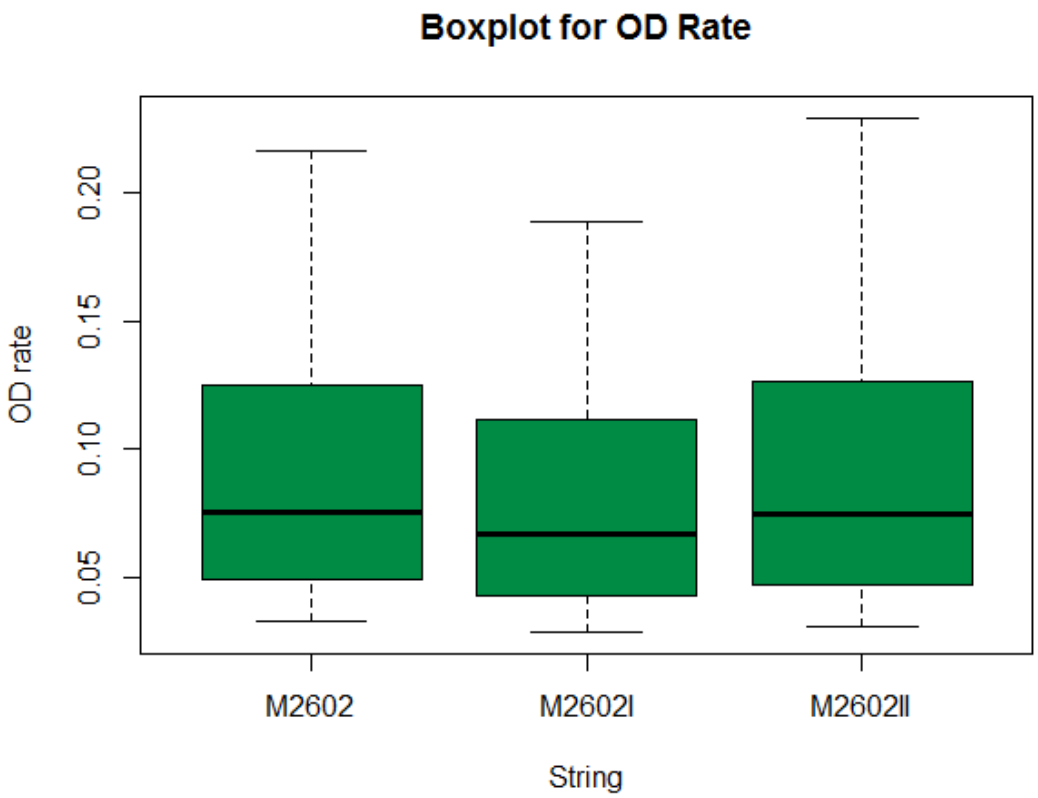

Figure g. Box plot for growth rate for ATCC 13880