AEM Accepts, published online ahead of print on 7 May 2010
Appl. Environ. Microbiol. doi:10.1128/AEM.01682-09

Assessing the Potential of Induced Mutation Strategy for Avermectins-Overproducers

Hong Gao[1†], Mei Liu[1†], Ying Zhuo[1, 2†], Xianlong Zhou[1], Jintao Liu[1, 2], Difei Chen[1], Wenquan Zhang[1], Zhongxuan Gou[1], Peng Shang[3], Lixin Zhang[1*]

1. Institute of Microbiology, Chinese Academy of Sciences (CAS), Beijing, People's Republic of China.

2. Graduate University of Chinese Academy of Sciences, Beijing, People's Republic of China

3. Key Laboratory for Space Bioscience and Biotechnology, Faculty of Life Sciences, Northwestern Polytechnic University, Xi'an, People's Republic of China

† These authors contributed equally to this work.

*Corresponding Author.

Mailing address: Institute of Microbiology, Chinese Academy of Sciences (CAS), Beijing, 100190, People's Republic of China

Phone/Fax: + 861062566511.

E-mail: lzhang03@gmail.com

Abstract

Mutant libraries of avermectins-producer *Streptomyces avermitilis* were constructed by different mutagenesis strategies. A metric was applied to assess mutation spectrum by calculating the distribution of average phenotypic distance of each population. The results showed for the first time that microgravity environment could introduce larger phenotype distribution and diversity than UV and NTG.

Induced mutagenesis is a classical and successful method for strain improvement to increase the productivity of commercial significant microbial metabolites. For evaluating different induced mutagenesis approaches Klein-Marcuschamer and Stephanopoulos presented a metric based on the quantification of phenotypic diversity to evaluate strain improvement approaches (14).

New approaches of induced mutagenesis emerged with the development of biotechnology, and among them, space-induced mutagenesis had led to great progress in strain improvement (6, 15, 26). In space, cosmic radial, high vacuum, intense magnetic field, and microgravity induced chromosome aberrance, further lead to microorganisms' genetic mutation (13). However, it is difficult to carry out space-induced mutagenesis extensively owing to the limitations of high cost and few chances to board spaceships. Therefore, ground-based simulated experiments have greater practical significance, and high magneto-gravitational experiments are a good choice to simulate the space environment.

Avermectins and its analogues, produced by *Streptomyces avermitilis*, are major commercial anti-parasitic agents for animal health, agriculture, and human infections (7). A variety of mutagenesis methods have been developed to increase its productivities (18-19, 21-23, 25). Though most of them can produce higher mutation rates, the potential of their success in strain improvement is different.

In this study, mutant libraries of *S. avermitilis* were constructed by three mutagenesis-inducing strategies: UV, NTG and HMGE (high magneto-gravitational environment). For each population, the distribution of average phenotypic distance was calculated based on the modified version of Klein-Marcuschamer's metric (15). The mutation rate was also calculated. A good correlation

between the distribution of average phenotypic distance and the percentage of improvement was found and analyzed. In this way, the potential of producing mutations among different induced mutagenesis approaches was evaluated to find the most effective one for *S. avermitilis* breeding.

The industrial producing strain *S. avermitilis* 3-115, and the mutants derived from 3-115, were grown on YMG agar medium (10). For diversity quantification and preliminary screening, fermentation was carried out in high-throughput format at 28°C. For results confirmation and secondary screening, mutants exhibited higher-yield than the wild type strain were inoculated into shake flask (10-11). High magneto-gravitational experimental platform using the large gradient superconducting magnet was described in detail by Qian et al (20).

The mutant libraries were prepared from *S. avermitilis* 3-115 with three different mutagenesis strategies. Spore suspension was prepared in sterile water (10^6 spores/ml). For UV-induced mutagenesis, 4 ml aliquot of the spore suspension was transferred into sterile petri dishes of 80 mm diameter. The petri dishes were then exposed to UV light in a "Dispensing – Cabinet" fitted with 15 w lamps with about 90% of its radiation at 265 nm. The dishes were placed at a distance of 30.0 cm away from the center of the UV light source and exposed to UV light for 15, 30, 45, 60, 75 and 90 seconds respectively. The UV-exposed aliquots were then stored in dark overnight to avoid photo reactivation. For NTG-induced mutagenesis, to 9 ml of spore suspension was added 1 ml of sterile solution of NTG (3 mg/ml in phosphate buffer, freshly prepared 1 hr before use). Samples were shaken at 28°C for 30 min, immediately centrifuged for 10 min at 5000 rpm and the supernatant was decanted. Cells were washed three times with sterile water and resuspended in 10 ml of sterile phosphate buffer (pH 7.2). All of the experimental samples were serially diluted with sterile water and plated over YMG plate. For HMGE-induced mutagenesis,

the superconducting magnet generated three different magnetic force fields in different places, which corresponded to three apparent gravity (0, 1, and 2 g) and three magnetic induction intensity (12, 16 and 12 T), respectively, that is: 0-g group (0g, 12T); 2-g group (2g, 12T); and 1-g group (1g, 16T). The YMG plates (plated with 4 ml spore suspension) were placed at the corresponding places in the platform for 7-day at 28°C to simulate strains grew in space flight. For all of above induced mutagenesis, the single colonies were transferred into 96-well plates to be cultivated when they were visible on YMG.

To quantify the avermectins production, UV absorbance of culture was measured on a multiplate reader (11) and all experiments were repeated twice except where specifically noted. More than 165 clones from each library were screened. The phenotypic distributions of five different populations (including the control) were quantified. We used optical density at 245nm as the phenotype for diversity quantification of each mutant library. All data were analyzed with MATLAB (MathWorks, USA). Average phenotypic distance (d) was calculated as:

$$d=\langle d_{i,j}\rangle$$

$$d_{i,j}=|P_i-P_j| \qquad (1)$$

where the brackets indicate an average over all pairs of members of the population and P_i is the phenotype of colony i. In this case, the logarithm of the OD_{245} was used as the phenotypic measure (Oi) because it was found to be lognormal distributed:

$$P_i=\ln O_i \qquad (2)$$

The strains from improved wells with respect to the control OD_{245} were cultured in shake flasks to verify the results. The production of avermectins was determined by HPLC (Agilent 1200, USA) (8, 11). The positive mutants were defined as the avermectin B1a production was

increased by more than 10% when compared with the original strains. The negative mutants were defined as the avermectin B1a production was decreased by more than 10% comparing with the original strains. The mutation rate was calculated as the number of either positive or negative mutants divided by the total number of screened mutants, and the calculation was based on the results of preliminary screen.

The distributions of the average phenotypic distances of five different populations were calculated. Bootstrapping was used to derive these distributions, and the results were displayed in a histogram (Fig. 1a). To see the statistical significance of the difference between the different groups, the mean and standard deviation of each histogram in Fig 1a were calculated (Fig. 1b). Homogeneous populations had small average phenotypic distances, whereas diverse populations had larger ones. Larger distance implied larger phenotypic dissimilarity among members of a population. Fig. 1a and 1b showed that phenotypic diversities in decreasing order were HMGE, NTG and UV. Among HMGE-induced mutagenesis libraries, phenotypic diversities in decreasing order were (0g, 12T), (2g, 12T), and (1g, 16T). For comparison, traditional evaluation index, mutation rate was also calculated (Fig. 1c). From Fig. 1c, the positive mutation rate of NTG is the highest, while that of UV is the lowest. Among HMGE-induced mutagenesis libraries, mutation rate in decreasing order were (1g, 16T), (2g, 12T), and (0g, 12T).

Fig. 2 shows the percentage of mutants exhibited higher yield in both of preliminary and secondary screening (percent improved), considering the instability of mutants in induced mutagenesis. The results parallel the findings of the diversity metric (Fig. 1b), not the mutation rate (Fig. 1c). To investigate the predictability of the divergence for improved phenotypes, the correlation between the divergence (average phenotype distance) and the occurrence of a mutant

with improved production was investigated. Fig. 3 shows the correlation between divergence and the improved percentage. A sigmoid fit and a correlation of R^2~1 were obtained. The results indicated that, for medium divergence (0.6<divergence<0.8), improved diversity increased rapidly with the probability of isolating mutants with improved phenotype; and for divergence which was less than 0.6 or more than 0.8, the correlation of divergence with the probability of finding improved mutants was relatively low.

Traditionally, positive mutation rate is used to evaluate mutation spectrum. However, positive mutation rate only describe the mutation do occur, but can not describe the extent of mutation and how broad the mutation spectrum is. Therefore, in this study divergence of mutant libraries was calculated to assess the mutation effect, and the divergence was applied to evaluate the effect of different induced mutation strategies, including HMGE-a new space simulated mutation strategy. The results indicated that: (*i*) HMGE-induced mutagenesis enhanced average phenotype distance and diversity better than UV and NTG mutagenesis, for *S. avermitilis*; (*ii*) microgravity introduced the largest diversity in the genome of *S. avermitilis* under HMGE conditions; (*iii*) for medium divergence (0.6<divergence<0.8), improved diversity increased the probability of isolating mutants with improved phenotype.

NTG was far more efficient than UV irradiation, an observation also concluded by many other researches, however in a study on the efficiency of mutagenesis to spectinomycin resistance in *Streptomyces fradiae*, it was reported that NTG was more efficient than UV (1-5, 17). The platform of HMGE was developed to simulate the space environment which has been reported to improve production of certain antibiotics in microorganisms (20). The results indicated that the simulated weightless environment significantly affected cell population, and suggested that

microgravity may cause the main mutagenic effects on the strains. Results reported here adds empirical support to the hypothesis that microgravity is the most important mutagen factor in space flight (12). Microgravity may speed microorganisms' growth rate; because under microgravity conditions, oxygen in air can be supplied to microorganism in all surfaces equally indicating an advantage in the production of biological matter in space (13). There was a hypothesis that microgravity may disturb the system of DNA repair, which blocks or delays the repair of DNA strand breakage. However, some researches (24) have demonstrated that is not true, and the mutation mechanism is still not clear.

Diversity has been reported to be correlated with the probability of finding improved mutants, and improved diversity would increase the probability of isolating mutants with improved phenotype (14). Results from our study partly supported this view. For medium divergence (0.6<divergence<0.8), there was a significant correlation between the diversity and the probability of isolating mutants with improved phenotype. An optimal mutation rate, which functioned as a balance between uniqueness and retention of function, was proved to exist (9). In addition, those findings demonstrated how optimal error-prone PCR mutation rates may be calculated, and indicated that 'optimal' rates depended on both the protein and the mutagenesis protocol. Our results concurred with the above findings and showed that no significant correlation was detected for divergence which is less than 0.6 or more than 0.8 and there existed an optimal mutation rate for medium divergence (0.6<divergence<0.8). The findings indicated the existence of a balance between mutation rate and improved phenotype, which implies that too high a mutation rate would cause dysfunction in some gene sequences, while certain mutation rates would produce a few of mutated gene sequences helpful for improving productivity.

We thank Fengzhu Sun, Yaqiao Li, Elizabeth Ashforth and Arnold Demain for providing helpful discussions. This work was supported in part by a grant from National 863 Project (No. 2006AA09Z402, 2007AA09Z443), National Basic Research Program of China (Project No. 2004CB719601), National Natural Science Foundation of China (Project No. 30560001, 30600001), National Key Technology R&D Program 2007BAI26B02, the National Science & Technology Pillar Program (No. 200703295000-02), Important National Science & Technology Specific Projects (No. 2008ZX09401-005), Science and Technology Planning Project of Guangdong Province, China (No. 2006A50103001), Key Project of International Cooperation (2007DFB31620). L.-X. Z. received funding from the Hundred Talents Program.

References

1. **Baltz R.H.** 1986a. Mutagenesis in *Streptomyces*, p. 184-190. *In* A.L. Demain and N.A. Soloman (ed.), Manual of Industrial Microbiology and Biotechnology. American Society for Microbiology, Washington, DC.
2. **Baltz R.H.** 1986b. Mutation in *Streptomyces*, p. 61-93. *In* S.W. Queener and L.E. Day (ed.), The Bacteria, vol. 9. Antibiotic-Producing *Streptomyces*. Academic Press, New York, NY.
3. **Baltz R.H.** 1999. Mutagenesis, p. 1819-1822. *In* M.C. Flickinger and S.W. Drew (ed.), Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis, and Separation. Wiley, New York, NY.
4. **Baltz R.H., and J. Stonesifer**. 1985a. Mutagenic and error-free DNA repair in *Streptomyces*. Mol. Gen. Genet. **200**: 351–355.
5. **Baltz R.H., and J. Stonesifer**. 1985b. Adaptive response and enhancement of *N*-methyl-*N*-nitro-*N*-nitrosoguanidine mutagenesis by chloramphenicol in *Streptomyces fradiae*. J. Bacteriol. **164**: 944–946.
6. **Benoit M.R., Li W., Stodieck L.S., Lam K.S., Winther C.L., Roane T.M., and D.M. Klaus.** 2006. Microbial antibiotic production aboard the international space station. Appl Microbiol Biotechnol. **70**: 403-411.
7. **Burg R.W., B.M. Miller, E.E. Baker, J. Birnbaum, S.A. Currie, R. Hartman, Y.L. Kong, R.L. Monaghan, G. Olson, I. Putter, J.B. Tunac, H. Wallick, E.O. Stapley, R. Oiwa, and S. Omura**. 1979. Avermectins, new family of potent anthelmintic agents: producing organism and fermentation. Antimicrob Agents Chemother. **15**: 361-367.
8. **Curdová E.,V. Jechová,J. Zima,and Z. Vaněk**. 1989. The effect of inorganic phosphate on the production of avermectin in *Streptomyces avermitilis*. J. Basic Microbiol. **29**: 341-346.

9. **Drummond D.A., B.L. Iverson, G. Georgious and F.H. Arnold.** 2005. Why high-error-rate random mutagenesis libraries are enriched in functional and improved proteins. J. Mol. Biol. **350**: 806–816.

10. **Gao H., M. Liu, J.T. Liu, H.Q. Dai, X.L. Zhou, X.Y. Liu, Y. Zhuo, W. Zhang and L.X. Zhang**. 2009. Medium optimization for the production of avermectin B1a by *Streptomyces avermitilis* 14-12A using response surface methodology. Bioresource Technology. **100**: 4012-4016.

11. **Gao H., M. Liu, X.L. Zhou, J.T. Liu, Y. Zhuo, Z.X. Gou, B. Xu, W.Q. Zhang, X.Y. Liu, A.Q. Luo, C.S. Zheng, X.P. Chen, and L.X. Zhang**. 2010. Identification of avermectin-high-producing strains by high-throughput screening methods. Appl. Microbiol. Biotechnol. **85:** 1219-1225.

12**. Halstead, T.W.** 1994. Introduction: an overview of gravity sensing, perception, and signal transduction in animals and plants. Adv Space Res. **14**: 315-316.

13. **Horneck G., H. Bucker, G. Reitz, H. Requardt, K. Dose, K.D. Martens, H.D. Mennigmann, and P. Weber.** 1984. Life Sciences: Microorganisms in the space environment. Science. **225**: 226-228.

14. **Klein-Marcuschamer D., and G. Stephanopoulos**. 2008. Assessing the potential of mutational strategies to elicit new phenotypes in industrial strains. PNAS. **105**: 2319–2324.

15. **Lam K.S., Gustavson D.R., Pirnik D.L., Pack E., Bulanhagui C., Mamber S.W., Forenza S., Stodieck L.S., and Klaus D.M.** 2002. The effect of space flight on the production of actinomycin D by *Streptomyces plicatus*. J Ind Microbiol Biotechnol. **29**: 299-302.

16. **Lu H.M., D.C. Yin, H.S. Li, L.Q. Geng, C.Y. Zhang, Q.Q. Lu, Y.Z. Guo, W.H. Guo, P. Shang, and N.I. Wakayama**. 2008. A containerless levitation setup for liquid processing in a superconducting magnet. Rev. Sci. Instrum. **79**, 093903.

17. **Mishra A.K. and D.N. Tiwari.** 1985. Enhancement of NTG mutagenesis by chloramphenicol in *Gloeotrichia ghosei*. Mol Gen Genet. **201**: 351-352.

18. **Pang C.H., K. Matsuzaki, H. Ikeda, H. Tanaka, and S. Omura**. 1995. Production of 6.8a-Seco-6.8a deoxy derivatives of avermectins by a mutant strain of *Streptomyces avermitilis*. J. Antibiot. **48**: 59-66.

19. **Petruk T.V., L.O. Biliavs'ka, V. Kozyryts'ka, and M.S. Mukvych**. 2004. Increasing biosynthesis of avermectins of *Streptomyces avermitilis* UCM Ac2161 under the effect N-methyl-N'-nitro-N-nitrosoguanidine. Mikrobiol. Z. **66**: 24-30.

20. **Qian A.R., D.C. Yin, P.F. Yang, B. Jia, W. Zhang and P. Shang.** 2009. Development of a ground-based simulated experimental platform for gravitational biology. IEEE Trans. Appl. Supercond. **19**: 42-46.

21. **Ren C, S.H. Zhao, and X.B. Ma**. 2006. Screening and optimizing of fermentation conditions of the effective avermecin B1a producing strain from *Streptomyces avermitilis*. Pharm. J. Chin. PLA. **22**: 365-368.

22. **Song Y, G.M. Cao, Z. Chen, and J.L. Li**. 2000. Selection of high avermectins producing strain and identification of avermectin B1. Sheng Wu Gong Cheng Xue Bao. **16**: 31-35.

23. **Stonesifer J., and R.H. Baltz**. 1985. Mutagenic DNA repair in *Streptomyces*. PNAS. **82**: 1180–1183.

24. **Takahashi A, K. Ohnishi, S. Takahashi, M. Masukawa, K. Sekikawa, T. Amano, T. Nakano, S. Nagaoka, and T. Ohnishi**. 2000. The effects of microgravity on induced mutation in *Escherichia coli* and *Saccharomyces cerevisiae*. Adv. Space Res. **28**: 555-561.

25. **Yan H.L., H.Z. He, and L.L. Liang**. 2002. Screening of high avermectin producing strains from *S. avermitilis* Y20. Biotechnology. **12**:16-17.

26. **Zhou J.Q., Sun C.H., Wang N.J., Gao R.M., Bai S.K., Zheng H.R., You X.F., and R.F. Li.** 2006. Preliminary report on the biological effects of space flight on the producing strain of a new immunosuppressant, Kanglemycin C. J Ind Microbiol Biotechnol. **33**: 707-712.

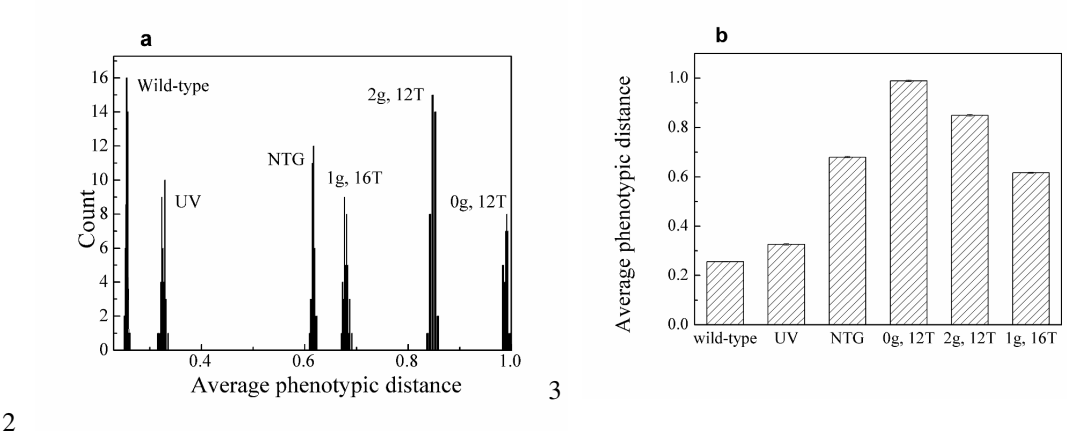
Figure legends

Fig. 1 Avermectin production spectrum evaluation of five different mutagenized populations and the untreated control. (a) Histograms that reflect the probabilistic distributions of the estimated average phenotypic distance (*d*, Eq. **1**); (b) To assess the statistical significance of this metric more directly, the means and standard deviation of each histogram in Fig 1a were calculated; (c) mutation rates of the five populations.

Fig. 2 Percentage of improved mutants which produce 10% more B1a than parent strain. The percentage represents the fraction of "successful" screening events (produce more avermectin B1a on secondary screening) and is a measure of the probability of finding improved mutants in a population.

Fig. 3 The correlation of divergence and percent improved. A sigmoid fit was used. $y = 41.681-30.3/ (1+\exp((x-0.75806)/dx))$. $R^2=1$.

1 **Fig. 1**

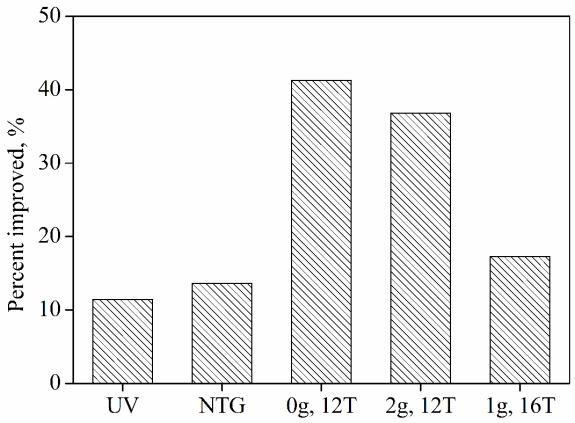


2 3

4

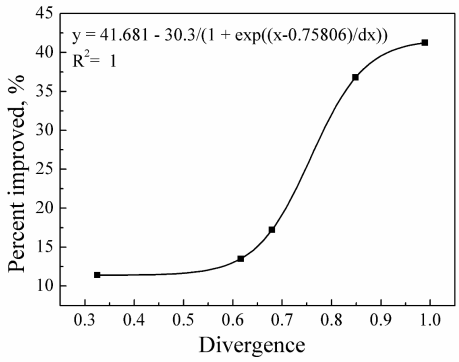
5

1 **Fig. 2**



2

1 **Fig. 3**



2

3