

Chapter-4

*Mode of Chromomycin Resistance in *Micrococcous luteus**

INTRODUCTION

Antibiotic resistance is an increasing problem for the treatment of infectious diseases. Bacteria have evolved diverse mechanisms (pathways) of resistance to antimicrobial agents. This is leading to shortage of antibiotics that can take care of multidrug resistant strains of pathogenic organisms. It is believed that only a small portion of total antimicrobials available in nature have been explored so far and that there is a huge scope of finding various new natural products, with new targets, to kill bacteria. At the same time, the fact is that after investing billions of dollars in resources and millions of valuable human hours, the success rate in new drug discovery is very frustrating. Since the introduction of the quinolone in 1962, only one antibiotic of a completely new structural class — the oxazolidinone linezolid (Zyvox; Pfizer), was approved in year 2000 and entered clinical use. While Daptomycin, the first among cyclic lipopeptides, discovered at Eli Lilly in the early 1980s has reached third phase of clinical trials. This leaves with only generating new analogs of the existing molecules; in particular, penicillins, cephalosporins, macrolides and quinolones — to ensure recovery of activity against those bacteria that have become resistant to the previous generation of drugs.

Another approach of combating menace of antimicrobial resistance is by developing resistance in bacteria at laboratory level, and finding out the mechanism of resistance. This approach emphasizes on increasing the life span of an existing drug, by supporting it with some inhibitors that can postpone resistance development. Thus, if a combination of resistance inhibitor is administered along with a drug; same drug can be effectively used for long without threat of pathogen developing resistance.

The test organisms used in the study to measure the bioactivity of chromomycin were two Gram positive bacterial species viz., *M. luteus* and *B. subtilis*. In the routine use of each of the strains, we noticed qualitative differences in terms of sensitivity of each of the organism towards chromomycin. Firstly, *B. subtilis* was more sensitive to chromomycin

than was *M. luteus*; secondly, *M. luteus* could very easily generate adaptive mutants to chromomycin whereas *B. subtilis* was excessively sensitive to concentration that was sublethal for growth of *M. luteus*. Though DNA acting antibiotics and drugs have limited clinical applications for treating infectious diseases, they are an important class of anticancer agents. However, it is now a fact of concern that cancer is increasingly becoming refractory to several natural and synthetic compounds, in many cases due to mutations in genes for P-Glycoproteins, functional homolog of bacterial MDR transporters. The sameness extends much more than this. The fact that bacteria, several pathogens included, like cancerous cells, have the ability to generate adaptive mutations, both stable point and unstable amplification mutations, can be equally worrisome in the clinical course of treatment. Understanding the origin and nature of adaptive mutations is valuable in designing strategies to counter the antibiotic resistance threat. We undertook studies on adaptive resistance in *M. luteus* also to find out correlation if any relatedness exists, between bacterial and cancer cells' mechanism of resistance to anticancer compound chromomycin. Thus, it becomes interesting to study, under given circumstances, how does a bacterium develop resistance.

The present study covers different aspects of chromomycin resistance in *M. luteus*. To maintain the flow of entire study, the work has been divided into three different parts in this chapter. First part is about generation of resistance in *M. luteus* and experiments associated with it. Second part deals with the mode by which chromomycin resistant mutant of *M. luteus* (Chromo^r) resists the toxic antibiotic environment. In third part, attempt has been made to work out the mechanism of resistance at molecular level, to further strengthen the results of first and second part.

RESULTS & DISCUSSION

4.3.1 Generation of Chromomycin Resistant Mutant of *M. luteus*

On culturing *M. luteus* cells in presence of chromomycin, spontaneous mutants could not be developed from 10^6 cells/ml and the result was unaltered even by culturing them till 10^{10} cells/ml. Some colonies could be obtained on chromomycin supplemented LA plates after prolonged incubation (> 3 days), but all colonies failed to grow when streaked on fresh LA with chromomycin. Thus, colonies did not attain resistance but represent result of a temporary phenomenon called persistence, which is a physiological state wherein few cells escape lethal action of the antibiotic, irrespective of its mode of action (Debbia et al, 2001). The population arising from these persisters show same susceptibility pattern as that exhibited by original inoculum.

Normal frequency of developing a desired spontaneous mutant in bacteria varies between 10^{-6} /cell and 10^{-8} /cell (Winkler, 1972). The ease of obtaining spontaneous mutants with a drug largely depends on the target of the drug (Wang et al, 2001) and the concentration of the drug used (Martinez et al, 2000). For chromomycin, the target site being GC rich minor groove of DNA double helix (Chakrabarti et al, 2001) there was little to no scope for drug target modification. So, the only scope of generating resistant mutant was by changing antibiotic concentrations.

Sub-lethal concentration, used for the assay, was the concentration of chromomycin that wild type cells could withstand without compromising its growth. Minimal inhibitory concentration of *M. luteus* being 120 ng/ml, as obtained in results of broth assays (Chapter - 3); starter culture was setup with an assured sub-lethal dose of 5ng/ml chromomycin in LB. Drug concentration was doubled at each subculturing stage. Since chromomycin concentrations were far below lethal levels, the cultures could grow as luxuriantly as

antibiotic untreated control during the early subculturing stages. Subsequently, there was a lag in growth observed after fifth subculture (80 ng/ml chromomycin) and incubation periods required for the culture to reach stationary phase increased with every subsequent sub-culturing. This delay could be attributed to the energy expenses incurred by the cell in withstanding the toxic environment, leading to a compromised growth rate (Santiago et al, 1999). An extra step of transfer to fresh medium without increasing chromomycin concentration ensured proper acclimatization of cells to withstand the selection pressure before being exposed to a higher concentration. Thus, a step-wise increased chromomycin exposure to cells, led to development of adaptive mutants (Chromo^r) that could endure more than 2.4 ug/ml of chromomycin concentration. Though there was a compromise in growth rate, these cells could withstand chromomycin 20 times its MIC values (120ng/ml). Inability of *M. luteus* to generate a resistant mutant spontaneously and development of the same by gradual adaptation could have occurred due to;

Case A - Either need of more than one favourable mutation in a single gene or a single favourable mutation in more than one gene, each directly imparting resistance.

Case B - Under influence of continuous stressful condition, altered regulation of particular gene to generate more transcripts and ultimately increased protein synthesis, imparting resistance.

Case C - Amplification of gene of interest to form multiple copies, thereby positively affecting protein synthesis.

4.3.2 Reversion Frequency:

Resistant phenotype was a result of gradual adaptation of wild type cells to withstand the toxic environment. Whether the change leading to resistance in Chromo^r was stable or in absence of selection pressure would be gradually lost, was not known.

Some colonies from Chromo^r culture grown in absence of chromomycin pressure failed to grow on LA supplemented with chromomycin, whereas its replica on LA without chromomycin could grow. Loss of resistance in those colonies was suggestive of their

reversion to wildtype phenotype. A reversion frequency of 3% was obtained for each generation. Though other cases cannot be ruled out, this initial result is indicative of the phenomenon to be following Amplification Mutation model (Case -C), which has similar reversion rate. This model was first proposed by experiments with *Salmonella* (Roth and Anderson, 2004). Also, the compromised growth rate observed in adaptive mutant, according to this model, is due to the deleterious effect of those genes in amplicon that are not involved in imparting resistance.

In both eubacteria and eukaryotes, gene amplification is a common mechanism of adaptation in response to different types of selective pressures. Most bacterial genomes are in a dynamic state with regard to gene copy number, and spontaneous duplications form and segregate at high rates. For example, in *S. typhimurium* under nonselective growth conditions, most genes are duplicated with a frequency of 10^{-5} to 10^{-2} , suggesting that at any given time 10% of all cells in a population have duplication somewhere in the chromosome (Sonti and Roth, 1989). The highest frequency and largest duplications are typically seen between *rrn* operons, where extensive homologies are available to form the duplication (Anderson and Roth, 1981). Certain duplications may be further amplified in response to a novel selection pressure, which could possibly confer a fitness advantage. Formation of the first duplication is probably the rate-limiting step in high level gene amplification (Reams and Neidle, 2004a). Because of the high intrinsic instability of tandem amplifications, haploid segregates will rapidly appear and take over the population when the selective condition disappears (Reams and Neidle, 2004b) which leads to the reversion phenomenon.

Thus, the gain and loss of this phenotype, over a period of time, could be probably involving gene amplification and deamplification phenomenon imparting resistance in Chromo^r.

4.4 Characterization of Mode of Chromomycin Resistance in *M.luteus*

Microbial resistance to antibiotics is manifested by changes in antibiotic permeability, alteration of target molecules, degradation of the antibiotics, and efflux of

antimicrobials from the cytosol. Bacteria and other microorganisms use all of these mechanisms to evade the toxic effects of antibiotics.

4.4.1 Alteration of the target site

Alternation in the site of action of antibiotic may prevent the interaction and hence the toxicity of antibiotics. These alterations comprise amino acid substitutions, which decrease the affinity for the drugs involved. Penicillin resistance can be caused by alterations in the penicillin-binding proteins (PBPs) that form irreversible complexes with penicillin thereby inhibiting their role in the peptidoglycan synthesis (Spratt, 1994). Erythromycin and tetracycline resistance can be mediated by covalent modifications of the ribosomes, which make them less susceptible to the action of these antibiotics. (Takata et al., 1970; Speer et al., 1992)

In case of Chromo^r , such mode of resistance can be ruled out based on two facts; firstly, the target being minor groove of DNA, it is very unlikely for the cell to undergo conformational change in its most basic structure – the double helix; secondly, the high reversion frequency shows the change has to be reversible and so one cannot expect the cell to keep on changing its DNA structure.

4.4.2 Drug inactivation

Drug inactivation is achieved by hydrolysis, group transfer, and redox mechanisms. While hydrolysis is especially important clinically, particularly as applied to β -lactam antibiotics, the group transfer approaches are the most diverse and include the modification by acyltransfer, phosphorylation, glycosylation, nucleotidylation, ribosylation, and thiol transfer. Redox reaction, a popular mammalian xenobiotic detoxification mechanism, is not known to be frequently exploited by pathogenic bacteria. These mechanisms have been reviewed in detail recently (Wright, 2005). Despite difference in methods of drug inactivation, a unique feature of all enzymes that physically modify antibiotics is that, these mechanisms alone actively reduce the concentration of drugs in the local environment.

Fig 4.1: Agar well assay for Chromomycin using a co-inoculum of wildtype and Chromo^r showing two distinct zones of inhibitions marked by difference in carotenoid levels of the two.



Inactivation of β -lactam antibiotics, like ampicillin is mediated by β -lactamases that catalyse hydrolysis of ring structure (Spratt, 1994). This phenomenon is routinely observed in transformation of plasmids with ampicillin resistance marker. Large number of non-transformed cells, popularly called satellite colonies, develops in vicinity of transformants due to antibiotic degradation. Based on this, and using pigment difference in wild type and Chromo^r as a marker, an assay was designed to study resistance mediated by drug inactivation.

An agar well assay of chromomycin was carried out using a co-inoculum of wildtype and Chromo^r. Fig – 4.1 shows two zones of inhibitions, a smaller zone ascribed to Chromo^r and an encircling larger zone of inhibition of wild type. The inhibition zone size matched to the values documented in Chapter -3. If the resistant cells were to cause chromomycin inactivation, wildtype cells' growth should encroach the restricted zone of inhibition. But presence of two distinct zones represented by marked colour difference is clearly suggestive of lack of any drug inactivation.

4.4.3 Curtailed entry of the antibiotic

It is a popular means of resistance as seen for most of the gram negative bacteria wherein their LPS layer forms a strong barrier against the entry of several toxic molecules

(Nikaido and Thanassi, 1993; Vaara, 1992). Alterations that influence permeability of drug barriers, like amount of outer membrane porins and/or lipopolysaccharides, can thus affect apparent resistance against drugs in gram negative bacteria (Jarlier and Nikaido, 1994).

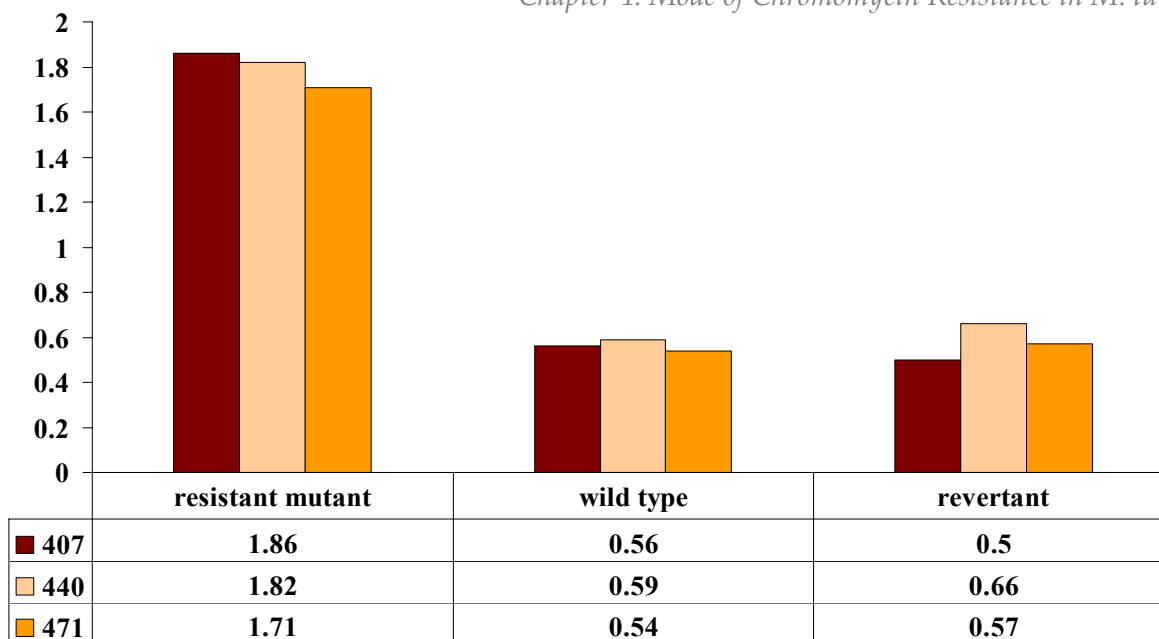
Gram positive bacterium on the other hand, does not possess any outer LPS layer or porins in the membrane, with exceptions like *Mycobacterium* that possess mycolata (Reiss et al, 2001), making them relatively more sensitive to toxic compounds. Instead, gram positive bacteria have been shown to alter membrane dynamics under extreme conditions (Khan et al, 1977).

4.4.3.1 Carotenoid estimation

During the adaptive change of Chromo^r, an interesting phenotype of change in colour intensity was observed, wherein characteristic pale yellow colour of wild type *M. luteus* (Medicharla et al, 1991) changed to turmeric yellow while acquiring resistance. This change was due to overproduction of pigment called sarcinaxanthin, which is a 50 carbon carotenoid present in its membrane (Hertzberg and Liaaen-Jensen, 1977). It is a polar carotenoid and an isomer of decaprinoxanthin, a pigment obtained from *Cellulomonas biazotea* (Weeks et al, 1980).

Pigment extraction and subsequent spectrophometric estimation of the same showed that there was over 200% increase in the pigment content at known absorbance wavelengths of sarcinaxanthin. Further, it was observed that the revertants for loss of chromomycin resistance, obtained in the earlier experiment, had also lost the phenotype of overpigmentation and were as pale yellow as the wild type (Graph-4.1). Thus, there could have been a direct link between chromomycin resistance and the overproduced polar carotenoid present in membrane of *M. luteus*.

Similar roles of carotenoids have been reported, as in case of *S. aureus* golden pigment present in the membranes that has been shown to be imparting resistance against neutrophils, oxidative stress (Liu et al, 2005) and antimicrobial agents like oleic acid



Graph 4.1: Carotenoid estimation from wild type, Chromo^r and revertants, as measured at three indicated wavelengths of absorption.

(Chamberlein et al, 1991). Alternatively, this membrane localized carotenoid might have an indirect role in chromomycin resistance, achieved by altering membrane dynamics. The effect of other polar carotenoids such as lutein, zeaxanthin, violaxanthin on the structure and dynamics of lipid bilayer membranes has been clearly demonstrated. (Subczynski et al, 1992 Subczynski et al, 1993; Wisniewska and Subczynski 1998).

4.4.3.2 Membrane fluidity assay:

In prokaryotes, the polar carotenoids serve as cholesterol equivalents of eukaryote and guide bacterial membrane fluidity (Subczynski et al, 1992a). Diphenyl Hexatriene (DPH) is a fluorescence probe, commonly used to study cholesterol guided membrane fluidity changes in mitochondria. Increased membrane rigidity (or reduced fluidity) do not allow DPH probe to loose its energy by undergoing rotational motions, when excited. Thus, the energy gets released in form of fluorescence which is directly proportional to the membrane rigidity.

DPH probe assisted fluorimetric study showed a significant difference between the membrane fluidity of wild type and Chromo^r. While the calculated average P value of wildtype was 0.186, Chromo^r was found to have 0.242. The increase in membrane rigidity can be attributed to elevated levels of 50 carbon characteristic polar carotenoid pigment – sarcinaxanthin present in *M. luteus*.

The increased sarcinaxanthin levels detected in Chromo^r in the earlier experiments, with reference to aforementioned findings suggests a direct correlation with the increased membrane rigidity. It has been observed that, membranes of extreme halophiles (Khan et al, 1977) and thermophilic bacteria (Yokohama et al, 1995) contain a fairly large amount of polar carotenoids. The psychrotropic strain of *M. roseus* possesses abundant of a C₄₁ unusual polar carotenoid instead of non-polar cathaxanthin, to maintain membrane rigidity (Medicharla et al, 1991). To withstand chromomycin stress, the cell, by incorporation of more carotenoid, might have developed rigid membranes that form barrier against entry of this molecule. It should be noted that in gram negative bacteria, chromomycin fails to cross strong membrane barrier (Lombo et al, 2006) and thus, the carotenoid mediated membrane rigidity in gram positive *M. luteus* might be mimicking similar resistance mechanism. Carotenoids stabilize both halves of the lipid bilayer like transmembrane “rivets” and increase membrane rigidity by ordering the alkyl chains of lipids. They also raise the membrane hydrophobic barrier for polar molecules and ions (Wisniewska and Subczynski, 1998) and the rigidity barrier for small non-polar molecules (Subczynski 1991; Subczynski, W.K. & Markowska, E. 1992). Chromomycin is strongly polar molecule and thus a candidate for becoming a victim to this adaptation. Thus, the results strongly support for a direct co-relation between membrane rigidity and chromomycin resistance.

4.4.3.3 Carotenoid inhibition using Diphenyl Amine

Diphenyl amine (DPA) is a known inhibitor of carotenoid biosynthesis pathway, inhibiting action of phytoene desaturatase that converts phytoene to lycopene in eubacteria (Pfander, 1994). The standardization assay with respect to *M. luteus* was carried out at three

Table- 4.1: Inhibition of pigment production in Chromo^r by Diphenyl Amine.

Wave length	Chromo ^r (control)	Chromo ^r + 12.5 ug/ml DPA	Chromo ^r + 25 ug/ml DPA	Chromo ^r + 50 ug/ml DPA
407	1.860	1.337 (6.3)	0.572 (60.0)	0.102 (92.9)
440	1.827	1.468 (15.1)	0.316 (81.7)	0.052 (96.5)
470	1.712	1.201 (25.3)	0.480 (70.1)	0.046 (97.1)

Figures in parentheses represent percentage inhibition over control.

different concentrations of DPA. Extraction of pigments from these treated samples clearly revealed, DPA dose dependent inhibition of sarcinaxanthin biosynthesis. There was an insignificant reduction at 12.5ug/ml DPA concentration. At 50ug/ml a commanding inhibition of carotenoid biosynthesis could be achieved turning the cells practically white in colour with marked <90% inhibition (table- 4.1) at all the wavelengths under study. There was no growth retardation in cultures at this concentration, suggesting it to be non-stressful for the cell.

Though we established, that sarcinaxanthin concentration in membrane governs membrane fluidity in *M. luteus*, its direct or indirect role in imparting resistance remains unexplored. Chromomycin agar well assay of Chromo^r in presence and absence of DPA, did not reveal any difference between their zones of inhibitions. If carotenoids were to play a direct role in imparting resistance against chromomycin, carotenoid suppressed (DPA treated) culture of Chromo^r should have shown larger zones of inhibition comparable to those of wildtype. But lack of sensitivity (persistence of resistance) suggests that carotenoids primarily do not impart resistance to the cell.

In light of the outcome of studies resulting in increased carotenoid content, reduced membrane fluidity and persistence of resistance in DPA treated Chromo^r, it appears that carotenoid overproduction does not cause resistance, but is rather a consequence of

resistance. Albeit, sarcinaxanthin alone might not alter the fate of resistance, but its significant impact on membrane fluidity suggests that the phenomenon might be helpful in checking the rate of chromomycin diffusion inside the cell and thereby reduce load on the primary resistance machinery.

4.4.4 Active drug efflux

Drug extrusion by the cell has been the most common method of resistance. There have been several such pump families, with varying specificities, discovered from large number of pathogenic isolates (Poole, 2005). They vary from being highly specific, like MefE pump of *S. pneumoniae* for Erythromycin (Tait-Kamradt, 1997), to a very broad range pumps such as MexAB system of *P. aeruginosa* mutant, that act on a range of antibiotics (fluoroquinolones, β -lactams, chloramphenicol and trimethoprim) and also triclosan, a commonly used household biocide (Chuanchuen et al, 2001).

4.4.4.1 MIC determination of *M.luteus* Chromo^r against different antibiotics :

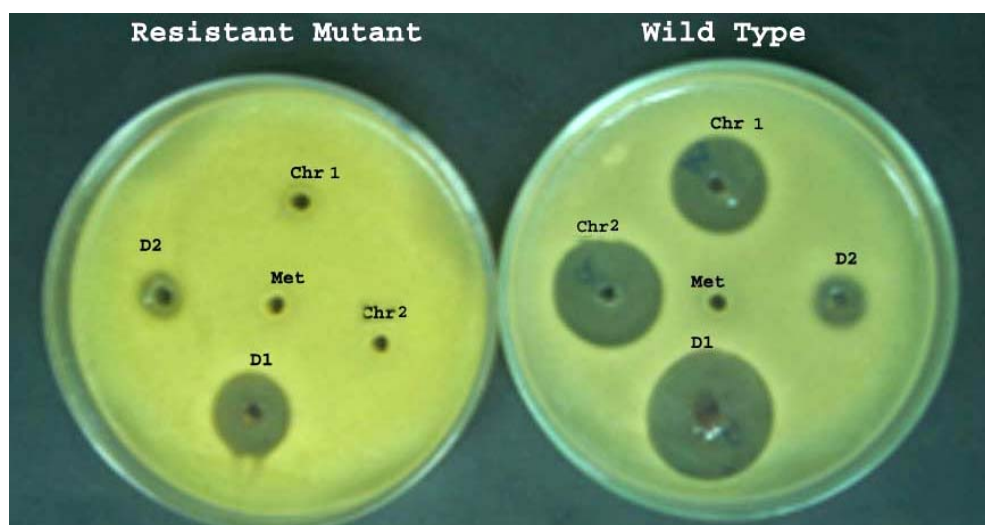


Fig - 4.2 : Difference in inhibition zones of Chromo^r vs wild type *M. luteus* against Chromomycin (Chr₁ = 0.25ug; Chr₂ = 0.5ug) and Daunomycin (D₁ = 0.5ug; D₂ = 1ug)

Owing to the remarkable ability of Chromo^r mutant to withstand supralethal concentrations of chromomycin, it was tested for resistance against various other antibiotics with varying bacterial targets (Fig- 4.2). A relative study of minimal inhibitory concentrations of different antibiotics on wild type and Chromo^r suggested that the later could quite effectively tolerate few other antibiotics as well (Table- 4.1). From the results of

Table – 4.2: Cross resistance of adaptive mutants against the group of antibiotics.

Antibiotic	Concentration	Wild type	Chromo ^r	Dauno ^r	Tet ^r
Tetracycline	0.5µg/ml	+	+++	+++	+++
	1µg/ml	-	++	++	+++
	2µg/ml	-	++	+	++
	3µg/ml	-	++	-	++
Erythromycin	10µg/ml	+	++	++	++
	20µg/ml	+	++	++	++
	30µg/ml	-	++	+	+
	40µg/ml	-	+	+	+
Chromomycin	500ng/ml	+	+++	+++	++
	1ug/ml	-	+++	++	++
	2ug/ml	-	+++	+	+
Daunomycin	1µg/ml	+	+++	+++	+++
	2µg/ml	-	+++	+++	++
	5µg/ml	-	++	+++	+
	10µg/ml	-	+	++	-

- = no growth; + = less growth ; ++ = moderate growth ; +++ = profuse growth

the agar well assay, the ability of Chromo^r to resist daunomycin, tetracycline and erythromycin becomes very apparent as shown in the (Table- 4.2). There was no significant difference in the MIC of wild type and Chromo^r generated by other antibiotics (ampicillin, apramycin, carbenicillin, chloramphenicol, gentamycin, kanamycin, mitomycin, rifamycin, streptomycin, spectinomycin, trimethoprim) covering a spectrum of targets used in the study.

Of the molecules against which Chromo^r resisted, daunomycin - belonging to anthracycline class of antibiotics, has same target as that of chromomycin. Macrolide antibiotic class members - erythromycin and tetracycline— on the other hand, binds to ribosomes. It is to be noted that all the four antibiotics are polyketide products but belong to different class of antibiotics. Thus, these are chemically and structurally different molecules against which the resistance is achieved. The common feature among them is that they all act on nucleic acid in one way or other (ribosome being a modified RNA).

Multidrug resistance (MDR) is a very prevalent feature of several pathogenic bacteria wherein structurally and functionally unrelated antibiotics are extruded out of the cell by efflux pumps (Poole, 2002). Though Chromo^r shows exactly same phenotype, the

Table- 4.3: Zone of inhibition of WT and Chromo^r against selected antibiotics

Antibiotic	Concentration	Wild type(cms)	Chromo ^r (cms)
Erythromycin	100µg	2.9	2.4
	10µg	2.4	1.5
Tetracycline	50µg	3.0	2.1
	5µg	1.8	Insignificant
Daunomycin	10 µg	1.7	1.0
	5 µg	0.8	Insignificant

generation of Chromo^r involved a passage of several generations during which there are chances that random spontaneous mutations might have occurred in Chromo^r genome. Resistance acquisition against tetracycline and erythromycin by spontaneous mutations are well documented in different bacteria (Takata et al., 1970; Speer et al., 1992). It could be such mutations, independent of Chromomycin resistance that could have imparted resistance to other antibiotics. Thus, it was essential to check whether the phenotype was achieved as a result of accumulation of several point mutations in unrelated genes conferring resistance to particular antibiotic individually or that the mechanism was common for all the antibiotics in question.

Adaptive mutants against daunomycin, tetracycline and erythromycin were generated independently, following exactly same protocol as one used for generating Chromo^r. When tested for MIC against various antibiotics, the three mutants resisted same set of antibiotics namely chromomycin, daunomycin, tetracycline and erythromycin (table- 4.4 and 4.5).

Table- 4.4: Difference in the inhibition zone of WT and Dauno^r against selective antibiotics

Antibiotic	Concentration	Wild type (cms)	Dauno ^r (cms)
Chromomycin	300 ng	2.6	1.6
	100 ng	1.8	1.0
Daunomycin	10 µg	1.7	1.0
	5 µg	0.8	Insignificant
Tetracycline	50 µg	4.0	2.8
	5 µg	1.5	0.8
Erythromycin	100 µg	3.6	2.2
	10 µg	3.5	2.2
Gentamycin	300 µg	2.7	2.2
	30 µg	2.0	1.6

Table- 4.5: Difference in the zone of inhibition of WT and Tet^r

Antibiotic	Concentration	Wild type(cms)	Tet ^r (cms)
Chromomycin	200 ng	2.4	1.9
Daunomycin	10 µg	2.0	1.5
	5 µg	1.1	0.5
Tetracycline	50µg	3.0	1.7
	5µg	3.0	1.7
Erythromycin	100µg	2.9	2.4
	10µg	2.6	2.0

If the resistance against different antibiotics in Chromo^r had resulted due to accumulation of independent mutations, then while repeating the same procedure for Dauno^r, Tet^r and Ery^r, there was equal probability for cells to generate resistance to other antibiotics and not the same set, since mutation is a random event. In view of the fact that this did not happen, it proved that resistance against unrelated antibiotics, developed in *M. luteus*, is not a chance event and there exist a common factor associated with resistance against this set of antibiotics. This confirms the phenotype of Chromo^r to be associated with MDR. The resistance imparted by each adaptive mutant against the same set of antibiotics is significant. The results obtained in agar well assays for all the resistant mutants were also confirmed by broth assays.

4.4.4.2 Reserpine assay

It has been observed that the multidrug resistance, in most cases, is due to an active efflux pump present in the membrane of these cells (Poole, 2005). Efflux pumps are able to extrude structurally diverse compounds, including antibiotics used in a clinical setting; the latter are rendered therapeutically ineffective. (Stavri et al, 2007).

Table- 4.6: Effect of selective antibiotics on the Chromo^r in presence of reserpine.

Antibiotic	Reserpine Concentration (µg/ml)	Chromo ^r + reserpine	Chromo ^r + antibiotic	Chromo ^r + antibiotic + reserpine
Chromomycin (0.2 µg/ml)	20	++	++	-
	10	++	++	-
	2	++	++	-
	0.2	++	++	-
Tetracycline (1µg/ml)	20	++	++	-
	2	++	++	-
	0.2	++	++	-
Erythromycin (10µg/ml)	20	++	++	-
	2	++	++	-
	0.2	++	++	-

Reserpine is an indole alkaloid obtained from an Indian plant *Rauwolfia serpentina*. It is classified as an antihypertensive drug since long (Sheldon and Kotte, 1957). It is also a popular efflux pump inhibitor (EPI) and is considered to inhibit members of resistance nodulation division (RND) family and the ATP binding cassette (ABC) family of efflux pumps (Braoudaki and Hilton, 2005). When tested over a range of concentrations, in isolation, neither reserpine nor the drug (chromomycin / tetracycline / erythromycin / daunomycin) proved inhibitory to Chromo^r growth, but the combination was found to be lethal even at 0.2 µg/ml concentration of reserpine used in the study (table- 4.6).

The antibiotic concentrations used in the study were comparable to the MIC concentrations of wild type. The results of the assay clearly suggest that there exists an efflux pump in Chromo^r, imparting MDR phenotype, and when blocked by reserpine, Chromo^r exhibits wildtype phenotype. If the dose of reserpine required in the assay, can be directly correlated with the abundance of efflux pumps present in the membrane, the

concentration required in this assay was considerably less than the standard concentration of 20µg/ml used in other cases. (Neyfakh, 1993; Schmitz, 1998; Ahmed et al, 1993; Ramon-Garcia et al, 2006).

Efflux of drug, as revealed from this experiment, could have been achieved as an opportunistic side effect of the normal and unique physiological function assigned to a protein in the cell (Neyfakh, 1997). To achieve such alteration the cell might need to undergo certain mutations. Over-expression of efflux pumps could result from mutations within local repressor genes (Adewoye et al, 2002; Wang et al, 2001; Webber et al, 2001) or might result from activation of a regulon regulated by a global transcriptional regulator (Alekschum and Levy, 1997; Pomposiello and Demple, 2000).

4.5 Studying resistance at molecular level:

4.5.1 Genomic library construction

Genomic library of Chromo^r was constructed in *E.coli* DH5α. *Sau3A* partially digested genomic DNA of 5kb to 9kb size were eluted and ligated with *Bam*HI site of pBluescript, making a convenient selection of blue-white on ampicillin- X-gal plate. Alternatively, *Bam*HI total digest of chromo^r genomic DNA was also used for library construction in pBSK. Chromomycin and daunomycin could not be used for screening of resistance phenotype, since these antibiotics act only against gram positive bacteria. Thus the screen was restricted to tetracycline and erythromycin which was used either in isolation or combination.

No positive clone could be screened from either library. Even low levels of expression could have proved sufficient for the phenotype due to high copy number of pBKS. The failure could be due to the fact that *M. luteus* (insert DNA) with one of the highest G-C containing genomes could have failed to express due to difference in codon preference (Ohama et al, 1989).

The other probable reason could be involvement of more than one gene, placed distantly in the genome, collectively involved in imparting resistance. Both genes might be necessary and in isolation, might not be sufficient to impart the resistance. The probability of two such fragments, containing the respective genes, getting integrated in the same plasmid backbone will tend to zero. Thus owing to the physical distance between the genes their cloning in the same fragment would not be possible. This hypothesis is also backed by results of earlier experiments showing inability to generate spontaneous mutants, since in such event two different genes of choice, in the same cell, need to undergo mutation and the frequency for such event is very rare. According to our estimate, with an average insert size of 7kb and five times coverage, 2000 colonies would be required in the genomic library of *M. luteus* (2.6Mb genome), in order to get desired insert (gene). Following this statistics, in order to obtain two desired fragments in the same clone, the library needs to have $2000 \times 2000 = 4 \times 10^6$ clones. And further, with an average ligation frequency of 10% integrents, the number goes to 4×10^7 transformants. This is an exhaustive number and is practically not feasible to achieve.

4.5.2 Restriction Digestion Pattern

The gradual adaptive nature and the reversion frequency in absence of chromomycin pressure, shown by Chromo^r suggested likelihood of gene amplification-deamplification theory discussed earlier.

Same amounts of genomic DNA of wild type and Chromo^r when completely digested with different restriction enzymes namely *EcoRI*, *XhoI*, *SalI* and *BamHI* and the bands for wild type and Chromo^r showed a characteristic signature pattern on agarose gel. Presence of any of these sites in the amplified region of DNA would lead to formation of several fragments of the same size as against a single copy in wild type, which shall light up on the gel. Owing to the GC rich nature of *Micrococcus luteus* genome, *BamHI* showed ideal digestion pattern with bands ranging in all lengths whereas *EcoRI* and *XhoI* could not generate a homogenous DNA smear pattern.

None of the restriction enzyme digestions revealed presence of any extra band of DNA in genomic DNA of Chromo^r against that of wildtype, except for one band of ~11kb size in Chromo^r DNA digested with *EcoRI*. The result appeared dubious since it DNA was incompletely digested. Clean digestions carried out with *EcoRI* later, did not show any difference in the gel patterns confirming absence of any extra band.

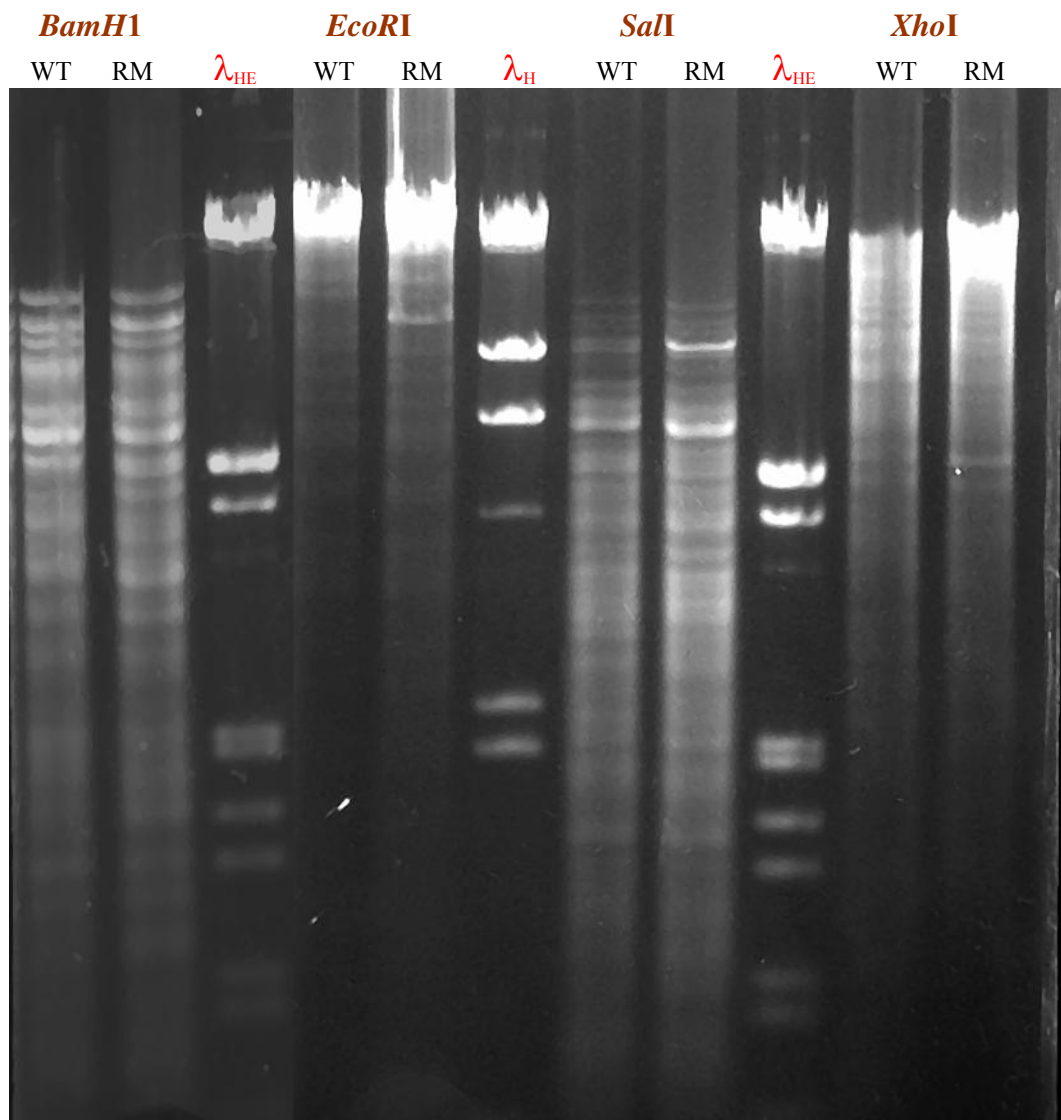


Fig- 4.3: Restriction digestion pattern using different restriction enzymes.

Lack of any accessory DNA band, in any of restriction digestions studied, negates presence of an amplified unit. Alternatively, if only duplication or low level of amplification is involved in imparting resistance as in case of sulfonamide and trimethoprim resistance of *Streptococcus agalactiae* (Brochet et al, 2008), then such minor difference would not get reflected in the gel.

4.5.3 Protein studies using SDS-PAGE:

There was difference in growth rate between wildtype and Chromo^r and the lag in growth of Chromo^r was directly proportional to chromomycin concentration. So for protein studies, instead of growing cultures in broth, they were plated on LA plates with/without antibiotic and colonies were scooped from overnight grown cultures. This was done because, in broth cultures all the cells remain at the same stage of growth and so temporal diversity of gene expression under chromomycin pressure is difficult to compare with wild type. While colonies on plate represent cells at all stages of growth, with a gradation of centre with stationary phase cells and actively dividing cells towards the periphery. Thus to obtain entire spectrum of expressed protein over a period of time, cells were directly scooped from overnight grown colonies on LA plates with/without antibiotic. The other advantage of this strategy was elimination of growth rate difference found between wildtype and Chromo^r at different antibiotic concentrations. Also, at a particular growth more than one protein might recognize the antibiotic stress and their expression might get altered. But in protein sample from colonies, such effect gets averaged out and only the protein with constitutive expression would appear. This would be helpful in targeting the protein of interest (Fig – 4.4).

Optical density measurement of the cultures was preferred over protein estimation method for quantification of sample concentration. Use of protein estimation by Folin's method, to load exactly same amounts of both protein samples in the gel, gave misleading results. For the same amount of protein loaded, the bands in Chromo^r sample could not match the concentration of its counterpart wild type sample (Fig- 4.4, B). This was because the overexpressing protein in the Chromo^r sample contributed to a significant fraction of

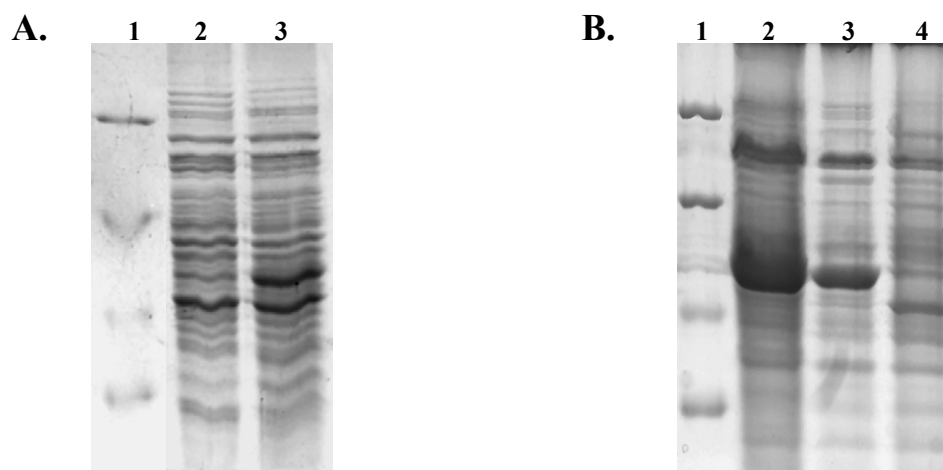


Fig – 4.4 : Comparison of SDS-PAGE gels of total proteins prepared from colonies (A) and broth (B).

A) 1-Marker, 2-wild type, 3-Chromo^r with normalized cell number

B) 1-Marker, 2- 2x Chromo^r , 3- Chromo^r, 4-wild type.

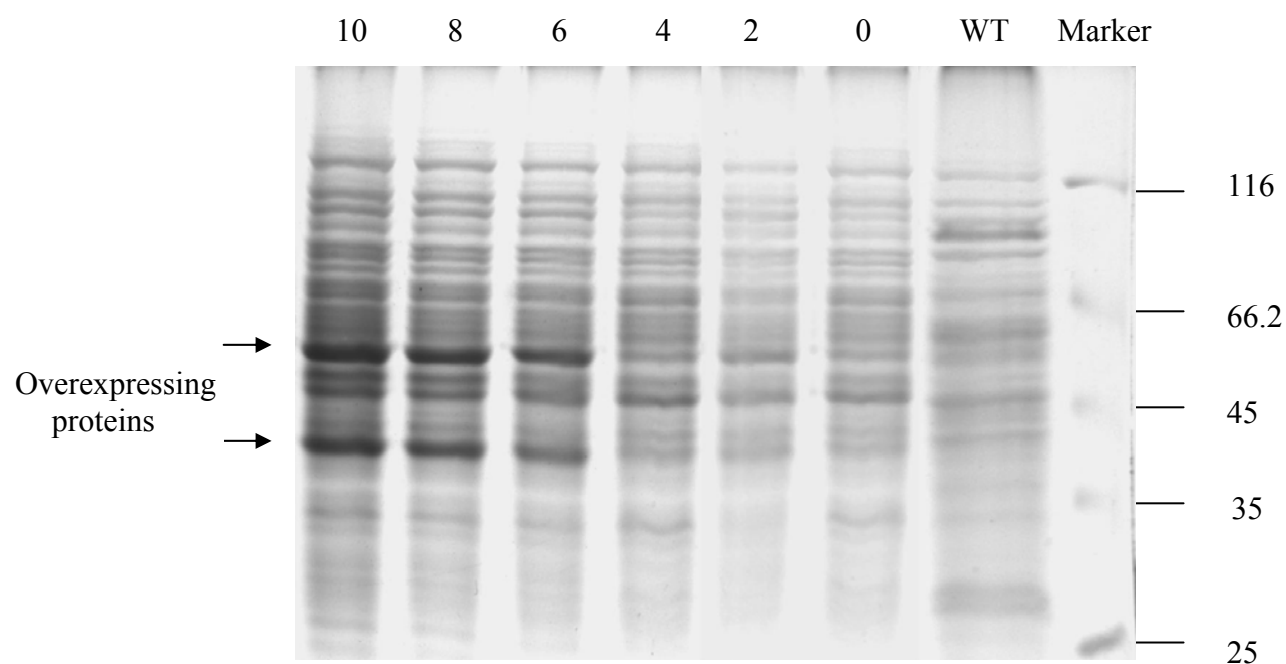


Fig-4.5: Effect of increasing chromomycin concentration (0, 2, 4, 6, 8, 10ug)/plate on Chromo^r. against wild type. Over expressing bands of size ~50KDa and ~40KDa as determined by protein molecular weight marker (#SM0431, Fermentas).

total cellular protein, and so equal amounts of total protein from wildtype and Chromo^r reflected different intensities for all the bands. On the other hand, O.D. measurement could ensure equal number of cells in both sets irrespective of the protein expression levels in them. Volumes of samples were adjusted with respect to OD₆₀₀ estimation to match cell concentration for sample preparation.

SDS-PAGE was carried out using gels of 10%, 12% and 15% of which 10% was found to be most suitable. Two highly conspicuous protein bands could be distinctly observed in Chromo^r against its wildtype equivalent. There was a very conspicuous protein band present in the Chromo^r for which no visible complement could be seen in the wild type sample. Of the two over-expressing proteins, one band was ~50kDa in size whereas the other was ~40kDa. The sizes were determined based on the protein molecular weight marker (#SM0431, Fermentas) with range 14.4KDa to 116KDa.

Interestingly, involvement of more than one protein in imparting resistance was hypothesized when repeated attempts of cloning the resistance gene from the genomic DNA library of Chromo^r had failed. The two overexpressing protein bands observed in Chromo^r needs to be studied in detail to find out if both collaboratively function to impart resistance or have independent mechanisms for it.

In earlier experiments, overproduction of carotenoids in Chromo^r was observed and there could have been an overexpression of one of the genes of carotenoid biosynthesis pathway. But there was no match between the two fold increase in carotenoid content and manifold increase of overexpressing proteins as seen in the gel. To ensure that the over expressed protein does not belong to carotenoid synthesis pathway, protein sample prepared from DPA treated Chromo^r cells were also run along with wild type and Chromo^r. No difference in band pattern and intensities between DPA treated and untreated Chromo^r ruled out the protein to be of carotenoid biosynthetic pathway.

Further, the dose dependent induction of the protein was also attempted. For this, the Chromo^r culture was grown overnight in absence of selection pressure (chromomycin). This

was done to nullify any residual induction effect of chromomycin. It was then used as source of inoculum for growing Chromo^r culture in increasing (0, 2, 4, 6, 8 and 10ug) concentrations of chromomycin. The increase in intensity of the protein bands in study, with increasing concentrations of chromomycin, depicted in the gel (Fig- 4.5), confirms the direct relation of the protein with resistance. Protein induction is directly proportional to the chromomycin concentration in the medium.

CONCLUSION

Adaptive mutations, be they stable point or unstable amplification mutations are a major cause of concern for bacterial diseases and cancer alike. Antibiotic resistant pathogenic bacteria and drug resistant cancerous cells arise as a result of permanent and dynamic changes in the genome respectively at a relatively high rate under selection pressure and result in incomplete failure of that drug being used in future treatments. The molecular mechanism of adaptive resistance is also apparently the same between the two.

P Glycoprotein variants in various drug resistant cancer cells and Multidrug Resistant (MDR) transporter proteins in bacterial systems share structural and functional similarities. We uncover that *M. luteus* possesses characteristic ability (not exhibited by *B. subtilis* and *S. aureus*) of adapting individually to increasing concentration of five antibiotics namely, tetracycline, erythromycin, daunomycin, chromomycin and actinomycin D and exhibiting cross resistance to remaining four. For example, adaptive resistant mutants each for chromomycin, daunomycin and tetracycline were generated and cross resistance to remaining unselected antibiotics was absolute in each case. Furthermore, again in each case, the mutation was unstable in that, in the absence of antibiotic pressure, the resistant phenotype reverted at ~3% frequency to wild type phenotype. Given the reversion frequency, gene amplification best explains the cause of resistance. A gene conferring low level of resistance to the subset of compounds used in this study is amplified to high copy for increased expression, a mechanism very commonly found in several bacteria and cancer cells in response to stress adaptation. The nature of the gene and the DNA amplicon is under investigation.

We attempted to clone the gene for resistance in *E. coli*, however without success, presumably due to poor expression of GC rich DNA of *M. luteus* in *E. coli*. Indeed, seldom in the literature we find that a *M. luteus* gene has been isolated by functional complementation in *E. coli*.

The second approach to identifying the amplicon was by comparing the restriction digestion pattern of the DNA of the resistant mutant with that of unadapted *M. luteus*. Though we did notice differences in the BamHI digested DNA of the mutant and the wild type DNA, we did not pursue this line of investigation.

In the protein profiling studies, two proteins of ~ 40 KDa and 50 KDa size were expressed to a high level. Although it is a common finding that amplicon bearing the phenotypic trait is often larger in size than is needed for expression of that trait, we expected more number of proteins to be affected than the mere two mentioned above. For example, in Chromo^r mutant but not in Dauno^r or Tet^r, there is concomitant overproduction of *M. luteus* specific carotenoid pigment in the resistant mutant. However, the protein profile of the Chromo^r, Dauno^r and Tet^r mutant was identical (data not shown), indicating that some of the proteins' overexpression may not be revealed on the protein gel. The MALDI-ToF analysis of the proteins is being done.

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