American-Eurasian J. Agric. & Environ. Sci., 6 (3): 294-297, 2009
ISSN 1818-6769

Effects of Basal Level *Serratia marcescens* Endonuclease on the Stability and *pHisNucSma* Plasmid Copy Number in *Escherichia coli* Recombinant Strains

Oleg Gimadutdinow, Ricardo Gudino, Kryakounova Elena, Moffat Malisheni and Raisa Khamidullina

Department of Genetics, Kazan State University, Kremlevskaya 18 St., 420008 Kazan, Russian Federation

Abstract: In the present work the activities of mutant *Serratia marcescens* nucleases at the basis level of expression their genes, which are being in the *pHisNucSma* plasmid were determined. Invert correlation between an elimination of *pHisNucSma* plasmids and an activity of the *S. marcescens* nuclease was established at the basis level of expression of their genes. It is shown, that as higher is the level of activity of the mutant *S. marcescens* nuclease at the basis level of expression their genes, the higher ampicillin resistance. Correlation between the level of ampicillin resistance, β-lactamase activity and an amount of plasmid copies in recombinant *E. coli* strains is also revealed.

Key words: Nuclease · Basal level of gene expression · Plasmid copies · β-lactamase · Plasmid elimination · Cleavage

INTRODUCTION

In recent years genetic engineering is used to solve a wide range of challenges in molecular biology and biotechnology. One of these tasks is the making of recombinant DNA strains that have the ability to synthesize the desired protein in large quantities. This over expression can be achieved either by increasing the gene dosage or by increasing its transcription activity[1].

Vector DNA adds new properties to the host cell, by giving it selective advantage. However replicative synthesis of plasmid DNA, over-synthesis of the product of a foreign gene has an extra metabolic load on the host cell [2] which makes it vulnerable to elimination from the cell in nonselective conditions. Therefore the stability of the vector is what is important in order to maintain high level gene expression of foreign DNA.

In recent years *Serratia marcescens* endonuclease is widely used in biochemistry, medicine, molecular biology and agriculture. Increasing demand in the use of *S. marcescens,* calls for the necessity of researches directed at acquiring new over-producers of this enzyme and the study of its properties [3,4]. This enzyme can be used for DNA and RNA degradation, protein purification and can also serve as a model for studying mechanisms of protein transport from the inside of cells into the extracellular environment in gram-negative bacteria [5].

Noncellular *Serratia marcescens* endonuclease has selective cleavage of phosphodiester bonds in the substrate molecule. In both, single and double stranded DNA, cleavage first takes place in GC-rich regions and then at a low rate in GA-rich regions. DNA/RNA hybrids are better substrates, likely because DNA strand in a hybridized duplex shows a-similar conformation [6].

The purpose of this work is to define the effects of basal level *Serratia marcescens* endonuclease expression on the stability and *pHisNucSma* plasmid copy number in the cells of *Escherichia coli* recombinant strains.

MATERIALS AND METHODS

In the present work were used strains of *Escherichia coli TGE900* and *LK111λ* carrying *pHisNucSmaHis89Ala*, *pHisNucSmaArg57Ala* and *pHisNucSmawt.* In these plasmids mutant variants of *Serratia marcescens* endonuclease genes are under the control of P_L promoter of phage 2 while the transcription activity is regulated by heat-sensitive *CI*-repressor [4].

In order to create selective conditions for culturing recombinant strains, ampicillin with a concentration of 150 μg/ml was added to LB media.

For Plasmid elimination 0.1 ml of fresh culture, grown in LB broth and ethidium bromide (EtBr) with concentrations of 50 μg/ml and 200 μg/ml, respectively

Corresponding Author: Ricardo Mauricio Gudino Carrillo, Department of Genetics, Kazan State University, Kremlevskaya 18 St., 420008 Kazan, Russian Federation

was added to 4.9 ml LB broth. At the same time inoculation of cultures not treated with elimination agents was conducted. Separately grown colonies were pricked in petri-dishes containing LB agar that contains ampicillin with a concentration of 125 μg/ml. Plasmid elimination was made according to the loss of resistance to ampicillin by the cells.

In order to determine non specific nuclease, activity indicator agar, the method suggested by *Meiss et al.* [7] was used.

The level of resistance was taken to be the minimum concentration of the antibiotic under which no visual growth occurred during an incubation period of 18-20 h at 28°C [8].

To determine β-lactamase activity the method suggested by Perret [9] was used.

RESULT AND DISCUSSION

Plasmid Elimination Using EtBr: In order to clarify how stable the given plasmid is inherited in a series of cell generations and also to show that variations in the level of resistance to ampicillin in recombinant strains is not connected with the mutations of the host strain itself or with transposition of a resistance gene from the plasmid in to the chromosome it is necessary to shown that plasmid loss is followed by the loss of the cell's ability to grow on media with ampicillin. For this we conducted *pHisNucSma* plasmid elimination from recombinant *E.coli TGE900* strains using EtBr.

It is known that *S. marcescens* endonuclease preferably cleaves DNA A-form compared to DNA B-form [10]. It is possible that the nuclease cuts the origin region that has been modified by EtBr to A-form, thus substituting the initiated replication action of DNA-topoisomerase. As shown from the data presented in Table 1, treatment with EtBr leads to decrease of the bacterial survival rate. Cell survival was analyzed on the lost ampicillin resistant. From Table 2 it is clear that with the increase of EtBr concentration the elimination percentage increases. However in recombinant *E.coli TGE900 pHisNucSmaHis89Ala* strains plasmid loss takes place at a higher frequency as compared to the two other strains. *pHisNucSmaArg57Ala* plasmids are eliminated at a lower rate than *pHisNucSmaHis89Ala* plasmids but at a higher rate when compared to *pHisNucSmaHis89Ala* plasmids. This can only be explained by the following there may be cells in the population of bacteria in which the protein-repressor Cl may be weakly bound to the operating region of the P_L promoter and therefore transcription of

Table 1: Survival rate of recombinant *E.coli* strains exposed to EtBr

Strain	*EtBr concentration*, μg/ml	Survival rate, %
TGE900 pHisNucSma	0	100.00
His89Ala	50	27.33
	200	1.36
TGE900 pHisNucSma	0	100.00
Arg57Ala	50	2.15
	200	0.04
TGE900 pHisNucSmawt	0	100.00
	50	1.35
	200	0.006

Table 2: Plasmid elimination of E.coli TGE900 cells by the action of EtBr

Strain	*EtBr concentration*, μg/ml	Resistance to Amp loss to, %
TGE900 pHisNucSmaHis89Ala	0	57.7
	50	100.0
	200	100.0
TGE900 pHisNucSmaArg57Ala	0	12.0
	50	20.0
	200	68.0
TGE900 pHisNucSmawt	0	7.0
	50	9.0
	200	28.0

S. marcescens endonuclease gene is under the control of P_L promoter (basal level gene expression). Because His89Ala mutant protein lacks nuclease activity while Arg57Ala mutant only has 35% nuclease activity as compared to the original protein (wild type) [11], the results only indicate that there exists inverse correlation between endonuclease activity and plasmid elimination. (Table 1, Table 2)

Determination of non Specific Nuclease Activity by Means of Indicator Agar: Non specific nuclease activity determination under basal level expression of genes was carried out using indicator media that contained toluidine blue and polymeric DNA [7]. (Figure 1).

As shown in Figure 1, nuclease activity only exists in lysate of strain cells (TGE900 *pHisNucSmawt* and TGE900 *pHisNucSmaArg57Ala*) that carry the plasmids with wild type nuclease genes (100% nuclease activity) and with 35% nuclease activity which explains the presence of pink zones around the deposited sample. The zone size directly depends on the level of nuclease activity: the wild type has a much bigger pink zone area around the sample as compared to the one with a 35% nuclease activity (*pHisNucSmaArg57Ala* plasmid). The lack of the pink

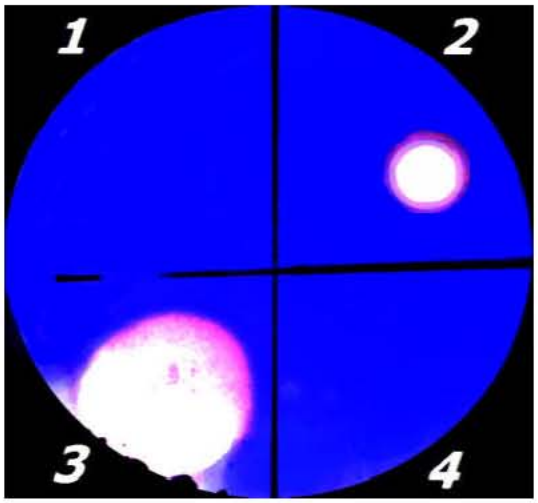

Fig. 1: Basal level gene expression of mutant endonuclease on indicator media: 1 - *TGE900 pHisNucSmaHis89Ala*; 2 - *pHisNucSmaHis89Ala;* 3 - *TGE900 pHisNucSmawt;* 4 - *TGE900*

zones around the *TGE900 pHisNucSmaHis89Ala* and *TGE900* cell lysates only tells us about the absence of nuclease activity that corresponds to the nuclease mutant forms with no activity of the given enzyme and a non plasmid strain. Nuclease activity shown by the above listed strains on indicator media corresponds to 100% and 36% of the wild type and Arg57Ala mutant respectively which is in line with literature data [11, 12].

Detection of the Level of Resistance to Antibiotics and β-lactamase Activity in Recombinant *E. Coli* Strains: It is known that resistance of bacterial cells to ampicillin is specified by β-lactamase that splits the antibiotic β-lactam ring.

In a series of experiments it was shown that there is a relationship between gene dosage, responsible for resistance to ampicillin and the expression. In other words, we observed a linier correlation between ampicillin resistance and enzyme synthesis and also between enzyme synthesis and gene *bla* copy number [14 -13]. It is also shown that increasing the number of plasmid copies lead to an increase in the level of ampicillin resistance [15 -14]. Thus change of the antibiotic resistance level and β-lactamase activity may serve as an indirect evidence of its genes quantity change. To prove the above statement, β-lactamase activity and level of resistance to ampicillin were determined in recombinant *E. coli* TGE900 strains that contained *pHisNucSma* plasmid. The results of the experiments are presented in Table 3.

Table 3: Relation between the cells resistance to ampicillin, β-lactamase activity and *pHisNucSma* plasmid copy number in the recombinant *E.coli TGE900* strains

Plasmid	Level of resistance to Amp, μg/ml	β-lactamase activity ($2 \cdot 10^5$ cells)	Approximate amount of plasmid copies
pAP42:Tn1	500	114,8	1-2
pHisNucSmaHis89Ala	1000	350,365	3-4
pHisNucSmaArg57Ala	13000	4191,74	40
pHisNucSmawt	>15000	10842,49	100

pAP42: Tn1 plasmid was used as a reference plasmid, with a known number of copies

In order to explain whether variation of resistance level to ampicillin correlates with β-lactamase activity variation, we measured the enzyme activity using the iodine titration method based on the ability of the products of the hydrolyzed β-lactam antibiotics to reduce iodine to iodide there Br causing decoloration of the starch-iodine complex [16 -15]. (Table 3).

As seen from Table 3, *E. coli* cells that carry the *pAP42:Tn1* plasmid grow on media with ampicillin with a concentration of not more than 500 μg/ml, *E. coli TGE900 pHisNucSmaHis89Ala* strains is resistant to an antibiotic concentration of 1000 μg/ml, *E. coli TGE900 pHisNucSmaArg57Ala* strain – 1300 μg/ml and *E. coli TGE900 pHisNucSmawt* strain - > 1500 μg/ml. in the reference plasmid copy number ranges from 1-2 per cell which corresponds to the minimal level of β-lactamase activity (114,8 units). In strains with *pHisNucSmaHis89Ala* plasmid the level of β-lactamase activity made up to 350,365 units which corresponds to 3-4 plasmid copies (≈ 100 activity units per copy). In *E. coli TGE900 pHisNucSmaArg57Ala* strains β-lactamase activity makes up to 4191,74 units ≈ 40 plasmid copies. β-lactamase activity in cells carrying the *pHisNucSmawt* plasmid was 10842,49 units ≈ 100 plasmid copies.

On the basis of the results, we can conclude that exists a direct dependence of cell resistance level to ampicillin to the amount of plasmid copies, which carry gene *bla*, in cells: the higher the antibiotic resistance, the higher the number of plasmid copies. It was observed that the increase of plasmid copies lead to the increase of nuclease activity, coded by the gene under basal level expression. It is likely that *S.marcescens* nuclease initiates replication of both plasmids and chromosomes by substituting the action of RNA-ase H which cuts RNA in DNA/RNA-hybrids at the start point of replication with the formation of 3′- ends that are used as primers for DNA synthesis.

We can conclude that basal level expression of endonuclease genes with high enzyme activity leads to an increase of the level of resistance to ampicillin, β-lactamase activity and amount of plasmid copies. According to the study of the plasmids, it was observed that the wild type was more stable than its mutant analogs.

REFERENCES

1. Gribskov, M. and R.R. Burgess, 1983. Overexpression and purification of the sigma subunit of Escherichia coli RNA polymerase. Gene, 26(2): 109-118.
2. Goldwin D. and J.N. Stater, 1979. The influence of the grouth on the syability of a drug resistance plasmid in Escherichia coli K-12. J. Gen. Microbiol., 111: 201-209.
3. Biederman, K., P.K. Jepsen, E. Riise *et al*, 1989. Purification and characterization of a *Serratia marcescens* nuclease activity. Carlsberg Rec. Commun, 54: 17-21.
4. Friedhoff, P., O. Gimadutdinow, T. Ruter, W. Wende, C. Urbanke, H. Thole and A. Pingoud, 1994. A procedure for renaturation and purification of the extracellular Serratia marcescens nuclease from genetically engineered Escherichia coli. Protein Expr. Purif., 5(1): 37-43.
5. Jepsen, P.K., E.K. Riise, Biedermann, P.Ch.R. Kristensen and C. Emborg, 1987. Two-level factorial screening for influence of temperature, pH and aeration on production of Serratia marcescens nuclease. Applied and Environmental Microbiology, 53(10): 2593-2596.
6. Gyi, J.I., G.L. Conn, A.N. Lane and T. Brown, 1996. Comparison of the thermodynamic stabilities and solution conformations of DNA/RNA hybrids containing purine-rich and pyrimidine-rich strands with DNA and RNA duplexes. Biochemistry, 35: 12538-12548.
7. Meiss, G., O. Gimadutdinow, P. Friedhoff and A. Pingoud, 2001. Microtiter-plate assay and related assays for nonspecific endonucleases. Methods Mol. Biol., 60: 37-48.
8. Barth, P.T., H. Richards and N. Datta, 1978. Copy number of coexisting plasmids in Escherichia coli K-12. J. Bacteriol., 135(3): 760-765.
9. Perret, C.J., 1954. Iodometric assay of penicillinase. Nature (London), 174(4439): 1012-1013.
10. Meiss, G., P. Friedhoff and O. Gimadutdinow, 1995. Sequence preferences in cleavage of ssDNA by the extracellular *Serratia marcescens* endonuclease. Biochemistry, 34(37): 11979-11998.
11. Friedhoff, P., O. Gimadutdinow and A. Pingoud, 1994. Identification of catalytically relevant amino acids of the extracellular Serratia marcescens endonuclease by alignment-guided mutagenesis. Nucl. Acids Res., 22(16): 3280-3287.
12. Friedhoff, P., B. Kolmes, O. Gimadutdinow, W. Wende, K.L. Krause and A. Pingoud, 1996. Analysis of the mechanism of the *Serratia nuclease* using site-directed mutagenesis. Nucl. Acids Res., 24(14): 2632-2639.
13. Uhlin, B. and K. Nordström, 1977. R-plasmid gene dosage effects in Escherichia coli K-12, copy mutants of the R-plasmid RI drd-19. Plasmid, 1: 1-7.
14. Ely, S. and W.L. Staudenbaner, 1981. Regulation of plasmid DNA synthesis: isolation and characterization of copy number mutant of mini RG-5 and mini F plasmids. Mol. Gen. Genet., 181: 29-35.
15. Livermore, D.M., 1991. Mechanisms of resistance to β-lactam antibiotics. Scand. J. Infect. Dis., 78: 7-16.