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RESISTANCE TO DAUNORUBICIN BY DrrC IN HETEROLOGOUS HOST, *ESCHERICHIA COLI*

PRIJA F[1] AND PRASAD R[1*]

1: Department of Genetic Engineering, School of Biotechnology, Madurai Kamaraj University, Madurai, Tamil Nadu, India

*** Corresponding Author: E Mail: ranjan_mku@yahoo.com; Telephone: +91 452 2458211; Fax: +91 452 2459105**

ABSTRACT

The protein, DrrC is the key determinant of self-resistance in *Streptomyces peucetius* which belongs to ATP-Binding Cassette (ABC) proteins. *S. peucetius* is the producer of anti-cancer drug, daunorubicin. Null mutation of *drrC* is lethal to *S. peucetius.* The gene *drrC* was cloned into expression vector, pQE30 and introduced into *E.coli* strains AB1157, M15pREP4 and N43. It was checked whether DrrC protein confers resistance to daunorubicin in *E.coli* strains. Protein profiling showed expression of DrrC in *E. coli* cell lysate. A daunorubicin gradient plate assay showed that *E. coli* AB1157/pQE30 and *E. coli* AB1157/pQE30-*drrC* were resistant up to 150 µg/ml of daunorubicin where as *E. coli* M15pREP4/pQE30 and M15pREP4/pQE30-*drrC* were resistant to daunorubicin up to 200 µg/ml where *E. coli* AB1157 and M15 pREP4 are wild strains with efflux pumps. *E. coli* N43/pQE30 that is deficient of efflux protein, AcrAB was sensitive to daunorubicin but *E. coli* N43 with resistant protein DrrC was resistant up to 2 µg/ml of daunorubicin. This reveals that DrrC of *S. peucetius* can confer resistance to daunorubicin in heterologous host, *E. coli* lacking native efflux pumps. Studying the function of DrrC would pave way to trace out the role of multi drug transporters which is homologous to DrrC.

Keywords: DrrC, *Streptomyces peucetius*, Self Resistance and *E. coli* N43

INTRODUCTION

Streptomycetes are Gram-positive, GC-rich, soil bacteria that produce spores and exhibit a morphological and physiological differentiation, which coincides with production of secondary metabolites. They also possess self resistance mechanisms to

overcome autotoxicity of these secondary metabolites that are co-regulated along with the genes responsible for antibiotic biosynthesis **[1]**. Self-resistance to the antibiotics may be either by inactivation of drug, modification of target site, drug efflux or drug sequestration by specific proteins **[2–4]**. *Streptomyces peucetius* synthesize anthracyclines, namely daunorubicin (DNR) and doxorubicin (DXR) with antitumor properties. Daunorubicin intercalates with DNA and inhibits topoisomerase I and II thereby halting DNA replication, transcription and translation **[5–7]**. Moreover there are conserved interactions between specific DNA bases (C and G) and aglycone moiety of daunorubicin molecule which is common to DNA binding drugs.

Mechanism of Resistance in *Streptomyces peucetius*:

S. peucetius has four genes that confer self-resistance against daunorubicin namely *drrA, drrB, drrC* and *drrD* which are expressed only during idiophase whereas *drrC* is transcribed earlier than *drrA* and *drrB* **[8]**. The ABC transporter, DrrA and DrrB **[9]** forms an ATP-dependent efflux pump for daunorubicin and doxorubicin in *S. peucetius* **[10]**. DrrA, the catalytic subunit forms a complex with integral membrane DrrB protein and their function and stability are biochemically coupled **[10]**. The efflux of daunorubicin by DrrAB is a primary mechanism for preventing build up of toxic drug within the cell. DrrC is a DNA-binding protein with strong sequence similarity to the UvrA proteins. UvrA of ABC family is involved in nucleotide excision repair mechanism of DNA **[11]**. The function of *drrD* is unknown however a study on DrrD in our lab has shown it to be a FAD binding protein helpful in self- resistance mechanisms (Unpublished data). On the other hand, a study on orthologue of *drrD*, namely *dauW* of *Streptomyces coeruleobidus* has shown *dauW* to be a novel class of regulator **[12]**.

Daunorubicin resistance protein: DrrC

Daunorubicin resistance protein, DrrC has 764 amino acids with a molecular weight of 83386 Da and calculated pI of 6.8 **[13]**. Null mutation of *drrC* is lethal to the organism **[13]**. The binding of DrrC to DNA at daunorubicin intercalated sites requires ATP and is enhanced by daunorubicin **[11]**. DrrC has zinc-finger motifs and the ATP binding sites as found in UvrA **[13]**. Being an UvrA-like protein we believe that it may help to repair damages caused by daunorubicin.

Multidrug resistance has become a challenge in chemotherapy. Multiple drug resistance or Multidrug resistance is a condition that enables a disease-causing organism to resist drugs of variety of structure and function targeted to eradicate the organism. Organisms displaying

multidrug resistance can be pathologic bacterial cells and neoplastic (tumor) cells. Multidrug resistance ATP binding cassette (ABC) transporters are a class of permeases that translocate drug molecules by coupling drug/lipid efflux with energy derived from the hydrolysis of ATP. Multidrug efflux transporters extrude structurally dissimilar organic compounds often providing resistance to multiple toxic chemotherapeutic agents. The first described multidrug efflux pump was mammalian P-glycoprotein, an ATP-driven pump that provides resistance to a broad spectrum of compounds including anticancer chemotherapeutic agents **[14, 15]**. In this study, we have checked whether the DNA-binding ABC protein, DrrC, is able to expel daunorubicin in heterologous host, *E. coli.* This preliminary work will be helpful to design experiments to study about multidrug resistant ABC transporter.

MATERIALS AND METHODS

E. coli strains used were AB1157 **[16]**, M15pREP4 (Qiagen) and N43 **[17]**. Total DNA from *S. peucetius* was isolated as described by **[18]**. Plasmid DNA was isolated by alkali lysis method as described by **[19]**. Restriction endonuclease digestion and ligation were carried out according to manufacturer's instructions. Agarose gel electrophoresis was carried out in a horizontal matrix of agarose with 1X TAE buffer as described by **[19]**.

Preparation of *E. coli* competent cells

Competent cells for electroporation were prepared as described by **[19]** with minor modifications. Fresh overnight cultures were made in 2 ml LB [(Luria broth) 10 g tryptone, 5 g Yeast extract and 10 g NaCl with pH adjusted to 7.2 and made up to 1000 ml with distilled water] and then subcultured in 100 ml of SOB [(Super Optimal Broth) 20 g tryptone, 5 g yeast extract, 0.19 g KCl and 0.58 g NaCl with pH adjusted to 7.0 and made up to 1000 ml with distilled water] and grown to an OD_{600} of 0.6. The cells were chilled on ice for 30 min, followed by centrifugation at 4,000 rpm for 20 min at 4°C. The pellet was resuspended in 100 ml of 10% glycerol and centrifuged at 4000 rpm for 20 min at 4°C. The pellet was resuspended in 50 ml of 10% glycerol and spun at 4000 rpm for 20 min at 4°C. After decanting, the pellet was dissolved in the residual glycerol and 80 µl aliquots were stored in sterile microfuge tubes at -70°C.

Transformation in *E. coli*

An aliquot of frozen competent cells was thawed on ice and was transformed as described by **[20]**. Hundred nanogram of DNA was mixed with the cells and kept on ice for 5 min. This suspension was transferred into an electroporation cuvette

(0.1 cm electrode gap; Bio-Rad) and electroporated using the following pulse conditions; voltage - 1.4 kV, resistance - 200 Ω; capacitance - 25 µF and pulse time - 15 s, which gave a time constant of 4.5 to 4.8. The mixture was diluted in 0.9 ml SOC (SOB medium (1000 ml) with 10 ml 2 M $MgCl_2$, 10 ml 2 M $MgSO_4$ and 10 ml 2 M glucose added just before inoculation) and kept on a shaker at 37°C for 45 min for the expression of antibiotic resistance. The culture was then plated on appropriate antibiotic containing LB plates and incubated at 37°C. The transformants were counted after 12 to 24 h.

Protein expression studies

E. coli/pQE30 and *E. coli*/pQE30-*drrC* was grown in 3 ml LB medium with appropriate antibiotics for 16 h at 37°C. From the 16 h culture 1% inoculum was subcultured in 20ml culture. At an optical density at 600 nm of 0.6, 1 mM isopropylthiogalactopyranoside (IPTG) was added and the cells were collected at 0 h, 2 h and 4 h time points. The cells were then suspended in sample lysis buffer (50 mM Tris-HCl [pH 6.8], sodium dodecyl sulfate [SDS], 0.1% [wt/vol]) and heated in boiling water bath for 5 min. The lysed cells were centrifuged at 12.000 rpm for 5 min and then the supernatant fractions were separated from the pellet. The supernatant was used to check the protein expression in 12 % SDS-PAGE. Proteins were resolved in SDS-polyacrylamide gel **[21]** as described by **[22]**.

Daunorubicin resistance assay in *E. coli*

Different strains of *E. coli*: AB 1157, M15pREP4 and N43 with plasmids pQE30 and pQE30-*drrC* were tested for levels of resistance to daunorubicin in the culture medium. LB plates with different concentrations of daunorubicin were prepared. The cells of *E. coli* AB 1157 and *E. coli* M15pREP4 with pQE30 and pQE30-*drrC* were grown in LB liquid for 8 h and 10 µl of the culture was placed on LB agar plates containing various concentrations of daunorubicin (0, 25, 50, 75, 100, 150 and 200 µg/ml) and were incubated for 12h and photographed to record growth inhibition. The cells of *E. coli* N43 with pQE30 and pQE30-*drrC* were grown in LB liquid for 8 h and 10 µl of the culture was placed on LB agar plates containing various concentrations of daunorubicin (as 0, 2, 4, 6, 8 and 10 µg/ml) and were incubated for 12 h and photographed to record growth inhibition.

RESULTS AND DISCUSSION

Cloning of *drrC* in pMAL-c5E

DrrC DNA fragment was amplified by PCR using Phusion DNA polymerase from genomic DNA template of *S. peucetius* and specific primer containing *Kpn*I site in the forward primer 5'-

AGATAA**GGTACC**ATGCTGATGCAGC AGCAGGC and *Hind*III site in the reverse primer 5'-GTATGT**AAGCTT**GGGACGCCGCTTA GCACC. The expected amplicon of 2.3 kb was digested with *Kpn*I and *Hind*III and cloned in pOK12. Presence of insert was confirmed by double digesting with *Kpn*I and *Hind*III **(Figure 1, lane 2).** This recombinant plasmid was named pOK12-*drrC*. The 2.3 kb *drrC* coding sequence was subcloned into pQE30, the expression vector with 6His-tag. pQE30 was digested with *Kpn*I and *Hind*III and ligated with *Kpn*I: *Hind*III digested *drrC* and transformed *E. coli* strain XL1blue by electroporation. The recombinant clones that displayed size increase were further confirmed by restriction digestion with *Kpn*I and *Hind*III. Double digestion with *Kpn*I and *Hind*III released a 2.3 kb fragment of *drrC* **(Figure 2: lanes 2, 3)**. The recombinant plasmid was sequenced to check the integrity of open reading frame and also to confirm in-frame fusion with histidine tag. This recombinant plasmid was named pQE30-*drrC*.

Bacterial transformation

E. coli strains AB1157, M15pREP4 and N43 were transformed with pQE30 and pQE30-*drrC*. The transformants were confirmed by checking the presence of plasmids.

Expression of pQE30-*drrC*

The expression of DrrC protein was induced in culture by addition of 1mM IPTG. The cells were lysed and the cell debris was pelleted. The supernatant was used to check the presence of the DrrC protein on SDS-PAGE. 30 μg protein was loaded on the gel and the gel was stained by colloidal Coomassie staining solution **(Figure 3)**.

***E. coli* AB1157/pQE30 and *E. coli* AB1157/pQE30-*drrC* are resistant to daunorubicin up to 150 μg/ml**

Single colony of *E. coli* AB1157/pQE30 and *E. coli* AB1157/pQE30-*drrC* were inoculated in LB and after 8 h of incubation were diluted up to 10^{-6}. Ten microlitres of the cultures were spotted on LB plates with daunorubicin. Initially a gradient of concentrations 0, 2, 4, 6, 8 and 10 μg/ml were used to check inhibition of growth (data not shown). As there was no growth inhibition, concentration of daunorubicin was increased to 25, 50, 75, 100, 150 and 200 μg/ml. The cells were very resistant up to 150 μg/ml as the host strain AB1157 has a strong drug efflux mechanism. The cells harbouring the plasmid control as well as the plasmid with the resistant gene were able to survive even in higher concentrations of daunorubicin **(Figure 4)**.

***E. coli* M15pREP4/pQE30 and M15pREP4/pQE30-*drrC* is resistant to daunorubicin up to 200 μg/ml**

The same series of assay was carried out for *E. coli* M15pREP4 with pQE30 and pQE30-*drrC*. **Figure 5** clearly illustrates that *E. coli* M15 pREP4/pQE30-*drrC* was able to grow up to 200 µg/ml of daunorubicin though there were few cells of M15pREP4/pQE30 which clearly implies that the excess resistance might be provided by the self resistance gene, *drrC* in the plasmid.

***E. coli* N43/pQE30-*drrC* is resistant to daunorubicin upto 2.7 µg/ml though *E. coli* N43/pQE30 is sensitive**

The above said experiment was carried out with *E. coli* N43/pQE30 and pQE30-*drrC*. The concentrations of daunorubicin used initially were 0, 2, 4, 6, 8 and 10 µg/ml. *E. coli* N43/pQE30 was very sensitive and there was no growth at 2 µg/ml but N43/pQE30-*drrC* was resistance to the daunorubicin at 2 µg/ml **(Figure 6)**. This is because the strain *E. coli* N43 lack the AcrAB system **[17]**. The AcrAB system of is a multidrug efflux system is composed of an RND-type transporter, AcrB and a periplasmic accessory protein AcrA pumps out a wide variety of lipophilic and amphiphilic inhibitors directly into the medium, through the TolC outer membrane channel **[23]**. Cells of N43 are hypersensitive to many antimicrobial agents and as anticipated, *E. coli* N43/pQE30-*drrC* became resistant to daunorubicin.

Restriction analysis of pOK12-*drrC*

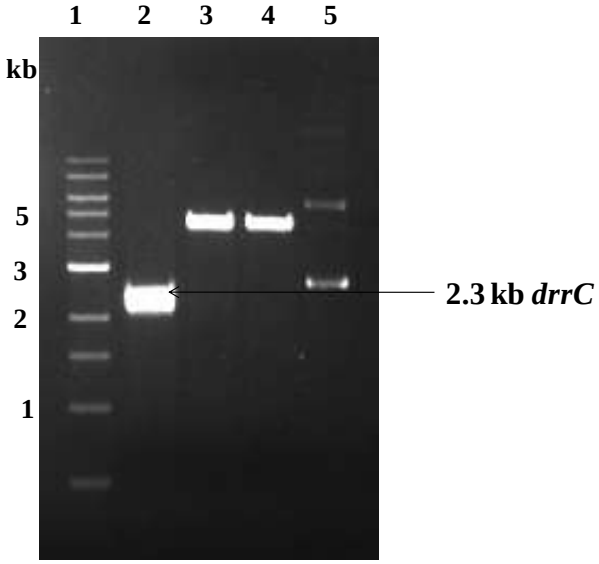

Figure 1: The recombinant plasmid DNA was digested with restriction enzymes and electrophoresed on a 0.8% agarose gel. Lanes: 1, 1kb ladder; 2, pOK12-*drrC* digested with *Kpn*I and *Hind*III; 3, pOK12-*drrC* digested with *Kpn*I; 4, pOK12-*drrC* digested with *Hind*III; 5, undigested pOK12-*drrC*

Restriction analysis of pQE30-*drrC*

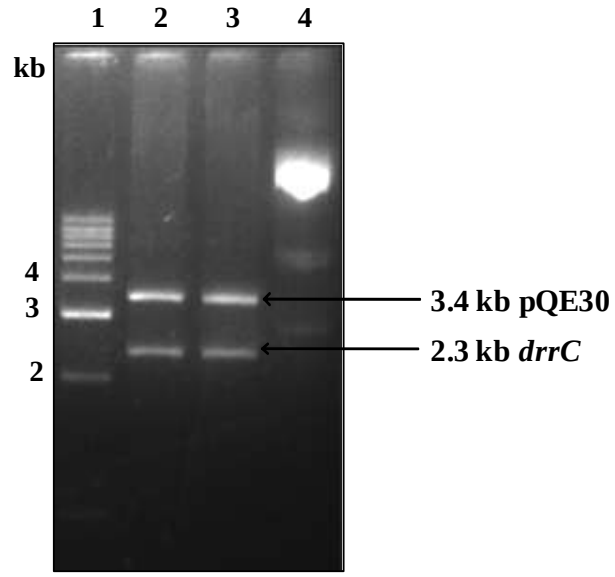

Figure 2: The recombinant plasmid DNA was digested with restriction enzymes and electrophoresed on a 0.8% agarose gel. Lanes: 1, 1kb ladder; 2 & 3, pQE30-*drrC* digested with *Kpn*I and *Hind*III; 4, undigested pQE30-*drrC*.

SDS-PAGE of *E.coli* cell lysate showing IPTG induced expression of DrrC protein

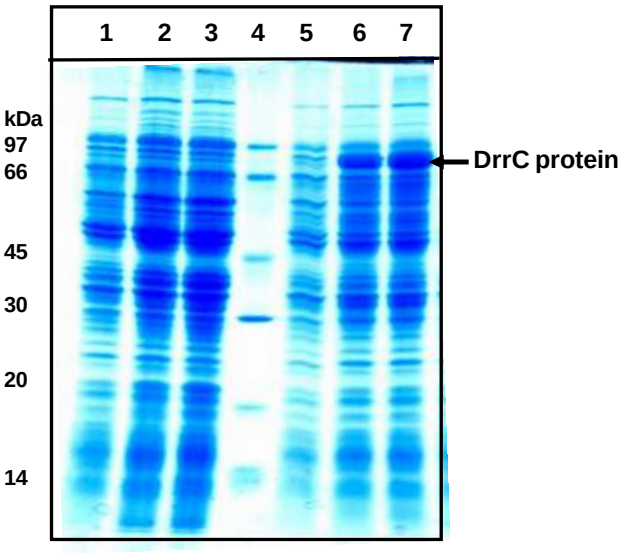

Figure 3: Colloidal Coomassie stained 12 % SDS-PAGE showing expression of pQE30-*drrC*. Lanes: 1, Uninduced control (pQE30); 2, 2 h IPTG induced control; 3, 4 h IPTG induced control; 4, Protein marker; 5, Uninduced pQE30-*drrC*; 6, 2 h IPTG induced pQE30-*drrC*; 7, 4 h IPTG induced pQE30-*drrC*.

Analysis of daunorubicin self-resistance in *E. coli* AB1157 by gradient plate

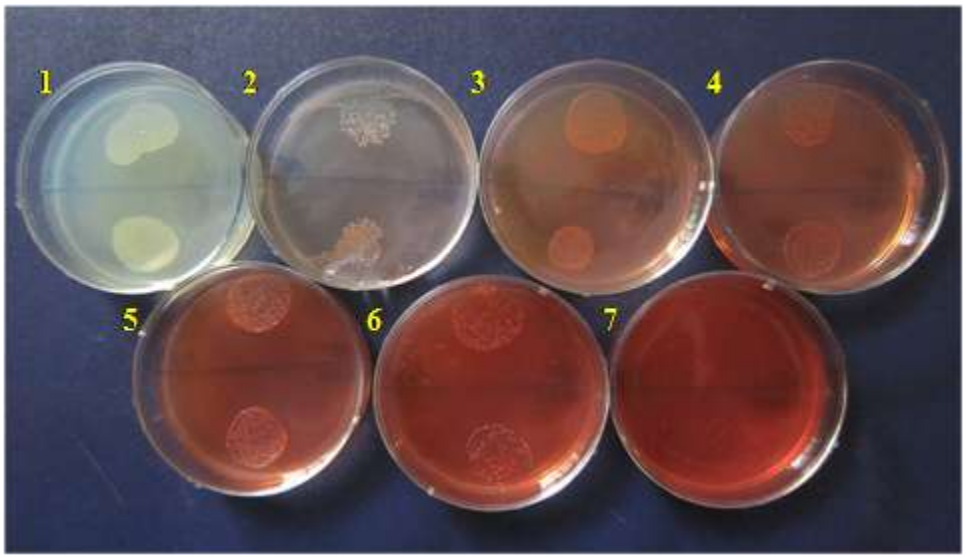

Figure 4: Plate 1= 0 µg/ml daunorubicin, Plate 2= 25 µg/ml daunorubicin, Plate 3= 50 µg/ml daunorubicin, Plate 4= 75 µg/ml daunorubicin, Plate 5= 100 µg/ml daunorubicin, Plate 6= 150 µg/ml daunorubicin, Plate 7= 200 µg/ml daunorubicin; A = *E.coli* AB1157/pQE30, B = *E.coli* AB1157/pQE30-*drrC*

Analysis of daunorubicin self-resistance in *E. coli* M15pREP4 by gradient plate

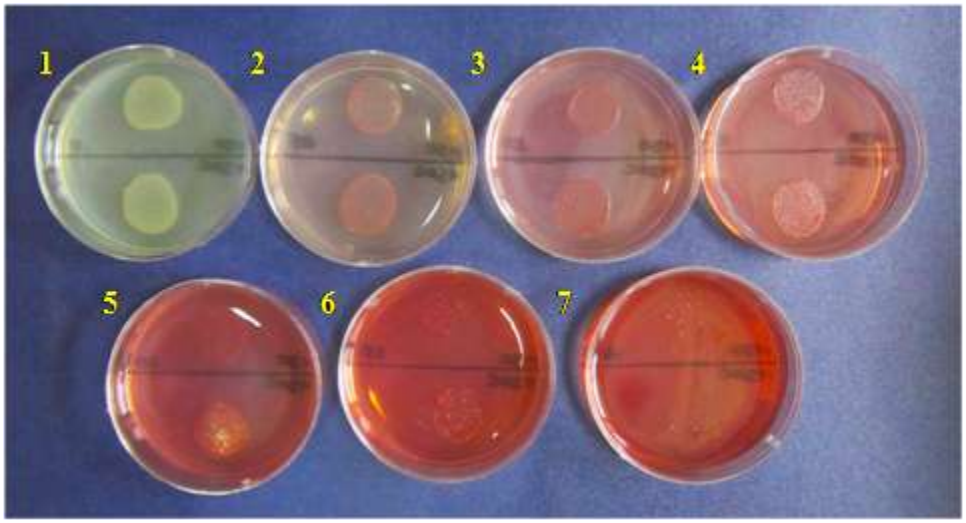

Figure 5: Plate 1= 0 µg/ml daunorubicin, Plate 2= 25 µg/ml daunorubicin, Plate 3= 50 µg/ml daunorubicin, Plate 4= 75 µg/ml daunorubicin, Plate 5= 100 µg/ml daunorubicin, Plate 6= 150 µg/ml daunorubicin, Plate 7= 200 µg/ml daunorubicin; A = *E.coli* M15pREP4/pQE30, B = *E.coli* M15pREP4/pQE30-*drrC.*

Analysis of daunorubicin self-resistance in *E. coli* N43 by gradient plate

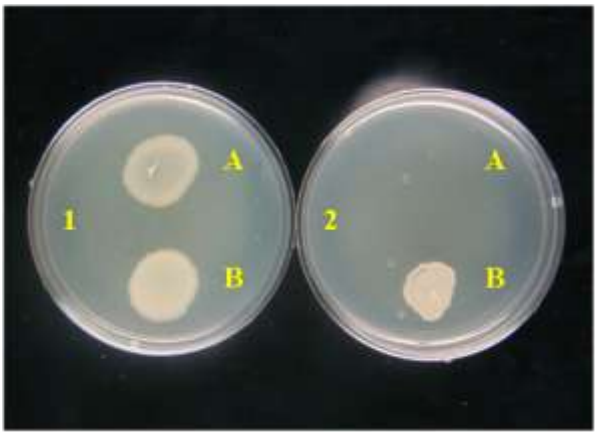

Figure 6: Plate 1= 0 µg/ml daunorubicin, Plate 2= 2 µg/ml daunorubicin; A = *E.coli* N43/pQE30, B = *E.coli* N43/pQE30-*drrC*

CONCLUSION

Thus DrrC the daunorubicin resistance protein in daunorubicin producer, *S. peucetius* shows resistance phenotype in *E. coli* lacking efflux pump. Similar type of protein might be present in multidrug resistant cancer cells that decrease the efficacy of anti-leukemic drug like daunorubicin by expelling it. Therefore studying identical proteins in human cancer cell lines and modifying either the protein or the drug targets might be helpful in combating multidrug resistance in chemotherapy.

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REFERENCES

[1] Bibb, M. J. Regulation of Secondary Metabolism in Streptomycetes. Curr. Opin. Microbiol. 2005, 8, p. 208–215.

[2] Cundliffe, E. Self-Protection Mechanisms in Antibiotic Producers. Ciba Found. Symp. 1992, 171, p. 199–208.

[3] Méndez, C.; Salas, J. A. ABC Transporters in Antibiotic-Producing Actinomycetes. FEMS Microbiol. Lett. 1998, 158, p. 1–8.

[4] Paulsen, I. T.; Brown, M. H.; Skurray, R. a. Proton-Dependent Multidrug Efflux Systems. Microbiol. Rev. 1996, 60, p. 575–608.

[5] Cutts, S. M.; Phillips, D. R. Use of Oligonucleotides to Define the Site of Interstrand Cross-Links Induced by Adriamycin. Nucleic Acids Res. 1995, 23, p. 2450–2456.

[6] Tewey, K. M.; Rowe, T. C.; Yang, L.; Halligan, B. D.; Liu, L. F. Adriamycin-Induced DNA Damage Mediated by Mammalian DNA

Topoisomerase II. Science 1984, 226, p. 466–468.

[7] Pilch, D. S.; Yu, C.; Makhey, D.; LaVoie, E. J.; Srinivasan, A. R.; Olson, W. K.; Sauers, R. R.; Breslauer, K. J.; Geacintov, N. E.; Liu, L. F. Minor Groove-Directed and Intercalative Ligand-DNA Interactions in the Poisoning of Human DNA Topoisomerase I by Protoberberine Analogs. Biochemistry 1997, 36, p. 12542–12553.

[8] Cundliffe, E.; Demain, A. L. Avoidance of Suicide in Antibiotic-Producing Microbes. J. Ind. Microbiol. Biotechnol. 2010, 37, p. 643–672.

[9] Guilfoile, P. G.; Hutchinson, C. R. A Bacterial Analog of the Mdr Gene of Mammalian Tumor Cells Is Present in Streptomyces Peucetius, the Producer of Daunorubicin and Doxorubicin. Proc. Natl. Acad. Sci. U. S. A. 1991, 88, p. 8553–8557.

[10] Pradhan, P.; Li, W.; Kaur, P. Translational Coupling Controls Expression and Function of the DrrAB Drug Efflux Pump. J. Mol. Biol. 2009, 385, p. 831–842.

[11] Furuya, K.; Hutchinson, C. R. The DrrC Protein of Streptomyces Peucetius, a UvrA-like Protein, Is a DNA-Binding Protein Whose Gene Is Induced by Daunorubicin. FEMS Microbiol. Lett. 1998, 168, p. 243–249.

[12] Yuan, T.; Yin, C.; Zhu, C.; Zhu, B.; Hu, Y. Improvement of Antibiotic Productivity by Knock-out of dauW in Streptomyces Coeruleobidus. Microbiol. Res. 2011, 166, p. 539–547.

[13] Lomovskaya, N.; Hong, S. K.; Kim, S. U.; Fonstein, L.; Furuya, K.; Hutchinson, R. C. The Streptomyces Peucetius drrC Gene Encodes a UvrA-like Protein Involved in Daunorubicin Resistance and Production. J. Bacteriol. 1996, 178, p. 3238–3245.

[14] Ambudkar, S. V; Dey, S.; Hrycyna, C. A.; Ramachandra, M.; Pastan, I.; Gottesman, M. M. Biochemical, Cellular, and Pharmacological Aspects of the Multidrug Transporter. Annu. Rev. Pharmacol. Toxicol. 1999, 39, p. 361–398.

[15] Paulsen, I. T. Multidrug Efflux Pumps and Resistance: Regulation and Evolution. Curr. Opin. Microbiol. 2003, 6, p. 446–451.

[16] Dewitt, S.; Adelberg, E. The Occurrence of a Genetic Transposition in a Strain of

Escherichia Coli. Genetics 1962, 47, p. 577–585.

[17] Nakamura, H.; Suganuma, A. Membrane Mutation Associated with Sensitivity to Acriflavine in Escherichia Coli. J. Bacteriol. 1972, 110, p. 329–335.

[18] Hopwood, D. A.; Bibb, M. J.; Chater, K. F.; Kieser, T.; Bruton, C. J.; Kieser, H. M.; Lydiate, D. J.; Smith, C. P.; Ward, J. M.; Schrempf, H. Genetic Manipulation of Streptomyces: A Laboratory Manual; John Innes Foundation, 1985.

[19] Sambrook, J.; Fritsch, E. F.; Maniatis, T. Molecular Cloning: A Laboratory Manual; 2nd ed.; Cold Spring Harbor: New York, Cold Spring Harbor Laboratory, 1989.

[20] [20] Dower, W. J.; Miller, J. F.; Ragsdale, C. W. High Efficiency Transformation of E. Coli by High Voltage Electroporation. Nucleic Acids Res. 1988, 16, p. 6127–6145.

[21] [21] Laemmli, U. K. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. Nature 1970, 227, p. 680–685.

[22] [22] Ausubel, F. M.; Brent, R.; Kingston, R. E.; Moore, D. D.; Seidman, J. G.; Smith, J. A.; Struhl, K. Current Protocols in Molecular Biology; NY: John Wiley and Sons., 1989.

[23] [23] Martinez, J. L.; Mart, L.; Fajardo, A.; Alvarez-ortega, C. Functional Role of Bacterial Multidrug Efflux Pumps in Microbial Natural Ecosystems. 2009, 33, p. 430–449.