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EXPERIMENTAL ARTICLES

An Efficient Mutational Method for Photosynthetic Bacteria[1]

J. Q. Lin[b], M. Xiao[a, 2], M. N. Long[b], B. Han[a], W. Qian[a], and J. Du[b]

[a] *State Key Laboratory of Microbial Technology, Shandong University, Jinan, P. R. China*
[b] *School of Life Sciences, Xiamen University, Xiamen, P. R. China*

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Abstract—The pigment and auxotrophic mutants of *Rhodobacter sphaeroides* Y6 were obtained by treatment with ethyl methanesulfonate (EMS) followed by lithium chloride (LiCl). Treatment with 0.081 MEMS and subsequent treatment with 0.071 M LiCl resulted in 12% higher frequency og than that by 0.081 mol/L EMS alone, and the same frequency of pigment mutations than application of 0.081 M EMS alone; the frequency of auxotrophic mutations increased 2.5-fold when treatment with lithium chloride was applied. A blue shift by10 nm was recorded in the absorption spectrum of carotenoids form YM5-3 green mutant; considerable accumulation of neurosporine was revealed by HPLC and mass spectrometry. The method is efficient for isolating the mutants of photosynthetic bacteria.

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Keywords: ethyl methanesulfonate (EMS), lithium chloride (LiCl), photosynthetic bacteria, pigment mutation, carotenoid, auxotrophic mutation.

INTRODUCTION

Ethyl methanesulfonate (EMS) is a mutagenic agent that acts as a powerful alkyl donor, providing an alkyl to guanine or thymine to produce AT ⟶ GC and GC ⟶ AT transition mutations [1]. EMS has a low lethal frequency but a higher efficient mutagenesis than many other physical and chemical mutagenic agents. Thus, it plays an important role in mutational breeding [2–4], and in some aspects it is better than N-methyl-N'-nitro-N-nitrosoguanidine (MNNG or NTG) [5].

Lithium chloride (LiCl) is a halide of alkali metals with hardly any mutagenicity that is effective in treating various neural diseases [6]. Because it inhibits the activity of RNase in yeast by the toxicity of Li^+ cations [7]), it may slow transcription, and may cooperate with mutagenic agents in the regulation of protein synthesis.

It has been reported that mutagenesis with ultraviolet rays (UV) and LiCl of *Streptomyces* sp. is effective at producing high-yield strains of tetracycline [8, 9]. This study describes a new method for isolating photosynthetic bacteria with pigment and auxotrophic mutations using EMS followed by LiCl.

MATERIALS AND METHODS

Bacteria strains and reagents. *Rhodobacter sphaeroides* Y6 was stored in the State Key Laboratory of Microbial Technology at Shandong University. EMS and lycopene were purchased from Sigma (Sigma Chemical Co., USA).

Culture media. Luria-Bertani (LB) medium, RCVBN medium [10], minimal medium (its growth factor was 10% of that in RCVBN medium), enrichment medium (Minimal medium plus one or two amino acids).

Ethyl methanesulfonate and LiCl treatments. A mid-logarithmic-phase culture containing approximately 1×10^6 cells/ml was centrifuged, washed, and resuspended in sodium phosphate (0.2 mol/L, pH 7.0–7.2). A portion of the suspension was diluted and 0.15 ml was dispensed onto LB plates, while appropriate volumes of EMS and LiCl solutions were added to the rest of the suspension to obtain a final concentration of 0.081 mol/L of EMS [11] and 0, 0.071, 0.118, or 0.212 mol/L of LiCl, respectively. The treatments were performed at 37°C in the dark for 15 min, 30 min, 45 min, and 60 min. To stop the mutagenic treatment, an equal volume of sodium thiosulfate (2% wt/vol) was added to the mixture and more LiCl was added to retain its concentration at 0 mol/L, 0.071 mol/L, 0.118 mol/L, or 0.212 mol/L respectively. The mutated bacterial suspensions were diluted and 0.15 ml was dispensed onto LB plates, which were incubated at 30°C in light for 3–5 days. The survival frequency was calculated according to the following formula: the survival frequency of different concentrations of EMS and LiCl (SF) = $(\alpha - \beta)/\alpha$ (α is the number of viable cells on the LB plate prior to mutagenic treatment and β is the num-

[1] The text was submitted by the authors in English.
[2] Corresponding author; e-mail: minxiao@sdu.edu.cn.

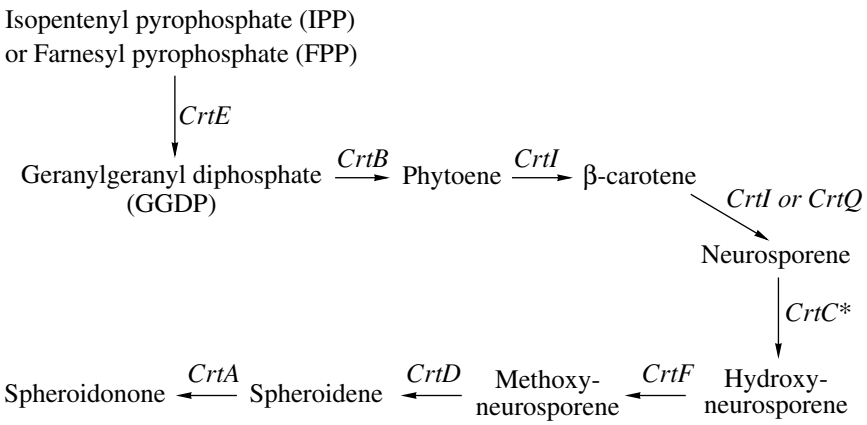

Fig. 1. Carotenoid biosynthetic pathway in the photosynthetic bacterium *Rhodobacter* sp.

ber of viable cells on the LB plate after EMS and LiCl treatments).

Enrichment incubation. After mutagenic treatment, the cell suspension was centrifuged at 10000 rpm for 30 min, washed, and incubated with LB medium at 30°C in the dark for several days until the number of cells increased to 10^7–10^8 ml^{-1}. Then the cells were centrifuged at 10 000 rpm for 30 min, washed, and resuspended in sodium phosphate (0.2 mol/L, pH 6.8–7.0). The suspension was diluted and 0.15 ml was plated on LB plates that were incubated at 30°C in light for 3–5 days.

Isolation of pigment mutants. Pigment mutants were identified and the pigment mutation frequency was calculated: The positive frequency (PF) = γ/β (β is the number of viable cells on the LB plate after EMS and LiCl treatment and γ is the numbers of pigment mutants in the same conditions on the control plates).

Isolation of auxotrophic mutants. All the colonies on the LB plates were transferred to semisolid minimal medium and incubated at 30°C in light for several days. The colonies that could not grow on the minimal medium were isolated and transferred to semisolid enrichment medium to determine which specific amino acid was required for growth. The positive frequency of auxotrophic mutation was also calculated: PF = δ/β (β is equal to the number of viable cells on the LB plate after treatment with specific concentrations of EMS and LiCl; δ is equal to the numbers of auxotrophic mutants in the control).

The absorption spectrum of the carotenoid extract. Cells (1.0 g) were collected, washed, resuspended in 10 ml sodium phosphate (0.2 mol/L, pH 6.8–7.0), and then somicated for 30 min. The suspension was mixed with an equal volume of ethyl acetate and stirred constantly for two hours at 30°C for carotenoid extraction. The mixture was centrifuged at 12 000 rpm for 20 min and the upper solution contained carotenoids. The carotenoid extract was diluted and scanned with a UV3100 spectrophotometer (Shimadzu Co., Japan) at 350–700 nm.

The HPLC analysis of the carotenoid extract. The carotenoid extract was analyzed with a SCL-10 Avp HPLC instrument (Shimadzu Co., Japan) using a KR100-5C18 chromatography column (150 × 4.6 mm, Kromasil Co., Sweden). The mobile phase was a mixture (methylenechloride : methanol : acetonitrile : water = 32 : 38 : 29 : 1). The flow rate was 1 ml/min. The sample volume was 10 μl and the temperature was 30°C.

Mass spectrum analysis of the carotenoid extract. The carotenoid extract was analyzed with a API4000 LC/MS/MS (AB Co., USA) at 30°C, and the mobile phase was methanol and acetonitrile (2 : 3).

RESULTS

Relationship between pigment mutation frequency (PMF) and survival frequency after EMS and subsequent LiCl treatment. Survival frequency and PMF were measured in repeated experiments (Fig. 2a). At longer treatment times, the survival of cells decreased while the PMF increased. At the 30 minute treatment period, the PMF was the highest among all samples and the conditions of 0.081 mol/L EMS followed by 0.071 mol/L LiCl were about 12% higher than with EMS alone.

Isolation and physical analysis of pigment mutant strains. Some pigment mutant strains (e.g., pink, orange, and green) were islolated and their absorption spectrum was measured. The absorption spectrum at 400–550 nm wavelength of green mutant YM5-3 was visibly different from that of the native strain Y6 (Fig. 3). Spectral analysis showed that absorbance peaks of the strain Y6 during 400–550 nm were 429.8, 456.0, and 487.2 nm, while that of YM5-3 appeared at 417.0, 441.4, and 471.0 nm; this blue shift of an average of 10 nm indicated that the pigment composition of YM5-3 was quite distinct from strain Y6.

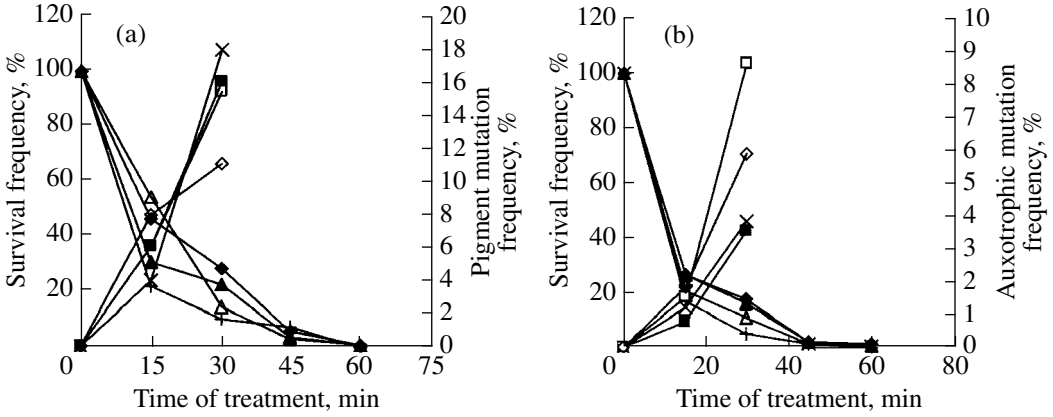

Fig. 2. Relationship between pigment mutation frequency (PMF) (a) or auxotrophic mutation frequency (AMF) (b) and survival frequency. Cells treated with 0.081 mol/L EMS followed by 0 mol/L–0.212 mol/L LiCl. The relationship between survival frequency and time (concentration of LiCl: 0 mol/L (◆), 0.071 mol/L (▲), 0.118 mol/L (△), 0.212 mol/L(+)); the relationship between PMF (a) or AMF (b) and time (concentration of LiCl: 0 mol/L (■), 0.071 mol/L (×), 0.118 mol/L (□), 0.212 mol/L (◇)).

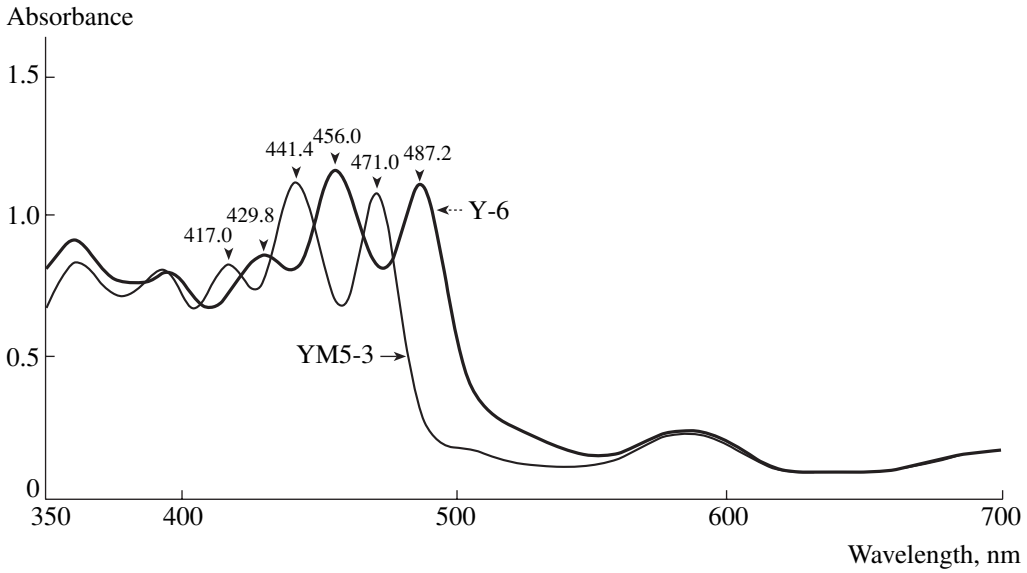

Fig. 3. The absorption spectrum of carotenoid extract: a, from strain Y6, b, from strain YM5-3. Scanning rate, 20 nm/s.

HPLC and mass spectrum analysis were used to examine the carotenoids in the mutant strains (Figs. 3, 4). The absorbance peaks of neurosporene are 416, 441, and 470 nm [11], and those of lycopene are 442, 470, and 500 nm [13, 14]. Neurosporene has a molecular weight of 538.9 Da, while lycopene has a molecular weight of 536.8 Da. From the HPLC results, compared to the peak value of lycopene in the standard sample, the value of lycopene/neurosporene (678055 volts, approximately 6.4 min) in strain YM5-3 was nearly 2000 times higher than that (309 volts) in strain Y6. Also, mass spectrum analysis revealed a peak at 538.7 Da, which is quite close to the published molecular weight of neurosporene. Thus, the neurosporene was dramatically accumulated in strain YM5-3. Carotenoid biosynthesis was altered by the chemical mutagenesis.

Relationship between auxotrophic mutation frequency (AMF) and survival frequency after EMS and LiCl treatment. The relationship between *AMF* and survival frequency (Fig. 2b) was similar to the relationship in pigment mutation, but the optimal conditions were different. AMF with 0.081 mol/L EMS followed by 0.118 mol/L LiCl was about 2.5 times higher than that with 0.081 mol/L EMS alone. This suggested that adding the LiCl treatment to the EMS treatment also produced many auxotrophic mutants and thus

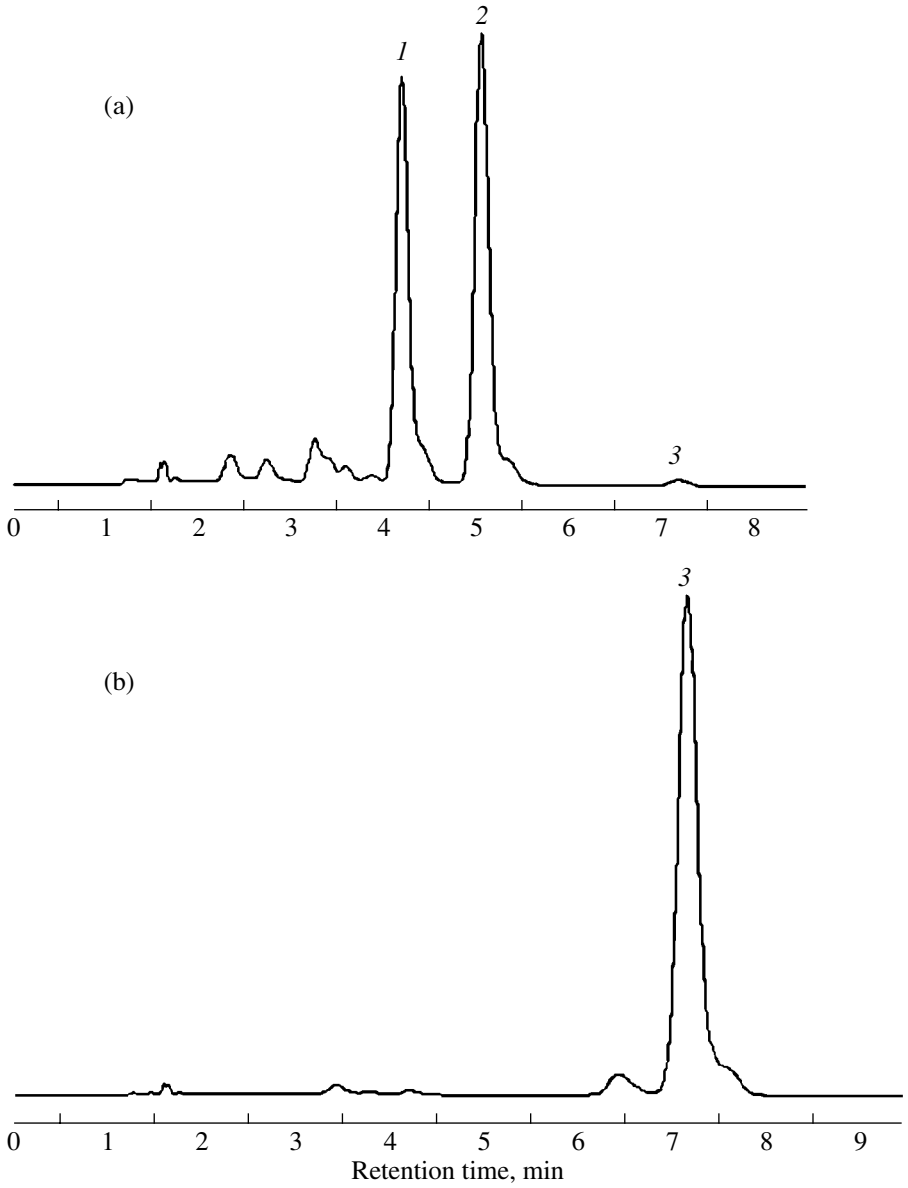

Fig. 4. HPLC spectrum of carotenoid. (a) carotenoid extract from strain Y6; (b) carotenoid extract from strain YM5-3. *1*—Spheroidonone, *2*—spheroidene, *3*—neurosporene.

many different amino-acid auxotrophic mutants could be isolated (the table).

DISCUSSION

In both industrial and research settings, physical and chemical mutagenesis induced by reagents such as EMS, MNNG, or NTG; ultraviolet rays (UV); and transposons has provided a useful method for producing ideal mutant strains. Mutant strains Hm68-5 was produced and had pigment production that was 30% higher than the parental strain. Mutagenesis of red yeast *Phaffia rhedozyma* [12] was also attempted with NTG and a mutant strain whose yield of carotenoid was 82.9% higher than the parent was obtained. Mutant strains producing high-yields of carotenoid or that have high growing capability have been isolated for industrial purposes using UV irradiation and transposons [13].

The number of isolated amino-acid auxotrophic mutants

Amino acid	*L*-Leu	*L*-Phe	*DL*-Trp	*L*-GLN	*L*-Ser	*L*-Met
Number of mutants	2	2	3	2	1	2

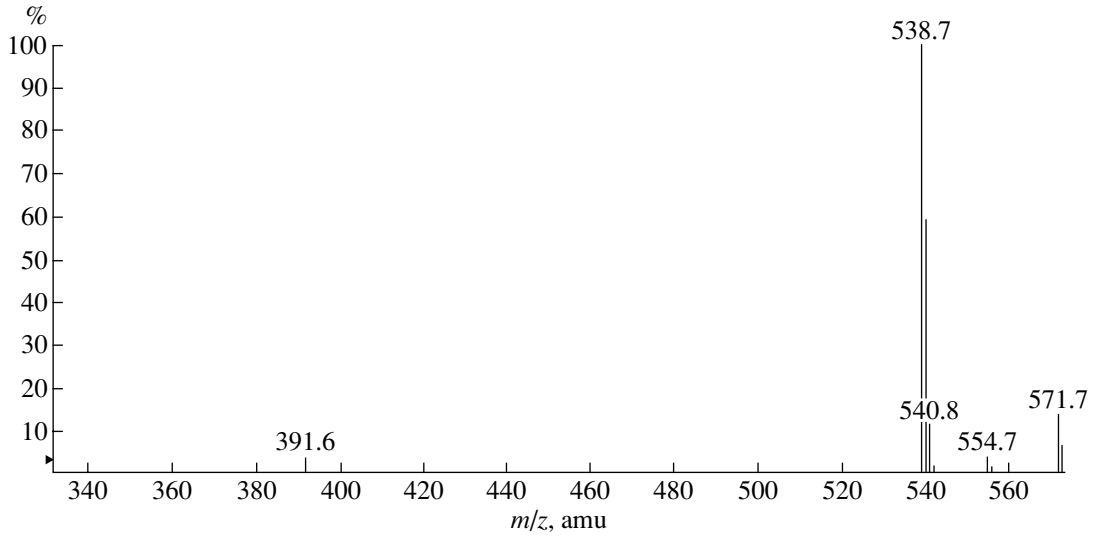

Fig. 5. Mass spectrum of the carotenoid extract from YM5-3 (only the 6.4-min peak area in HPLC is displayed).

Many different mutant photosynthetic bacteria have already been produced using the single or multiple mutagenic agents mentioned above. For example, a green mutant named GM309 was produced by EMS treatment providing a novel method for investigating the mechanism of excitation energy transfer in Light Harvesting Complex II(LH2) [11]. Similarly, mutants of *Thiocapsa roseopersicina* were isolated by transposon (Tn*5*) mutagenesis and the functions of several crucial genes that regulate the biosynthesis of carotenoid in cells were studied [7]. Also, complex mutagenesis with NTG and UV was applied to *Rhodopseudomonas* sp. to produce mutants with a high yield of 5-aminolevulinic acid (ALA) [14].

Our research on EMS and LiCl mutagenesis provides a new and efficient method for producing mutant photosynthesis bacteria. The relationships between positive mutation frequency and survival frequency were investigated using 0.081 mol/L EMS followed by different concentrations of LiCl (0 mol/L, 0.071 mol/L, 0.118 mol/L, 0.212 mol/L). The optimal conditions for chemical treatment were determined (0.081 mol/L EMS and 0.071 mol/L LiCl for 30 min for pigment mutations and 0.081 mol/L EMS and 0.118 mol/L LiCl for 30 min for auxotrophic mutations, Fig. 1).

It is noteworthy that the positive frequency may continue to increase with time using this method. This is consistent with related studies. Wartin and Prieur [15] treated *Pyrococcus abyssi* with 0.1 M EMS for 0 min, 30 min, 60 min, 90 min or 120 min, and showed that though the survival decreased from 100% to about 0.1%, the mutation frequency increased up to 1000-fold and continued to increase smoothly at extended treatment times. A similar phenomenon was observed in research by Lin et al., using complex mutagenesis of UV rays and LiCl [8]: when either the dose of UV or LiCl increased, the lethal frequency increased. There was a slight difference in our study in that the positive frequency increased when the concentration of mutagenic agents increased beginning at 30 min. This suggested that LiCl may play a distinct role under different treatment conditions (e.g., different mutagenic reagents or different strains). Since the lethal frequency increased with time (45, 60 min or even more), there were few colonies we could count on a plate, and thus, we concluded that the optimal relationship between survival and mutation frequency needed to be determined before ideal mutants for specific purposes could be produced.

In mutating the pigment of YM5-3, a composition alternation was suggested by the 10 nm (on average) blue shift of the carotenoid absorption spectrum (Fig. 3) and neurosporene was demonstrated to accumulated dramatically by HPLC and mass spectrum analysis (Figs. 4, 5). Since the pathway for carotenoid biosynthesis in photosynthetic bacteria has already been determined (Fig. 1), the accumulation of neurosporene may be caused by the inhibition of hydroxyneurosporene synthase (regulated by *crtC*), which induces the biosynthesis of hydroxyneurosporene in the downstream pathway. Also, various auxotrophic mutants were isolated (see Table) and this may indicate the blockage or even destruction of amino-acid biosynthetic pathways that was induced by efficient chemical mutagenesis.

In conclusion, we provided a novel method using EMS treatment and subsequent LiCl treatment that is efficient at producing mutants that may be useful for research or industrial purposes.

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