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ORIGINAL PAPER

Effect of mutations induced by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine on expression of penicillin G acylase and β-lactamase in wild-type *Escherichia coli* strains

Rubina Arshad · Shafqat Farooq · Syed Shahid Ali

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Abstract We introduced random mutations into three locally isolated strains of *Escherichia coli* using the mutagen *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) at concentrations ranging from 50 to 200 μg/ml. Viable colonies were screened for mutants with an increased production of penicillin G acylase (PGA) and a reduced production of β-lactamase, which are desired properties of industrial strains. Surviving colonies were recorded and, to determine the best level of mutagenesis, survival curves of *E. coli* strains exposed to different concentrations of MNNG for different time intervals were constructed. A total of 120 mutants were selected from mutagen-treated colonies and characterized for the response of surviving cells in terms of PGA and β-lactamase expression. Our results indicated that the frequency of β-lactamase-deficient mutants was significantly higher (73%) compared to PGA-deficient mutants (26%) in the mutants obtained. Fifty percent of the mutants exhibited higher PGA activity, 24% had PGA activity equal to or lower than the parent strain, while the remaining 26% had lost PGA activity due to the mutagenic effect of MNNG. The best MNNG-induced mutant strain, BDCS-N-W50^{M38}, exhibited considerably increased potential for PGA (four-fold higher) and negligible expression of β-lactamase compared to that of the parent strain. We estimated, for the first time, the average spontaneous mutation rate in PGA-producing wild-type parent and in the MNNG-induced mutants using the Luria-Delbruck fluctuation assay; this rate was found to range from 4.4×10^{-7} to 5.6×10^{-7} mutations per cell division.

Keywords *Escherichia coli* · Mutation · *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine · Penicillin G acylase · β-lactamase

R. Arshad (✉)
Nuclear Institute for Agriculture and Biology (NIAB),
P.O. Box 128, Jhang Road,
Faisalabad, Pakistan
e-mail: arshadrubina@hotmail.com

S. Farooq
Pakistan Atomic Energy Commission (PAEC),
P.O. Box 1114, Islamabad, Pakistan

S. S. Ali
Department of Zoology, University of the Punjab,
Quaid-e-Azam Campus, Canal Bank Road,
Lahore, Pakistan

Introduction

Penicillin G acylase (PGA) is an industrially important microbial enzyme used for hydrolysis of penicillin to produce β-lactam nucleus 6-aminopenicillanic acid (6-APA), which is a building block in the manufacture of semi-synthetic penicillin (Shewale et al. 1990). Several approaches have been used to improve PGA production from *Escherichia coli*, in particular mutagenesis, genetic and protein engineering techniques (Chou et al. 2000; Dai et al. 2001). Various other strategies, such as site-directed mutagenesis, have also been developed for high-level gene expression in *E. coli* for the production of PGAs (Alkema et al. 2000; Abian et al. 2004; Gabor and Janssen 2004).

In random mutagenesis, chemical mutagens such as hydroxylamine, nitrous acid, acridines or *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) are used to improve the productivity of bacterial strains (Fantini 1975). The action of certain chemical mutagens such as *N*-nitroso methyl urea, *N*-nitroso ethyl urea, *N*-nitroso dimethyl urea and *N*-nitroso

methyl biuret, as well as physical mutagens such as UV light on *E. coli* producing PGA has been studied, and high mutagenic activity has been achieved with *N*-nitroso methyl biuret (Kochetkova et al. 1986). However, among the mutagens tested, higher levels of mutagenesis (Adelberg et al. 1965; Miller 1992; Chandel et al. 2008) and PGA hyper-producer mutants from *E. coli* have been obtained using MNNG (Morita and Iwata 1984; Niersbach et al. 1995). Since β-lactamase limits PGA yield (Ghuysen 1991), we have made previous efforts to overcome this problem by inducing mutants with enhanced production of PGA and inactivation of β-lactamase through treatment of wild *E. coli* cells with acridine orange (Arshad et al. 2006b) and UV radiation (Arshad et al. 2010). In the present study, we assessed the potential of MNNG as a mutagen to induce random mutations in wild *E. coli* strains. The goal of this classical whole genome mutagenesis was to obtain mutants with high level expression of PGA and reduced or eliminated expression of β-lactamase. We also carried out the Luria-Delbruck fluctuation assay (Luria and Delbruck 1943) to determine the mutation rate in *E. coli* strains. To our knowledge, the results presented here provide the first experimental evidence of the frequency of MNNG-induced mutations in PGA- and β-lactamase-producing *E. coli* strains.

Materials and methods

Bacterial strains, medium and growth conditions

Three wild *E. coli* strains (BDCS-N-FMu10, BDCS-N-S21 and BDCS-N-W50) displaying PGA and β-lactamase activity were obtained from the Bacterial Diversity Culture Collection (Arshad et al. 2006a). Cultures were grown in 50 mL BD Difco™ Luria-Bertani (LB) Broth, and incubated overnight at 37°C with shaking (150 rpm).

Treatment with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine

Overnight grown cultures (1.2×10^8 cells/mL) were centrifuged at 3,000 *g* for 15 min at 4°C. Cells were washed twice with 0.1 M citrate buffer (pH 5.5) and centrifuged after each wash. The cells were re-suspended in 25 mL citrate buffer, placed on ice for 10 min and treated with MNNG following a modified method (Miller 1992). The MNNG stock solution was prepared in acetone and diluted in 0.1 M citrate buffer (pH 5.5). Different concentrations of MNNG ranging from 50 to 200 μg/mL were prepared from stock solution. For mutagenic treatment, 0.1 mL MNNG solution was added to 1.9 mL re-suspended cells; for controls, 0.1 mL citrate buffer was used. The cell suspension was incubated at 37°C in shaking water bath for different time intervals (5, 10, 15, 30, 45, 60, 75 and 90 min) to determine the best level of mutagenesis. After incubation, the cells were harvested and washed twice before re-suspension in 2 mL phosphate buffer (pH 7.0). Serial dilutions of viable cells (10^{-4}, 10^{-5}, 10^{-6}) were prepared in phosphate buffer and 100 μL aliquots of diluted samples (treated and control) were plated on LB agar for viable cell counts. These plates were incubated overnight at 37°C, colonies grown on plates were counted and the percentage of cells killed after exposure to mutagen was determined by comparing the number of viable cells after MNNG treatment to the number of viable cells originally present. After different treatments, the cell survival rate was determined and a survival curve for each strain was made by plotting MNNG concentration and time of exposure versus percent survival.

Selection and characterization of mutants for β-lactamase and PGA

The mutagenized cultures were grown overnight to allow expression of β-lactamase and PGA. The surviving colonies were screened for β-lactamase-deficient mutants by replica plating on nutrient agar containing 1% soluble starch followed by staining with iodine reagent, and for evaluation of PGA activity by *Serratia marcescens* overlay plate assay and spectrophotometric method as described previously (Arshad et al. 2006b). One unit of PGA activity was defined as the amount of 6-APA produced h^{-1} mg^{-1} wet cells under assay conditions. Data regarding PGA activity in wild and parent *E. coli* strains were expressed as the mean of three independent experiments, and differences were determined by one-way analysis of variance (ANOVA) followed by Duncan's multiple range (DMR) test.

Luria-Delbruck fluctuation assay

The fluctuation test (Luria and Delbruck 1943) was performed to determine the mutation rate in PGA-producing wild-type *E. coli* strain BDCS-N-W50 and mutant strain BDCS-N-$W50^{M38}$. For the assay, fresh overnight grown cultures (1.1×10^8 cells/mL) of wild and mutant strains were diluted 10^{-6}-fold in nutrient broth. A small number of cells (1×10^3) from the diluted culture were inoculated into bulk culture and ten parallel cultures were inoculated per strain. The cultures were incubated overnight at 37°C, and 10 μL from each culture was then plated on selective media for mutant colonies producing PGA- or β-lactamase, respectively (Arshad et al. 2006b). The total number of viable cells was determined by plating 10^{-6} dilution of bulk culture and pooled parallel cultures

on non-selective medium. The resulting colony counts were used to calculate the mutation rate (m) based on the Poisson distribution (P_0) method of Luria and Delbruck (1943).

$$m = \frac{-\ln(P_0)}{N}$$

where P_0 is the number of culture tubes with 0 mutants and N is the final number of cells per parallel culture at the time of application of the selective agent.

Results

Effect of MNNG on survival

The survival percent of three wild *E. coli* strains (BDCS-N-FMu10, BDCS-N-S21 and BDCS-N-W50) treated with 50 μg/mL MNNG for 5, 15, 30, 45, 60, 75 and 90 min in triplicate experiments is shown in Fig. 1. In BDCS-N-FMu10, the survival rates fluctuated between 90.4±0.6% and 8.9±0.23% in dilutions ranging from 10^{-3} to 10^{-5} at 50 μg/mL MNNG (Fig. 1a). At a dilution of 10^{-6}, the MNNG lethal effect was high, leading to a percent survival of 68.11±1.03% to 4.2±0.47%; thus, dilution of 10^{-6} was selected to obtain a high frequency of mutants. A significant difference in the viable count of the cultures before (~10^8 cells/mL) and after (~10^3 cells/mL) MNNG treatment was observed. Survival percentage at 10^{-6} dilution was relatively high in BDCS-N-S21 (5.5%) as compared with BDCS-N-FMu10 (4.2%) and BDCS-N-W50 (4.1%). A time interval of 90 min was found suitable for selection of mutants. Figure 2 shows the survival percentage of the three *E. coli* strains treated for 90 min with varying concentrations of MNNG (50–200 μg/mL). The number of surviving colonies decreased in a concentration-dependent manner, and the decrease in survival percentage was associated with the increase in concentration of MNNG. At the lowest concentration (50 μg/mL), 4–5% survival was observed, whereas at the highest concentration (200 μg/mL), survival declined to (0.01–0.03%). Survival rate also differed among strains; for all concentrations of MNNG the lowest survival was recorded in BDCS-N-W50.

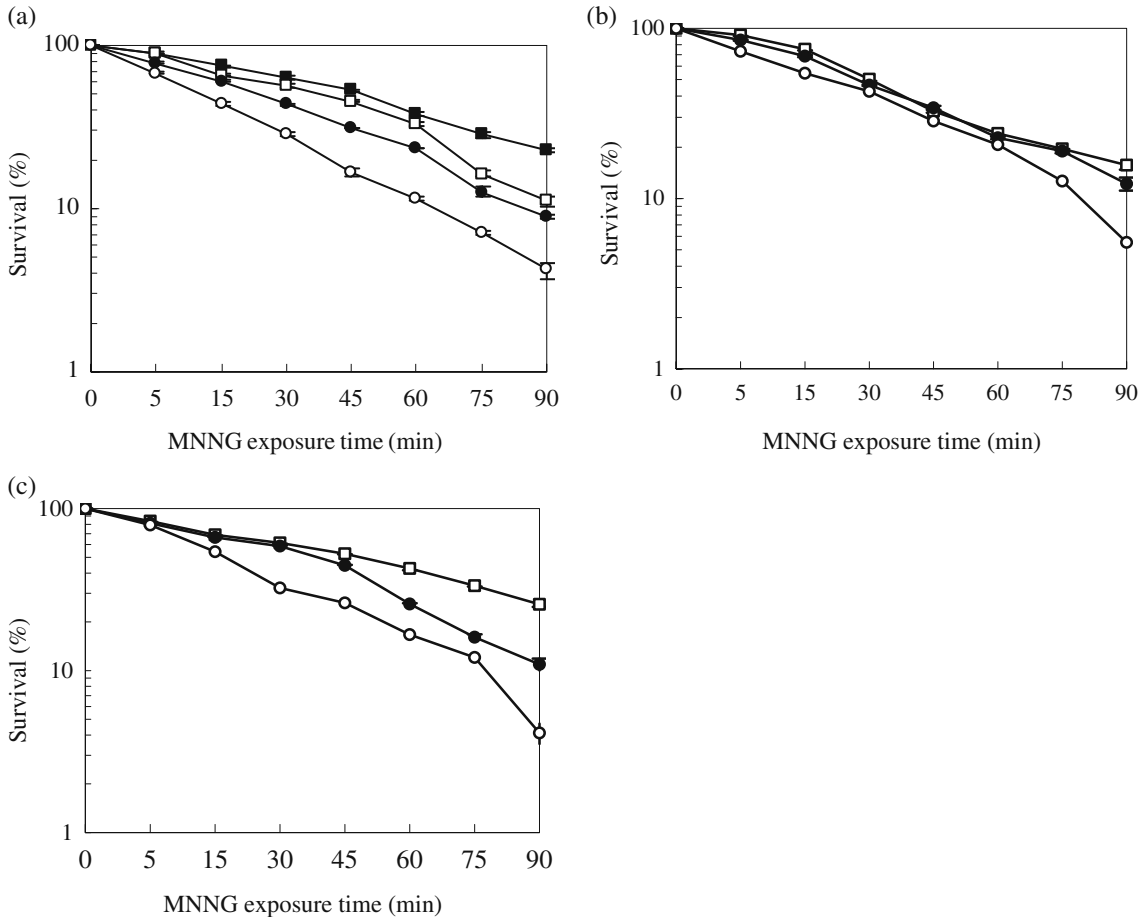

Fig. 1a–c Survival percentage of three strains of *Escherichia coli* at different dilutions (■ 10^{-3}, □ 10^{-4}, ● 10^{-5}, ○ 10^{-6}) after various time of exposure to 50 μg/mL *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG). **a** BDCS-N-FMu10, **b** BDCS-N-S21, **c** BDCS-N-W50

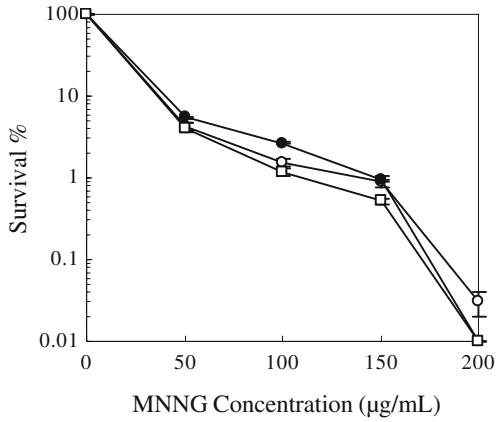

Fig. 2 Survival percentage of three *E. coli* strains after exposure to different concentrations of MNNG for 90 min. ○ BDCS-N-FMu10, ● BDCS-N-S21, □ BDCS-N-W50

Effect of MNNG on PGA and β-lactamase activity in *E. coli*

A total of 120 survivors isolated from MNNG-treated populations of the three wild strains were screened for enzyme activity. Only eight (17.8%) mutants in BDCS-N-FMu10 manifested higher β-lactamase activity than the parent. Overall, BDCS-N-S21 produced the highest number of β-lactamase-deficient (91.4%) as well as PGA-hyper-producing (65.7%) mutants (Table 1). The overlay plate assay performed for evaluation of PGA activity in parent and mutant strains revealed PGA activity in 89 mutants while the remaining 31 had lost PGA activity due to the mutagenic effect of MNNG. The parent strains exhibited small inhibition zones of 16 mm or less, indicating low PGA activity, whereas 30 mutants demonstrated higher activity with inhibition zone diameters in the range of 20–31 mm compared with the zones of inhibition produced by the parent strain (Table 2). Of the four mutant strains (BDCS-N-W50^{M38}, BDCS-N-W50^{M40}, BDCS-N-FMu10^{M101}, BDCS-N-FMu10^{M104}) for which growth zone diameter was equal to or greater than 28 mm, BDCS-N-W50^{M38} appeared to be the best, with a maximum inhibition zone diameter of 31 mm indicating the highest PGA activity. However, minimum inhibition zones of 20 mm were observed in three mutants (BDCS-N-W50^{M37}, BDCS-N-W50^{M48}, BDCS-N-S21^{M71}). Thus, growth inhibitory zones of *S. marcescens* confirmed the presence of PGA, whereas lack of such zones indicated negligible PGA activity.

The levels of PGA activity expressed by 89 PGA-positive mutants recovered from the three strains varied extensively from 1.7 mg 6-APA h^{-1} mg^{-1} wet cells to 24.4 mg 6-APA h^{-1} mg^{-1} wet cells (data not shown). Sixty mutants showed PGA activity levels one- to four-fold higher than their respective parent strain; 30 promising mutants exhibiting greater PGA activity together with negligible β-lactamase expression are shown in Table 2. The best mutant strain, BDCS-N-W50^{M38}, exhibited PGA activity four-fold higher (24.4 mg 6-APA h^{-1} mg^{-1} wet cells) than the parent strain (BDCS-N-W50: 6.3 mg 6-APA h^{-1} mg^{-1} wet cells). Another mutant BDCS-N-W50^{M40} retained from the same parent also showed considerably enhanced PGA activity (20.6 mg 6-APA h^{-1} mg^{-1} wet cells). One-way ANOVA test revealed a highly significant difference among mutants and parent ($F_{32,66}$=1655.7, P<0.05). DMR test (P<0.05) indicated that the mean PGA activity was significantly higher in mutants BDCS-N-W50^{M38}, BDCS-N-W50^{M40}, BDCS-N-FMu10^{M101} and BDCS-N-FMu10^{M104} than in parent strains, and PGA was over-produced in BDCS-N-W50^{M38} (Table 2).

Luria-Delbruck fluctuation analysis

Table 3 shows the results of three replicate fluctuation experiments performed with ten cultures for each sample.

Table 1 Percentage frequency of mutants for penicillin G acylase (PGA) and β-lactamase (β-lac) expression after 90 min of 50 µg/mL *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) treatment in three wild type strains of *Escherichia coli*. Values correspond to the number of mutants (percentages). Numbers with different letters in the same row are significantly different at $P \leq 0.05$ with Duncan's multiple range (DMR) test

Enzyme[d]	Strains			
	BDCS-N-FMu10	BDCS-N-W50	BDCS-N-S21	Total
PGA$^{+>}$	11 (24.4) b	26 (65) a	23 (65.7) a	60 (50) a
PGA$^{+<}$	3 (6.7) c	14 (35) b	12 (34.3) b	29 (24.2) c
PGA^{-}	31 (68.9) a	0 (0) c	0 (0) c	31 (25.8) b
β-lac$^{+>}$	8 (17.8) c	0 (0) c	0 (0) c	8 (6.7) c
β-lac$^{+<}$	10 (22.2) b	12 (30) b	3 (8.6) b	25 (20.8) b
β-lac^{-}	27 (60) a	28 (70) a	32 (91.4) a	87 (72.5) a

[d] +> greater than parent, +< less than parent, - nil

Table 2 Relative PGA and β-lac activities of three *E. coli* parent and mutant strains after 90 min of treatment with 50 μg/mL MNNG. Data correspond to the mean (± SD) of three independent experiments for each strain. Means with different letters are significantly different with DMR test at $P \leq 0.05$. *6-APA* 6-Aminopenicillanic acid

Parent *E. coli* strain	Mutant #	Enzyme activity		
		PGA		β-lac
		Inhibition zone diameter (mm)	mg 6-APA h^{-1} mg^{-1} wet cells (mean activity±SD)	Zone around colony (status)
BDCS-N-W50		16	6.3 t±0.17	White (+)
	BDCS-N-W50^{M34}	22	10.7 n±0.1	Nil (-)
	BDCS-N-W50^{M35}	21	10.2 qr±0.1	Nil (-)
	BDCS-N-W50^{M36}	23	11.4 l±0.1	Nil (-)
	BDCS-N-W50^{M37}	20	10.5 nop±0.17	Nil (-)
	BDCS-N-W50^{M38}	31	24.4 a±0.17	Nil (-)
	BDCS-N-W50^{M39}	23	11.5 l±0.2	Nil (-)
	BDCS-N-W50^{M40}	29	20.6 b±0.26	Nil (-)
	BDCS-N-W50^{M45}	26	13.3 i±0.26	Nil (-)
	BDCS-N-W50^{M46}	24	12.6 j±0.1	Nil (-)
	BDCS-N-W50^{M48}	20	10.1 r±0.17	Nil (-)
	BDCS-N-W50^{M49}	22	11.4 l±0.1	Nil (-)
	BDCS-N-W50^{M50}	21	10.3 pqr±0.1	Nil (-)
	BDCS-N-W50^{M51}	24	12.1 k±0.1	Nil (-)
	BDCS-N-W50^{M52}	25	13.4 i±0.1	Nil (-)
	BDCS-N-W50^{M53}	26	15.2 e±0.26	Nil (-)
	BDCS-N-W50^{M55}	21	10.3 pqr±0.1	Nil (-)
BDCS-N-S21		15	6.5 st±0.1	White (+)
	BDCS-N-S21^{M60}	25	14.3 fg±0.1	Nil (-)
	BDCS-N-S21^{M63}	22	10.2 qr±0.17	Nil (-)
	BDCS-N-S21^{M66}	23	12.7 j±0.1	Nil (-)
	BDCS-N-S21^{M69}	25	13.5 hi±0.1	Nil (-)
	BDCS-N-S21^{M71}	20	10.4 opq±0.1	Nil (-)
	BDCS-N-S21^{M72}	22	11.1 n±0.1	Nil (-)
	BDCS-N-S21^{M74}	21	10.6 no±0.2	Nil (-)
	BDCS-N-S21^{M84}	22	11.3 lm±0.1	Nil (-)
BDCS-N-FMu10		16	6.7 s±0.1	White (+)
	BDCS-N-FMu10^{M99}	24	12.3 k±0.2	Nil (-)
	BDCS-N-FMu10^{M101}	28	16.1 d±0.15	Nil (-)
	BDCS-N-FMu10^{M103}	25	14.4 f±0.1	Nil (-)
	BDCS-N-FMu10^{M104}	28	17.2 c±0.1	Nil (-)
	BDCS-N-FMu10^{M105}	23	13.7 h±0.17	Nil (-)
	BDCS-N-FMu10^{M106}	25	14.1 g±0.17	
One-way analysis of variance (ANOVA) between parent and mutants			F=1,655.7 d.f. = 32.66 $P<0.05$	

The number of viable cells was determined in two series of samples, one series representing parallel cultures and the other series taken from the same bulk culture. The average spontaneous mutation rate of PGA-producing wild-type (BDCS-N-W50) cells in parallel cultures was found to be 4.4×10^{-7} per cell division. In contrast, the mutation rate per cell division determined in bulk culture was much lower (2.1×10^{-6}). The number of mutations per cell division in mutant BDCS-N-W50M^{38} was 1.5×10^{-6} and 5.6×10^{-7} for bulk and parallel cultures, respectively. Based on the results in Table 3, the maximum mutation rate was found in BDCS-N-W50M^{38}, which produced 56 mutants in parallel

Table 3 Fluctuation test to determine the *E. coli* mutation rate in PGA- and β-lactamase-producing wild and MNNG-induced mutant strains

Strain	No. of cells plated (N) [a]	Variance	P_0 [b]	Mutation rate (m)
Wild-type *E. coli* BDCS-N-W50				
Bulk culture	1.1×10^{6}	231.6	0.1	2.1×10^{-6}
Parallel cultures	2.1×10^{6}	777.4	0.4	4.4×10^{-7}
Mutant BDCS-N-W50^{M38}				
Bulk culture	1.5×10^{6}	175.3	0.1	1.5×10^{-6}
Parallel cultures	8.8×10^{6}	545.6	0.1	5.6×10^{-7}

[a] Final number of cells per culture obtained by averaging the number of cells from each culture

[b] Proportion of cultures without mutants

cultures during growth from a single cell to a population of 10^7 cells. The variance calculated from the number of PGA-producing cells in ten samples taken from the same (bulk) culture was 175.3 (BDCS-N-W50M^{38}) and 231.6 (BDCS-N-W50), while the variance for parallel cultures of BDCS-N-W50M^{38} and BDCS-N-W50 was 545.6 and 777.4, respectively.

Discussion

Site-directed mutagenesis is an efficient way to improve the synthesizing capacity (Alkema et al. 2000) and stabilization of PGA (Abian et al. 2004) for biocatalytic application (Tianwen et al. 2006). Alhough a rapid way to create a large collection of mutants and achieve a high mutation rate, this method requires a series of optimization steps and sequence analysis (Neylon 2004). Moreover, it is a costly and labor-intensive technique that is economically inaccessible for resource-deficient laboratories especially in Third World countries. In the present study, we used a relatively simple and inexpensive "whole genome mutagenesis" approach for inducing genetic alterations in three wild *E. coli* strains that were collected from different ecological niches (Arshad et al. 2006a). The results presented here indicate a dose- and exposure-dependent decrease in survivor colonies, and show 30–40% killing of cells after 30 min of treatment with 50 μg MNNG/mL in three *E. coli* strains. Reports on MNNG-mediated random mutation have shown 50% bacterial cell death after 30 min of 100 μg MNNG/mL treatment in an *E. coli* K12 derivative strain (Adelberg et al. 1965), whereas treatment with a low concentration (50 μg MNNG/mL) for the same time period led to 95% killing in another *E. coli* strain (Miller 1992). It is evident from the present study that MNNG-induced mutation in our *E. coli* strains required longer exposure of bacterial cells to MNNG; significantly high (95%) killing of cells was achieved when cells were exposed to 50 μg MNNG/mL for 90 min. Since 4–5% survival was observed at concentration of 50 μg MNNG/mL, this concentration was selected for mutagenesis. This mutagen is known to bind rapidly to bacterial cells (Adelberg et al. 1965), thus the increase in killing due to longer exposure (90 min) of cells to MNNG may reflect the time required for completion of this binding process. The toxic effects of MNNG were clearly observed at a dilution order of 10^{-6} after an exposure of 90 min and, consequently, fewer viable cells per milliliter of culture were recorded in treated samples (~10^3 cells/mL) as compared to untreated controls (~10^8 cells/mL). In addition, percent survival decreased significantly with increasing exposure time and dose of the mutagen as well as with increasing order of dilution. It is likely that the low survival rate was due to the delayed expression of lethality in mutagenized cultures. Since the observed delayed lethality effect on survival was eliminated by increasing the dilution of the original mutagenized culture before overnight growth, a dilution of 10^{-6} was selected to obtain the highest frequency of mutants.

Based on our results, 50% of the mutant derivatives (selected randomly from the MNNG-treated population) exhibited enhanced PGA activity together with a considerable decrease in β-lactamase expression. The frequency of PGA hyper-producing mutants (50%) was two-fold higher than that of low PGA-producing mutants (24%). This is probably due to statistical reasons, in that random mutation is not focused close to the active site and has a larger impact on the catalytic performance. Such mutations may result in a relatively low frequency of mutants with significantly altered enzyme activity. However, mutations in the active site are often too drastic and lead to loss of activity. It seems likely that the loss of PGA activity in 25.8% of mutants obtained from BDCS-N-FMu10, and the loss of β-lactamase activity in 60, 70 and 91% of mutants developed from BDCS-N-FMu10, BDCS-N-W50 and BDCS-N-S21, respectively, were due to the mutagenic effect of MNNG on catalytic performance. MNNG is thought to act preferentially at certain sites in the genome and to induce closely linked multiple mutations (Guerola et al. 1971). Our results support the conclusion that complete

loss of enzyme activity and expression of the mutant were attributable solely to random mutation in the active site of the enzyme.

The average number of mutations calculated by the Luria-Delbruck test (Luria and Delbruck 1943) provided a novel insight into the effects of MNNG on PGA-producing *E. coli* strains. Fluctuation assay demonstrated that β-lactamase-deficient mutants exhibited an increased mutation rate (5.6×10^{-7} mutations per cell division) for PGA expression. Analysis of spontaneous PGA mutants also indicated that the number of mutants varied independently among parallel and bulk cultures. The variance among parallel independent cultures was greater than the variance among samples taken from the bulk culture. Moreover, the time of occurrence of a mutation in a series of parallel cultures was subject to random variation. This finding supports the spontaneous-mutation hypothesis (Luria and Delbruck 1943) that cultures with early mutation contain large numbers of PGA-producing cells whereas cultures with late mutation have only a small number of such cells. The mutation hypothesis thus leads to the prediction that there will be larger fluctuations in the number of PGA-producing mutants from culture to culture in a parallel series, than from a series of samples taken from the same culture. Random mutations that are produced independently of selection are important for the survival of an organism. We used the P_0 method to better estimate the mutation rate, which is the method required when the number of mutants in a culture is difficult to ascertain but the presence or absence of mutants in a culture can be determined (Zheng 2005). Our estimates of the number of mutations per cell division in wild-type and mutant cells under similar growth conditions indicate that mutants may grow faster than their parent.

The spectrum of MNNG-induced mutants studied here is consistent with a previous finding (Morita and Iwata 1984) suggesting that high PGA activity in mutants is not attributable to the permeation of penicillin but rather to an increased level of regulatory proteins in the bacterial cell. The magnitude of the increase in PGA production was four-fold higher in the best mutant strain BDCS-N-W50^{M38} (24.4 mg 6-APA h^{-1} mg^{-1} wet cells) than those from the parental *E. coli* strain (BDCS-N-W50: 6.3 mg 6-APA h^{-1} mg^{-1} wet cells). Similarly, another mutant strain BDCS-N-W50^{M40} retained from the same parent (BDCS-N-W50) supported higher PGA yield (20.6 mg 6-APA h^{-1} mg^{-1} wet cells) with the functional inactivation of β-lactamase. In a previous study, PGA production by an acridine orange (AO)-treated mutant derivative was improved two-fold over its parental *E. coli* strains (Arshad et al. 2006b). Comparison of these chemical mutagens shows that MNNG is a more potent chemical mutagen that can increase the mutation frequency and PGA yield in *E. coli* strains. To our knowledge, our results provide the first experimental measurements of MNNG-induced mutation rate in PGA-producing wild-type *E. coli* strains. The PGA hyper-producing *E. coli* mutant derivatives and novel information generated in this study may have potential for application to local industry.

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