African Journal of Microbiology Research Vol. 7(6), pp. 471-476, 5 February, 2013
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR12.1642
ISSN 1996-0808 ©2013 Academic Journals

Full Length Research Paper

Biodegradation of ethidium bromide by *Bacillus thuringiensis* isolated from soil

Pharanai Sukhumungoon[1]*, Pattamarat Rattanachuay[2], Fadeeya Hayeebilan[1] and Duangporn Kantachote[1]

[1]Department of Microbiology, Faculty of Science, Prince of Songkla University, Hat-yai, 90112, Thailand.
[2]Faculty of Science and Technology, Prince of Songkla University, Pattani Campus, 94000, Thailand.

Accepted 7 February, 2013

Ethidium bromide (EtBr) is a powerful carcinogen. Most molecular biology laboratories use EtBr in the research for nucleic acid visualization. However, EtBr waste seems to be efficiently degraded by only chemical method to date. In this study, *Bacillus thuringiensis* isolated from soil sample uncontaminated with EtBr was investigated for its ability in EtBr degradation. *B. thuringiensis,* strain PSU9 demonstrated the ability to degrade EtBr shown by clear zone formation on EtBr-supplemented Tryptic soy agar and thin layer chromatography (TLC). In TLC experiment, the results suggested that the large portion of EtBr could be degraded within 18 h using bacterial culture as well as cell-free supernatant of *B. thuringiensis* PSU9. These results may suggest the promising solution using microorganism to solve the problem of EtBr waste in the laboratory for the decrease of pollutant in the environment.

Key words: Ethidium bromide, biodegradation, *Bacillus thuringiensis.*

INTRODUCTION

Ethidium bromide (EtBr) is widely used in biochemistry and molecular biology laboratories for a long period of time. It is well-known to be used for the nucleic acids and proteins observation (Lunn and Sansone, 1987; Dvortsov et al., 2006). EtBr intercalates between DNA strands of nucleic acids. After activation by UV-light, it emits the light which allows nucleic acids to be observed. Although some newer chemicals were recently applied for nucleic acids detection such as cyanine dyes (Hilal and Taylor, 2008), however, EtBr is still the most widely used in the laboratories because of its higher sensitivity in nucleic acids detection. EtBr is a strong mutagen which is able to cause high frequency of frame shift mutation to the microorganisms when it is processed by rat liver extract (Singer et al., 1999; Ohta et al., 2001; MacGregor and Johnson, 1977). The potency to cause mutation of EtBr is approximately 5-fold greater than benzo-(a)-pyrene and 10-fold higher than *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) (Quillardet and Hofnung, 1988). Moreover, EtBr waste is not easy to be destroyed and because of its toxicity, it should not be disposed as a normal liquid waste in a municipal water system. Lunn and Sansone (1987) used the sodium nitrite and hypophosphorous acid in the reaction to destroy EtBr. Although, the EtBr degradation was shown to be more than 99.8%, these two chemicals are also toxic.

Biological methods are thought to be the greater ways to establish EtBr removal and degradation. Phytoremediation by plants was employed for EtBr removal from the environment (Uera et al., 2007). However, those plants removed EtBr by absorbing the substance into themselves and they also need to be destroyed by chemical processes. Thus, microorganisms are thought to be the choice of interest for degrading EtBr. Although, there was evidence showing that the biological activities in some microorganisms were inhibited by EtBr (Tomchick and Mandel, 1964), there is the possibility to find the EtBr-resistant microorganisms from nature. Hence, this study aimed to seek for bacteria

*Corresponding author. E-mail: pharanai82@gmail.com. Tel: (66) 74-288322. Fax: (66) 74-446661.

possessing the activity in EtBr destruction, which suggested the safe and efficient promising way to cope with the EtBr waste in the future.

MATERIALS AND METHODS

Bacterial screening and isolation

Soil samples were randomly collected in Songkhla Province, Thailand, on October, 2011. One gram of soil was suspended in 0.9% NaCl solution (NSS). Ten-fold serial dilution of suspended solution were carried out and 100 µl of each dilution was spread on EtBr (Nacalai Tesque, Kyoto, Japan) -supplemented Tryptic soy agar, E-TSA (Merck, Germany) plate (30 µg/ml). The plates were incubated at 37°C in dark place, preventing the uncontrollable factors that may be involved in EtBr degradation such as the light, for 18 h. Then, the plates were briefly exposed to UV-light for image capture and the colonies showing clear zone of EtBr degradation were marked and selected. The selected colonies were cultured in Tryptic soy broth, TSB (Merck, Germany), at 37°C, 150 rpm agitation for 6 h. Morphological study of isolated bacteria was performed by Gram staining. The bacteria were subsequently kept in stock at -80°C.

Screening for bacteria with EtBr degradation activity

To screen for bacteria that give the highest EtBr degradation activity, isolated bacteria were cultured on TSA plate for 18 h at 37°C. Pin-point colony of each of isolate was inoculated on E-TSA by spotting, using microbiological needle. The plates after inoculation were incubated at 37°C for 6, 9 and 24 h in the dark to monitor the clear zone of EtBr at each time point. The related bacterial species, *Bacillus subtilis* and unrelated bacterial species *Escherichia coli* O157:H7 strain EDL933 were used as positive and negative controls, respectively. The relationship between EtBr degradation and bacterial growth was investigated by the shift of temperature from 37°C to room temperature for delaying the bacterial growth and then monitoring the clear zone as compared to the plate that was incubated at 37°C for 24 h.

Identification of bacterial isolate by 16S rDNA sequencing

Genomic DNA of a selected bacterial isolate was obtained by boiling method based on the work of Vuddhakul et al. (2000) with slight modification. Briefly, a single colony of bacteria was inoculated into 3 ml of TSB and then incubated at 37°C for 6 h with shaking at 150 rpm. Subsequently, one milliliter of culture broth was taken to boiling at 100°C for 10 min and immediately immersed on ice for 5 min. Centrifugation at 11,000 x*g* was applied to obtain a supernatant portion. Supernatant was diluted 10 folds to prepare the template for PCR. Amplifications of 16S rDNA were carried out using bacterial universal primers, BSF8/20 (5'-AGAGTTTGATCCTGGCTCAG-3') and REVB (5'-GGTTACCTTGTTACGACTT-3') (Kanokratana et al., 2004). Amplicons were run on 1% agarose gel electrophoresis and purified by Gel/PCR DNA fragment extraction kit (Geneaid, Taiwan). DNA sequencing was performed by ABI Prism 377 (Applied Biosystems, USA). For sequencing reactions, BSF8/20 was used to investigate the nucleotide sequences from 5' terminal of amplicons and REVB was used to obtain the sequences from 3' regions of PCR product for confirmation of the sequencing results. The resulting nucleotide sequences were searched for homology using program from NCBI website.

Investigation of EtBr degradation by bacteria using thin layer chromatography

Single colony of bacteria was inoculated into 3 ml of TSB and cultured at 37°C, 150 rpm shaking for 18 h and used as a starter. The number of bacterial cells was adjusted to 0.5 McFarland turbidity standards and 1 ml of adjusted cells was taken to a mixture of 10 ml of NSS and 10 ml TSB. EtBr was added to a final concentration of 30 µg/ml. The mixture was incubated at 37°C with 150 rpm shaking for 18 h prior to application of thin layer chromatography (TLC). TLC was performed using TLC silica gel $60F_{254}$ (Merck, Germany) as a stationary phase and n-butanol : acetic acid : water (4:1:1) as a mobile phase at room temperature (Olmsted III and Kearns, 1977). Twenty microliters of samples were spotted on a TLC silica gel. TLC was run under dark condition. Dried TLC silica gel was taken to UV-transilluminator to monitor EtBr and the image was captured. EtBr including the unidentified substances in the mixture were also monitored using the light at wavelength 365 nm. *Vibrio cholerae* PSU 401, which has been preliminarily found to provide comparable clear zone for EtBr degradation to PSU9 (unpublished data), was used as a positive control.

EtBr degradation by bacterial cell-free supernatant

In order to test whether the decrease of EtBr in the broth medium was not from the factors of bacterial cells, cell-free supernatant portion after 18 h culture was separated and used for examining the degradation of EtBr. In brief, the protocol of growing bacteria was performed as in the section above. Then, the culture broth was filtered through 0.45 µm filter. One milliliter of cell-free supernatant was added by EtBr (final concentration of 30 µg/ml). The mixture was incubated at 37°C with 150 rpm shaking for 18 h in the dark. Then, 20 µl of the mixture was applied to TLC with the condition exactly the same as mentioned in TLC experiment with the presence of bacterial cells.

RESULTS

Bacterial isolation

On the processes of bacterial screening from soil sample, several colonies showed clear zone of EtBr (Figure 1, blank arrow) on E-TSA plate. Six isolates (PSU8 to PSU13) were randomly selected from the plate due to their production of immense clear zone.

Screening of EtBr degradation on agar plate

In order to screen for the isolate that is potent to degrade EtBr, six isolates were chosen to test the ability to destroy EtBr at 3 time points. The tested colonies (PSU8 to PSU13) were spotted on E-TSA plate and incubated at 37°C for 6, 9 and 24 h to monitor the capability of destroying EtBr by clear zone forming. In this experiment, the clear zone was initially observed at 6 h post inoculation and then they continued to form a larger zone at 9 and 24 h for all tested isolates (Figure 2A to C). Moreover, we recognized that clear zone formation was independent on the growth of bacteria because the

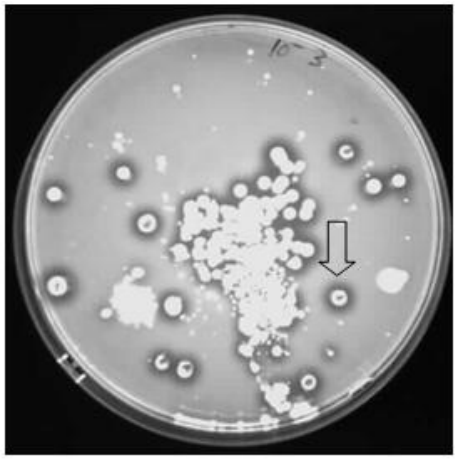

Figure 1. Isolation of EtBr-degrading bacteria from soil. The suspensions of soil in NSS were spread on E-TSA and incubated at 37°C for 16 to 18 h in the dark. Plates were flashily illuminated by UV-light. Blank arrow indicates an example of bacterial colony forming clear zone.

isolates that grew at room temperature (around 25°C) facilitated the production of comparable clear zone as those grown at 37°C (Figure 2C and E). *E. coli* O157:H7 EDL933 was shown to form narrower clear zone at 24 h. *B. subtilis* was incapable of growing on both E-TSA (Figure 2) and in TSB supplemented with EtBr (data not shown).

16S rDNA sequencing

All tested isolates showed comparable clear zone of EtBr in the experiment of EtBr degradation by plate method. Thus, one of the isolates (PSU9) was randomly selected to identify the bacterial species by 16S rDNA sequencing method. Using genomic DNA of PSU9 as a PCR template, two amplicons (approximately 1,200 bp in size), obtained using both BSF8/20 and REVB universal oligonucleotide primers, were introduced to DNA sequencing processes. It was found that PSU9 was identified to be *Bacillus thuringiensis* strain IAM 12077 by DNA sequencing of 16S rDNA gene (99% identity).

Investigation of EtBr degradation by thin layer chromatography

To clearly illustrate the degradation of EtBr by isolated bacteria, thin layer chromatography using a silica gel was employed to qualitatively monitor the depletion of EtBr. Since all isolates showed the comparable efficiency in clear zone formation, *B. thuringiensis* PSU9 was thus used in this experiment. The 18 h culture of PSU9 in E-TSB along with control groups were applied on TLC silica gel and subsequently illuminated under UV-light to monitor EtBr. The result demonstrated that the main portion of EtBr disappeared in the presence of PSU9 while abiotic control (E-TSB) clearly showed high quantity of EtBr (Figure 3B). *Vibrio cholerae* PSU 401 which previously showed large clear zone on E-TSA (unpublished data) was used to compare the destruction of EtBr. However, the same phenomenon as abiotic control was seen in the reaction with *V. cholerae* PSU 401 (Figure 3B, lane 4). Long wavelength (365 nm) was also applied to confirm the position and the existence of EtBr (Figure 3A), including other substances engendered during bacterial growing phase. The position and rough quantity of EtBr when monitored by the light at 365 nm wavelength were consistent with the result monitored by UV-illumination (Figure 3A).

Investigation of EtBr degradation using cell-free supernatant of PSU9 by thin layer chromatography

To show that the decrease of EtBr was not as a result of the presence of bacterial cell of *B. thuringiensis* PSU9, cell-free supernatant of PSU9 was obtained by filtration through 0.45 µm filter and used in this experiment. It was found that cell-free supernatant of PSU9 still show a similar phenomenon as in the experiment that used bacterial cultured cells of *B. thuringiensis* PSU9. Large portion of EtBr was depleted when compared with abiotic control (Figure 4B). When the wavelength at 365 nm was applied to visualize a portion of EtBr on TLC silica gel, a similar trend of result was obtained (Figure 4A).

DISCUSSION

Soil contains a microbial community which has a broad range of microorganisms. These microorganisms harbour a divergence of abilities useful for several aspects such as bioremediation of heavy metals (Hu et al., 2007; Rajkumar et al., 2008) and biodegradation of toxic substances, azo dyes, dichloroaniline, which are toxic waste from industry for instance (Khan, 2011; Yao et al., 2011; Ahmed et al., 2010).

Ethidium bromide is a mutagen which is able to cause high genotoxicity to the organisms when it is processed in the presence of rat liver extract (Ohta et al., 2001; Macgregor and Johnson, 1977). It affects the cellular organelles of yeast (Keyhani, 1987) and inhibits mitochondrial replication and transcription in animal cells (Nass, 1970). A work by Tomchick and Mandel (1964) also showed the inhibition of growth on *E. coli* strain B and *Bacillus cereus* strain 569 H. They demonstrated that the low level of EtBr (approximately 5 to 10 µM) could inhibit the growth of *E. coli* strain B and more effectively, *B. cereus* strain 569 H. EtBr affected DNA synthesis of

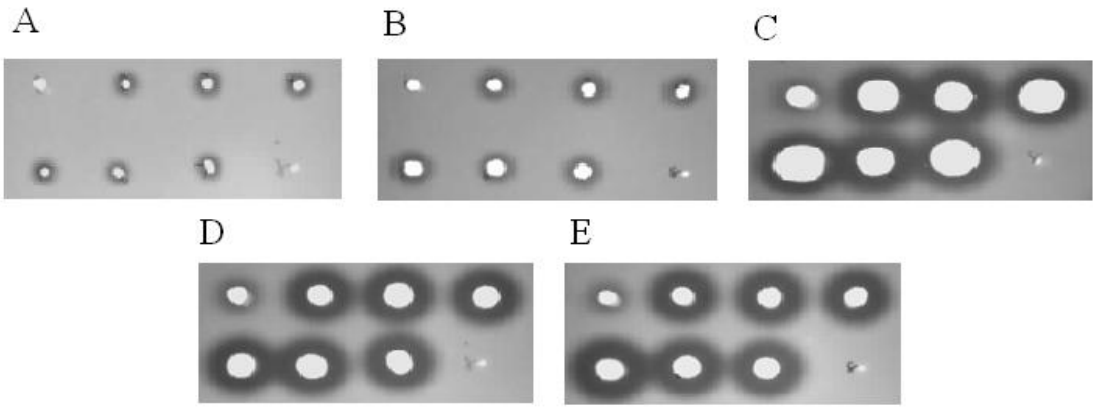

Figure 2. Screening of EtBr degradation activity of isolates by agar plate. Tested isolates were spotted on E-TSA plates and incubated in various conditions. A, 37°C for 6 h; B, 37°C for 9 h; C, 37°C for 24 h; D, 37°C for 6 h followed by room temperature for 18 h; E, 37°C for 9 h followed by room temperature for 15 h. Spot 1 to 8 (left to right and up to down panel, respectively) are *E. coli* O157:H7 EDL933, PSU 8, PSU 9, PSU 10, PSU 11, PSU 12, PSU 13 and *B. subtilis*, respectively.

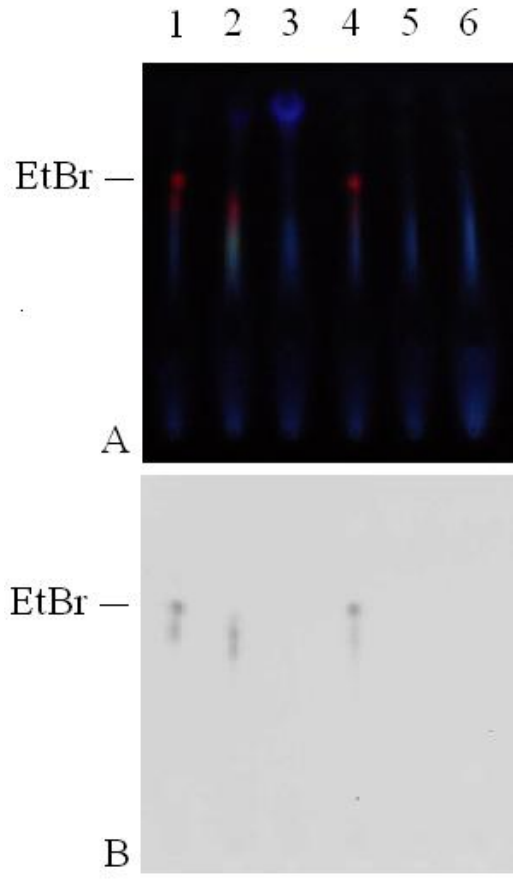

Figure 3. Detection of EtBr degradation using bacterial culture by thin layer chromatography. Lane 1, abiotic control (E-TSB); lane 2, PSU9+E-TSB; lane 3, PSU9 in TSB; lane 4, *Vibrio cholerae* PSU401 + E-TSB; lane 5, *Vibrio cholerae* PSU 401 + TSB; lane 6, TSB. A: Detection of EtBr by long wavelength (365 nm); B: Detection of EtBr by UV-illumination.

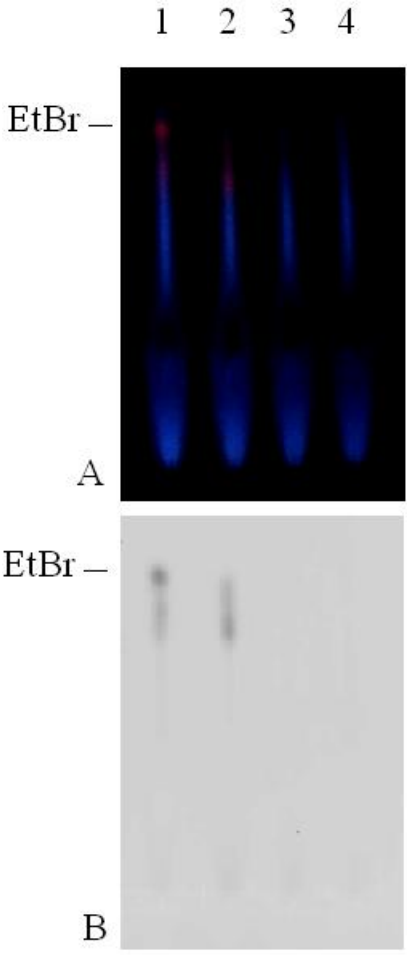

Figure 4. Detection of EtBr degradation by thin layer chromatography using cell-free supernatant. Lane 1, abiotic control (E-TSB); lane 2, PSU9 + E-TSB; lane 3, PSU9 + TSB; lane 4, TSB. A: Detection of EtBr by long wavelength (365 nm); B: Detection of EtBr by UV-illumination.

the bacteria but not RNA content and slightly affected protein biosynthesis (Tomchick and Mandel, 1964). In this study, *E. coli* O157:H7 strain EDL933 was used as a negative control for EtBr degradation and when it was evaluated with bare eyes, it was shown that *E. coli* O157:H7 EDL933 showed only a slightly slower growth rate after 24 h incubation at 37°C on E-TSA as compared to isolates from soil collected in the present study (Figure 2). Moreover, *E. coli* O157:H7 EDL933 also provided a small clear zone of EtBr (Figure 2). *V. cholerae* PSU 401 was also used as an unrelated bacterial species in detection of clear zone of EtBr. It was shown that *V. cholerae* PSU 401 provided the immense clear zone of EtBr (unpublished data). Contrarily, *B. subtilis* isolated in our laboratory was used as a positive control because it belongs to a related species as *B. thuringiensis* PSU9.

Surprisingly, it could not show the sign of growth when the bacterial colony was spotted on E-TSA. Similar phenomenon on the susceptibility of *B. subtilis* to EtBr was documented by a study from Bishop and Brown (1973). They demonstrated the susceptibility of *B. subtilis* Marburg strain to various concentrations of EtBr. It was shown that the growth of Marburg strain in MG medium (Spizizen's minimal supplemented with 0.5% glucose), in the presence of 2.5 µg/ml of EtBr, was completely inhibited. Hence, the ability of the microorganisms in EtBr degradation may depend on individual bacterial isolates.

In this study, although the exact mechanism that bacteria used to degrade EtBr was not unraveled, it has been thought that the possible approach of how *B. thuringiensis* cope with EtBr is the production of substance to destroy EtBr. In TLC experiment, we observed the huge extra dark blue stripe produced by *B. thuringiensis* PSU9 (Figure 3A, lane 3), while *V. cholerae* PSU 401 cultured in the same conditions as PSU9 did not illustrate this extra stripe (Figure 3A, lane 5). In addition, only small amount of dark blue stripe appeared in the presence of EtBr and PSU9 (Figure 3A, lane 2). This may suggest that this dark blue substance might be responsible for EtBr degradation. The production of substance by *B. thuringiensis* PSU9 is independent upon the growth of bacteria. As illustrated in Figure 2, although the bacterial colonies on E-TSA plate were left at room temperature for 15 to 18 h (Figure 2D and E) which showed the smaller colonies as compared to the plate incubated at 37°C for 24 h (Figure 2C), the size of clear zone is comparable.

EtBr-degrading bacteria were screened from soil that has not been contaminated with EtBr. The normal concentration of EtBr used for agarose gel staining in the laboratory is approximately 1 µg/ml. However, in order to concretely obtain the EtBr-degrading bacteria, we used EtBr in a much higher concentration than normal concentration in our screening process (30 µg/ml). Several kinds of bacterial colonies were observed even in the high concentration of EtBr. Some of them were capable of depleting EtBr (Figure 3). It was noted that if we collected bacteria from the EtBr contaminated soil, we would obtain the bacterial species that provide more potency in EtBr depletion than our present collected bacteria.

B. thuringiensis is a bacterium that has been found to produce many substances that are useful in biodegradation and bioremediation (Lin et al., 2012; Dave et al., 2012). In this study, we found that *B. thuringiensis* PSU9 qualitatively decreased EtBr. This may be the promising solution to manage EtBr waste although the quantitative measurement of EtBr has not been performed to gain the results in real application. Furthermore, the strains that provide a greater ability to destroy EtBr may be found in nature. This will be useful for the treatment of small amount of EtBr contamination before releasing into the environment; such as EtBr residues in destaining water used for destaining of EtBr

from agarose gel in the laboratory.

Conclusion

B. thuringiensis PSU9 was isolated from soil and showed the sign of EtBr degradation by clear zone formation on EtBr-supplemented agar plate. The efficient ability of the bacterial isolate in EtBr degradation was observed through thin layer chromatography method. Bacterial cell culture as well as cell-free supernatant of *B. thuringiensis* PSU9 were clearly shown to have activity in EtBr depletion. This might provide a possible solution to manage EtBr waste in the future.

ACKNOWLEDGEMENTS

This work was funded in part by Grant-in-Aid from the Faculty of Science, Prince of Songkla University. We thank Anussara Ratanaburee for technical assistance.

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