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Identification of *Clostridium perfringens* AB&J and Its Uptake of Bromophenol Blue

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Abstract Several microorganisms from rat and human feces and rumen fluid of cows were screened for their ability to decolorize the synthetic dyes. Consequently, a novel dye-degrading strain AB&J was isolated. Taxonomic identification including 16S rDNA sequencing and phylogenetic analysis indicated that the isolate had 99.9% homology in its 16S rDNA base sequence with *Clostridium perfringens*. After 27 h incubation with the strain, brilliant blue R, bromophenol blue, crystal violet, malachite green, methyl green, and methyl orange were decolorized by about 69.3%, 97.7%, 96.3%, 97.9%, 75.1%, and 97.2%, respectively. The triphenylmethane dye, bromophenol blue, was decolorized extensively by growing *Clostridium perfringens* AB&J cells in liquid cultures under anaerobic condition, although their growth was strongly inhibited in the initial stage of incubation. This group of dyes is toxic, depending on the concentration used. The dye was significantly decolorized at a relatively lower concentration of below 50 μg ml^{-1}, however, the growth of the cells was mostly suppressed at a dye concentration of 100 μg ml^{-1}. The decolorization activity in cell-free extracts was much higher in cytoplasm than in periplasm and cytoplasmic membrane. Therefore, the enzyme related uptake of bromophenol blue seemed to be localized in cytoplasm. The optimal pH and temperature of bromophenol blue uptake for decolorization activities were 7.0 and 40°C, respectively.

Key words: Bromophenol blue, *Clostridium perfringens*, decolorization, phylogenetic analysis

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Dyes are released into the environment *via* industrial effluents from two major sources, the textile and dyestuff industries [4]. Synthetic dyes are extensively used for dyeing and printing in industries, and there are more than 100,000 commercially available dyes with over 7×10^5 tons of dye-stuff produced annually [29, 40]. Synthetic dyes can be classified into four types by their chemical structure; Azo, triphenylmethane, heterocyclic, and polymeric dyes [40]. A necessary criterion for the use of these dyes is that they must be highly stable in light and during washing. Therefore, they are not readily degradable. The treatment of spent textile dyeing wastewater by traditional methods has proven to be ineffective for many wastewater treatment facilities. Nevertheless, the antibacterial action of triphenylmethane dyes against various bacterial species and microbial N-demethylation reaction of the dye by *Phanerochaete chrysosporium* have been reported [1, 4]. The decolorization of triphenylmethane dyes including crystal violet by *Bacillus subtilis* [34] and *Nocardia corallina* [35] has been investigated. Kwasniewska [14] demonstrated that *Rhodotorulae* sp. and *Rhodotorulae rubra* had a high biodegradation potential against crystal violet. The ability of *Cyathus bulleri* to decolorize crystal violet, malachite green, and bromophenol blue was also reported [31]. *Aspergillus sojae* B-10 [24] was reported to be biodegradation activity of azo dyes such as Amaranth, Sudan III, and Congo red. On the other hand, various azo dyes were shown to be decolorized by anaerobic sludge [2, 3], anaerobic sediment [32], and by pure cultures of bacteria incubated aerobically [10, 18]. Dye decolorization was also reported in continuous anaerobic reactions with immobilized biomass [39]. The isolation of microorganisms from human intestinal microflora was previously attempted

[23, 26]. Therefore, intestinal microflora is important in the metabolism and enterohepatic circulation of drugs [27]. The bacteria flora of human gastrointestinal tract is complex with 10^{11} to 10^{13} bacteria per g of feces [19]. Any compound taken orally or entering intestine *via* biliary tract, bloodstream, or secretion directly into lumen is a potential substrate for metabolism by the intestinal microflora [6]. In the present work, we isolated microorganism exhibiting decolorization activities on bromophenol blue from rat and human feces as well as from rumen fluid of cows, and identified microorganism which decolorized several dyes anaerobically. In addition, a new potent bacterium, *Clostridium perfringens* AB&J, capable of biodegrading bromophenol blue was reported, and structures to obstruct absorption of bromophenol blue and characteristics of its related decolorization activities were investigated.

MATERIALS AND METHODS

Chemicals and Microorganism

Brilliant blue R, bromophenol blue, crystal violet, malachite green, methyl green, and methyl orange were purchased from Sigma Chem. Co. U.S.A., and used without further purification. Its purity was examined by TLC and confirmed to be homogeneous. Valio Company in Finland kindly provided *E. coli* B44, used as a control for bromophenol blue uptake.

Isolation of Microorganisms to Decolorize Synthetic Dyes

Various samples were collected from different sources, such as rat and human feces and rumen fluid of cow, and screened for dye decolorizing microorganisms. A total of 30 specimens were collected and 0.5 g samples were mixed with 4.5 ml distilled water and then left for 2 h to settle down. The mixed suspensions were diluted up to 10^{-1}–10^{-9}. One ml sample was transferred to plates and BL agar (beef extract 3 g, liver extract 5 g, yeast extract 5 g, proteose peptone 10 g, tryptone 5 g, iso-peptone 0.5 g, glucose 10 g, K_2HPO_4 1 g, KH_2PO_4 1 g, $MgSO_4$ 0.00674 g, L-cysteine HCl-H_2O 0.5 g, $FeSO_4$ 0.01 g, tween 80 1 g, blood 50 ml, and agar 15 g per 1 l) containing 30 g sodium propionate, 0.1g paromomycin sulfate, 0.4 g neomycin sulfate, and 6 g per 100 ml lithium chloride was added. This was incubated anaerobically in the BBL anaerobic jar at 37°C for 48 h. From the plates containing 30–300 colonies, each colony was streaked on a BCP (yeast extract 2.5 g; peptone 5.0 g, glucose 1.0 g, tween 80 1.0 g, L-cysteine 0.1 g, bromocersol purple 0.04 g, and agar 15.0 g per 1 liter) plate to purify it further by single colony isolation. The organisms were selected on the basis of a clear zone on the agar plate. The strains growing on the plates and decolorizing dye were selected and incubated in LB medium (tryptone 10 g, yeast extract 5 g, and NaCl 10 g per 1 liter) containing 200 μg/ml dyes such as brilliant blue R, bromophenol blue, crystal violet, malachite green, methyl green, and methyl orange at 37°C for 48 h in an AnaeroPack system (Mitsubisui Gas Co., Tokyo, Japan).

Determination of Biochemical and Physiological Characteristics of Dye-Decolorizing Microorganism

The morphological and biochemical characteristics were determined, including carbon utilization, optimum pH, and temperature for growth, a hemolysis test, homo-hetero fermentation test, and Gram straining.

Identification of Strain AB&J

A phylogenetic analysis based on 16S rDNA sequence was performed for the identification of strain AB&J. The chromosomal DNA was isolated and purified according to the method described previously [36], and the 16S rDNA was amplified by PCR using two universal primers, as described elsewhere [37]. The PCR product was purified using a QIAquick PCR purification kit (Qiagen). The purified 16S rDNA was sequenced using an ABI PRISM Big Dye Terminator cycle sequencing ready reaction kit (Applied Biosystems). The purified sequencing reaction mixtures were automatically electrophoresed using the Applied Biosystems model 377 automatic DNA sequencer. The alignment of sequences was carried out with the CLUSTAL W software [28]. Gaps at the 5' and 3' ends of the alignment were omitted from further analyses. The evolutionary distance matrices were calculated by using the algorithm of Jukes and Cantor [13] with the DNADIST program within the PHYLIP package [8]. A phylogenetic tree was constructed by using the neighbor-joining method [25] as implemented within the NEIGHBOR program of the same package.

Measurement of Decolorization Activity

The decolorization activity was estimated as follows: 100 μl of strain AB&J grown on BCP broth medium was inoculated into LB medium supplemented with 200 μg/ml concentration of each dye, and then incubation was carried out at 37°C for 27 h in anaerobic jar. The absorbance was measured at 588 nm for brilliant blue R, 590 nm for bromophenol blue, 590 nm for crystal violet, 616 nm for malachite green, 631 nm for methyl green, and 453 nm for methyl orange using a spectrophotometer (Specord 10, Carl Zeiss Technology, Germany). The decolorization activity was calculated as follows [22]:

$$\text{Decolorization activity (\%)} = 100 - (\text{O.D. value after treatment} / \text{O.D. value before treatment}) \times 100$$

Culture Conditions and Preparation of Cell-Free Extracts
The strains used for this study were precultured in LB medium containing tryptone 10 g; yeast extract 5 g; NaCl 5 g; deionized water 1 liter. The preculture was incubated at 37°C and pH 7.0 for 10 h in LB medium and then 1% of precultured cells were put into Erlenmeyer flask containing the same medium under the same condition for 24 h. The cells were harvested by centrifuging at 5,000 rpm for 30 min and then suspended in 10 mM phosphate-citrate buffer (pH 7.0). The bacterium was homogenized with a pestle and mortar for 40 min at 4°C. The cellular debris was removed by centrifugation (15,000 rpm for 2 h at 4°C). Acetone was added at 10% (v/v) to the clear supernatant (crude extract) and the mixture was used immediately for the assay of decolorization activity. Cell-free extracts for experiment of bromophenol blue uptake into cell were prepared by the method of Gustafsson *et al.* [9]. The cells were suspended in the same buffer at approximately 1.3 O.D. at 660 nm and then the cell suspensions were treated with 10 mM EDTA or lysozyme (400 μg ml^{-1}) at 37°C for 10 min. After the reaction, the cells were centrifuged at 5,000 rpm at 4°C and then resuspended in 10 mM phosphate-citrate buffer (pH 7.0) after treatment with EDTA. The spheroplast was stored in the same buffer containing 30% sucrose. Absorbance of the prepared cell suspensions was adjusted to 1.3 O.D. at 660 nm.

Preparation of Periplasm
Periplasm fraction was prepared by the method of van der Western *et al.* [30]. The bacterial cells were suspended in 10 mM phosphate-citrate buffer (pH 9.0) containing 50 mM EDTA and the suspension was incubated for 20 min at 37°C. Supernatants as periplasm fractions were obtained by centrifuging the cells at 15,000 rpm for 30 min under 4°C.

Cell Fractionation
Cell-free extracts and membrane fraction were separated according to the method of Niviere *et al.* [20]. After periplasm fraction was separated, the harvested cells were homogenized with a pestle and mortar in 10 mM phosphate-citrate buffer (pH 7.0) for 40 min at 4°C. The homogenates were centrifuged for 30 min (15,000 rpm, 4°C), and the supernatants were further centrifuged at 150,000 rpm for 5 h to prepare cytoplasmic fraction. The cell paste suspended in the same buffer was used as cytoplasmic membrane fraction.

Effects of Environmental Factors on Bromophenol Blue Decolorization
The decolorization of bromophenol blue by cell-free extracts of *Clostridium perfringens* AB&J was examined at different temperatures, pH's, and concentrations of the dye. The cell-free extracts in 10 mM phosphate-citrate buffer (7.0) containing 60 μM (25 μg ml^{-1}) bromophenol blue were incubated at different temperatures and pH values. The samples taken from each mixture were then examined for decolorization activities on bromophenol blue. The effect of concentration of bromophenol blue on its decolorization was also tested at 37°C and pH 7.0

Assay of Bromophenol Blue Uptake
Bromophenol blue up taken into cells was assayed by the method of Gustafsson *et al.* [9]. The cell suspension was incubated at 37°C for 30 min with the same buffer containing 10 μg ml^{-1} bromophenol blue. Bromophenol blue concentrations of the supernatant and cell pellet were determined after 30 min incubation. The remaining dye concentrations in the supernatant obtained by re-centrifugation (5,000 rpm, 30 min) were measured with a spectrophotometer. The pellets were washed twice with 0.85% NaCl, re-suspended in 10 mM phosphate-citrate buffer (pH 9.0) containing 50 mM EDTA, and the suspension was incubated for 1 h at 37°C. The incubated cells were disrupted by sonication for 20 min at 4°C, and then centrifuged under the same conditions described above. The concentration of bromophenol blue extruded from intact cells into the supernatant was determined by a spectrophotometer. *E. coli* B44 was treated with the same procedure used for a control. The uptake ratio of bromophenol blue was calculated as follows:

Uptake ratio (%)
=[(Initial concentration of bromophenol blue)
-(Concentration of bromophenol blue after reaction for 30 min)/
(Initial concentration of bromophenol blue)]×100

Measurement of Decolorization Activity on Bromophenol Blue
Decolorization activities were assayed by measuring the amounts of reduced bromophenol blue after the cell-free extract was incubated with the dye at 37°C for a certain period of time. Decrease of absorbance was measured at 590 nm after the reaction. Enzyme activity to reduce 1.25 μM bromophenol blue min^{-1} was defined as one unit. Thus, reaction mixture containing 890 μl of 10 mM phosphate-citrate buffer (pH 7.0), 10 μg of bromophenol blue, and 100 μl of cell-free extracts was made up to 1,000 μl. To find the optimal temperature to decolorize bromophenol blue, decolorization activities were measured after reaction at 4, 37, 60, and 70°C for 60 min. To find the optimal pH to decolorize bromophenol blue, 50 mM phosphate-citrate buffer was used for pHs 5–8, and 50 mM phosphate-glycerin buffer for pHs 9 and 10. The concentration of protein was determined by the method of Bradford [5] with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Isolation and Characterization of Strain AB&J

A number of bacteria were isolated from rat and human feces, and rumen fluid of cow. Only nine isolates exhibited an ability to decolorize various dyes on the agar plates, and among them, strain AB&J was the strongest in decolorizing several dyes. The characteristics of strain AB&J are shown in Table 1. The strain AB&J was found to be very similar to *Clostridium* sp. in its biochemical and physiological characteristics. Decolorization of triphenylmethane groups of dye including crystal violet by *Bacillus subtilis* [11] and *Nocardia corallina* [12] was investigated. Kwasniewska [14] demonstrated that *Rhodotorulae* sp. and *Rhodotorulae rubra* have a high biodegradation potential against crystal violet. The ability of *Cyathus bulleri* to decolorize crystal violet, malachite green, and bromophenol blue was also reported [31]. There have been some reports on the biodegradation of azo and triphenylmethane dyes by fungi such as *Trametes* sp. [21], *Phanerochaete chrysosporium*, [7, 13, 15, 26], and *Aspergillus sojae* [24].

Phylogenetic Analysis of Strain AB&J Based on Its 16S rDNA Sequence

The 16S rDNA sequence of strain AB&J was analyzed to determine which species of the strain matched with the highest homology in the GenBank. The phylogenetic analysis based on its 16S rDNA sequences showed that the strain AB&J fell within the evolutionary radiation comprising *Clostridium* species (Fig. 1). The sequence data were aligned to construct a phylogenetic tree, and the phylogenetic position of strain AB&J was then compared with certain *Clostridium* species and related taxa in a dendrogram. In the phylogenetic tree, strain AB&J was closest to *C. perfringens* ATCC 13124 and part of a robust monophyletic cluster with *C. barati* ATCC 27638 and *C. absonum* DSM 599 (Fig. 1). The level of sequence similarity of strain AB&J within the monophyletic cluster was greater than 94.4% (Table 2). In particular strain AB&J showed high degree of relation to the type strain of *Clostridium perfringens*, sharing 99.9% 16S rDNA similarity.

Table 1. Biochemical and physiological characteristics of strain AB&J.

Cell type	Rod
Gram staining	Positive
Biological characteristics	
Catalase	Negative
Hemolysis	Negative
Motility	Negative
Homo-hetero fermentation	Hetero
Carbon source utilization	
Amygdalin, arabinose, esculin, fructose, galatose, glucose, lactose, maltose, mannitol, mannose, melezitose, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, sucrose, trehalose, xylose	Positive
Optimum pH for growth	6.0
Growth at 4	-
Growth at 5	+
Growth at 7	+
Growth at 8	+
Growth at 9	+
Growth at 10	+
Optimum temperature for growth (°C)	37°C
Growth at 5	-
Growth at 15	+
Growth at 40	+
Growth at 50	+
Growth at 60	-

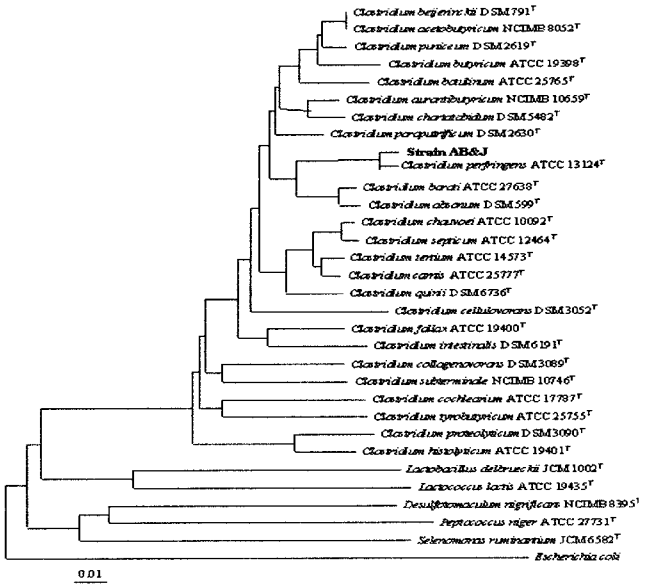

Fig. 1. Phylogenetic tree based on 16S rDNA sequences showing the positions of strain AB&J, some *Clostridium* species, and the representatives of some other related taxa.
Scale bar represents 0.01 substitutions per nucleotide position.

Change of Decolorization Activity of Various Dyes

The decolorization of various dyes by strain AB&J is shown in Fig. 2. Bromophenol blue, malachite green, and crystal violet, were decolorized most rapidly after 9 h of incubation. The decolorization for bromophenol blue, crystal violet, malachite green, methyl green, and methyl orange were 97.7%, 96.3%, 97.9%, 75.1%, and 97.2%, respectively. Kim and Yoon [15] reported that *Streptomyces* sp. exhibited the highest decolorizing rate of 72.9% for alizarin sodium sulfonate into heterocyclic dye and the lowest 14.6% for crystal violet for 20 days. According to Yoon and Choi [38], when *Iupex zonatus* BN2 was incubated with each dye for 10 days, the decolorization rates of azo, triphenylmethane, and heterocyclic dyes were over 94.0%. Hence, *Clostridium*

Table 2. Level of 16S rDNA similarity between strain AB&J, type strains of certain *Clostridium* species, and representatives from other related taxa.

Strain	% of Similarity:																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1 **Strain AB&J**																		
2 *Clostridium perfringens* ATCC 13124^T	99.9																	
3 *Clostridium barati* ATCC^T	95.2	95.0																
4 *Clostridium absonum* DSM 599^T	94.8	94.4	98.9															
5 *Clostridium butyricum* ATCC 19398^T	93.0	92.8	94.3	94.2														
6 *Clostridium acetobutyricum* NCIMB 8052^T	93.9	93.8	95.9	95.6	97.2													
7 *Clostridium aurantibutyricum* NCIMB 10659^T	93.0	92.9	95.4	95.3	94.8	96.4												
8 *Clostridium paraputrificum* DSM 2630^T	94.1	93.7	96.1	96.4	95.4	96.3	96.4											
9 *Clostridium csrnis* ATCC 25777^T	93.9	94.0	95.9	95.9	94.4	95.4	96.3	97.0										
10 *Clostridium cellulovorans* DSM 3052^T	91.5	91.3	92.9	93.3	93.1	93.2	95.4	95.8	93.3									
11 *Clostridium septicum* ATCC 12464^T	93.3	93.3	95.4	94.8	93.8	94.5	93.2	93.9	98.3	92.5								
12 *Clostridium intestinalis* DSM 6191^T	92.4	92.3	92.1	92.1	91.9	92.9	94.5	94.8	93.2	91.9	93.5							
13 *Clostridium cochlearium* ATCC 17787^T	90.6	90.6	90.7	91.2	90.7	91.2	92.9	93.3	90.5	91.2	90.3	90.3						
14 *Clostridium histohlyticum* ATCC 19401^T	91.0	91.0	91.0	91.2	90.8	92.0	91.2	90.8	89.8	91.1	90.0	90.6	90.7					
15 *Lactobacillus delbrueckii* JCM 1002^T	83.3	83.0	83.6	83.5	82.3	82.7	92.0	90.8	83.5	82.8	83.6	83.0	82.2	83.0				
16 *Lactococcus lactis* ATCC 19435^T	81.7	81.9	83.3	83.7	82.6	82.5	82.7	83.6	83.7	82.7	83.0	82.1	81.1	83.4	85.6			
17 *Desulfotomoculum nigrificans* NCIMB 8395^T	82.2	82.3	82.5	82.5	83.2	83.0	82.5	83.3	82.7	80.8	83.7	82.6	81.9	82.9	82.0	82.0		
18 *Peptococcus niger* ATCC 27731^T	81.3	81.1	81.7	81.7	82.2	82.2	83.0	83.0	82.5	80.2	82.3	83.5	82.3	83.2	80.9	80.1	83.7	
19 *Escherichia coli*	77.5	77.5	78.3	78.3	77.8	77.2	82.2	82.7	77.7	78.7	78.4	78.8	77.3	77.2	77.9	77.2	78.0	78.1

^{T}Type strain.

perfringens AB&J showed higher decolorization abilities over those of *Streptomyces* strains and *I. zonatus* BN2.

Effect of Environmental Factors on Decolorization Activity of *Clostridium perfringens* AB&J

The effects of certain environmental factors on decolorization of bromophenol blue were examined and the results are showed in Table 3. In order to determine the relationship between decolorization activities and the concentration of bromophenol blue, degradation activities of the dye by cell-free extracts of *C. perfringens* AB&J which had been cultivated in 10 mM phosphate-citrate buffer (pH 7.0) containing 10, 25, 50, 100, and 250 μg ml^{-1} bromophenol blue were measured. Decolorization activities were strongly inhibited by higher (100 and 250 μg ml^{-1}) concentrations

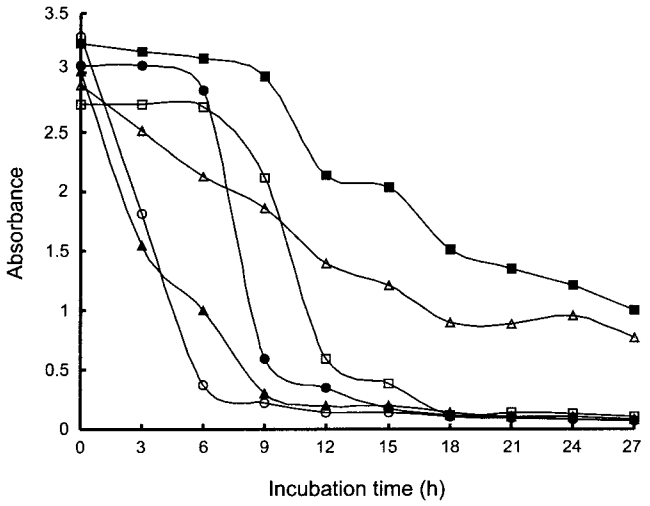

Fig. 2. Decolorization of various dyes by strain AB&J at 37°C. Symbols are (●) Bromophenol blue; (■) Brilliant blue R; (▲) Crystal violet; (○) Malachite green; (△), Methyl green; (□) Methyl orange.

Table 3. Effects of environmental factors on the degradation of bromophenol blue by cell-free extract of *Clostridium perfringens* AB&J incubated in 10 mM phosphate-citrate buffer for 48 h.

Environmental factor	Degradation (%)
Temperature (°C)	
20	28
30	42
40	96
50	86
60	36
70	3
pH	
5	25
6	62
7	95
8	48
9	31
10	19
Concentration of dye (μg ml^{-1})	
10	100
20	100
50	76
100	26
250	7

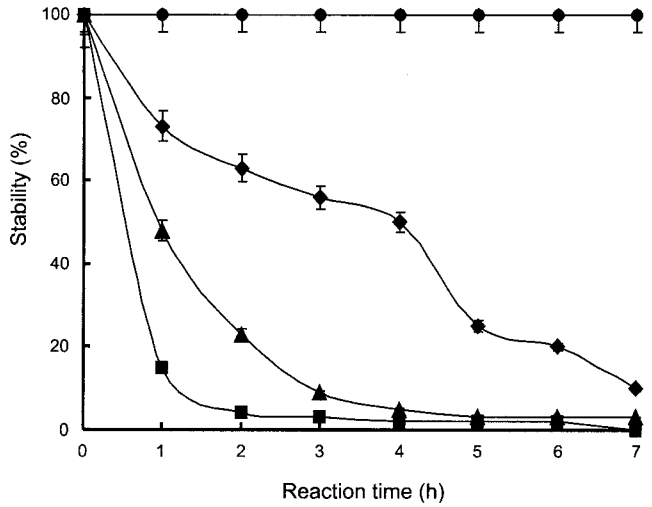

Fig. 3. Stabilities of decolorization activity on bromophenol blue by cell-free extracts of *Clostridium perfringens* AB&J.
Cell-free extracts were incubated at various temperatures in 10 mM phosphate-citrate buffer (pH 7.0) and the remaining activities were determined. Symbols are (●) 4°C; (◆) 37°C; (▲) 60°C; (■) 70°C.

of the dye (Table 3). The growth and decolorization activities of *Nocardia corallina* were reported to be completely inhibited in 7 μM of dye concentration [12], *Bacillus subtilis* in 10–20 μM [11], and *Pseudomonas pseudomallei* 13NA in 50 μM [33]. It seemed that toxicity of triphenylmethane dye strongly suppressed enzymatic activities and also inhibited synthesis of proteins. Decolorization of bromophenol blue by cell-free extract reached about 90% in 24 h incubation at lower concentration of bromophenol blue. To determine the stability of decolorization activities of bromophenol blue, the cell-free extract was reacted at different temperatures and the results are shown in Fig. 3. Stability of decolorization activities was maintained 100% at 4°C for 7 h. At 37°C, it was generally reduced, and most of the enzyme were inactivated after 7 h. When incubated at 70 and 60°C, however, the decolorization activities completely vanished after one and three hours of incubation, respectively. To investigate the optimal temperature and pH for decolorization activities on bromophenol blue, the cell-free extracts were treated at various temperatures and pH values for 48 h in 10 mM phosphate-citrate buffer. As shown in Table 3, *C. perfringens* AB&J was found to have the highest degradability of bromophenol blue at 40°C and pH 7.

Uptake of Bromophenol Blue by *C. perfringens* AB&J

The uptake ratio of bromophenol blue by the cell suspensions of *E. coli* B44 and *C. perfringens* AB&J are shown in Table 4: 1.36 μg ml^{-1} concentration of the dye was taken up into the *E. coli* B44 cell but only 0.04 μg ml^{-1} of the dye was degraded in the cell and 8.64 μg ml^{-1} remained in the supernatant. *C. perfringens* AB&J took up 5.93 μg ml^{-1} of the dye and degraded almost all of it, and only 0.83 μg ml^{-1} was in the cell and 4.07 μg ml^{-1} was in the supernatant after reaction. The uptake ratio of *C. perfringens* AB&J was 59.3% and it was four times that of *E. coli* B44. Although negatively-charged compounds of the cell surface of Gram-negative bacteria *E. coli* B44 easily cohere with bromophenol blue, immoderate bromophenol blue usually was reported to inhibit the cohesion [16].

Table 4. Comparison of bromophenol blue uptake by *E. coli* B44 and *Clostridium perfringens* AB&J.

Strain	Bromophenol blue concentration (μg ml^{-1})			Uptake ratio (%)
	Supernatant	Pellet	Total	
E. coli B44	8.64	1.32	9.96	13.6
C. perfringens AB&J	4.07	0.83	4.90	59.3

Cells were (1×10^8 cell ml^{-1}) suspended in 10 ml of 10 mM phosphate-citrate buffer (pH 7.0) containing 1 μg/ml bromophenol blue and incubated at 37°C for 30 min. Bromophenol blue concentrations of the supernatant and cell pellet were determined after 30 min uptake to the cells. The uptake of bromophenol blue into intact cells was measured. The uptake ratio of bromophenol blue was calculated as follows: Uptake ratio (%) [(Initial concentration of bromophenol blue)−(Concentration of bromophenol blue after treatment of strain)/(Initial concentration of bromophenol blue)]×100.

In this study, *C. perfringens* AB&J was found to be Gram-positive bacteria, and differences of uptake ratio between *E. coli* B44 and *C. perfringens* AB&J were due to the carbohydrate content of the cell surfaces. Despite that 5.93 μg ml^{-1} bromophenol blue was taken up, only 0.83 μg ml^{-1} dye was in the cells after reaction, due to enzymatic degradation of bromophenol blue in the cells. Whereas *E. coli* B44 had no degradability of the dye and 99.6% was not degraded, 59.3% of dye was taken up and only 0.9% survived in the cell of *C. perfringens* AB&J. In the case of *C. perfringens* AB&J, almost all of the bromophenol blue taken up into the cell were subjected to cleavage by enzymes.

Uptake Characters of Bromophenol Blue

Influence of outer membrane and peptidoglycan on the uptake of the dye into the cells was tested in 10 mM phosphate-citrate buffer (pH 7.0) containing 10 μg ml^{-1} bromophenol blue (Fig. 4). Uptake ratio of bromophenol blue by the cell whose outer membrane was removed with EDTA reached 58% in 40 min after reaction, and this procedure was thought to prevent absorption of the dye into the cell. Furthermore, 98% of bromophenol blue was taken up into the cell, whose spheroplast was treated with EDTA and lysozyme, indicating that peptidoglycan and outer membrane interfered with absorption of bromophenol blue. Therefore, bromophenol blue was first absorbed into the cell and then degraded by the enzyme inside of the cell. Since the uptake ratio of the dye into the cell was markedly increased when the spheroplast of *C. perfringens* AB&J

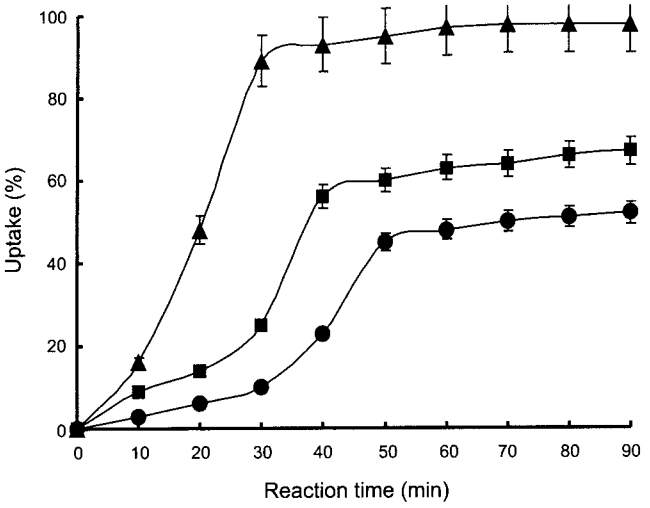

Fig. 4. Uptake of bromophenol blue by *Clostridium perfringens* AB&J treated with EDTA or lysozyme.
At zero time, 25 μg ml^{-1} of bromophenol blue was added in culture. Intact cell suspensions and the cells treated with EDTA, or EDTA and lysozyme, were incubated at 37°C. Symbols are (●) intact cells; (■) EDTA; (▲) EDTA+lysozyme.

was treated, the structures to obstruct absorption of the triphenylmethane group dye were considered to be outer membrane and peptidoglycan.

Cellular Localization of the Enzyme Related to Bromophenol Blue Uptake

Cell culture fraction and cell-free extract of *C. perfringens* AB&J were treated with bromophenol blue to calculate dye decolorization activities. Cell-free extract had 1.98 units mg^{-1} decolorization activities on bromophenol blue, while cell culture fraction had not (Table 5). Yatome *et al.* [35] found that cell suspension of *Nocardia corallina* was able to degrade triphenylmethane and suggested that the enzyme for degradation of triphenylmethane was located in the cell of *Bacillus subtilis* [34]. In this study, the enzyme to degrade bromophenol blue was found inside of the cell.

Decolorization activities of bromophenol blue by periplasm, cytoplasm, and cytoplasimic membrane were tested to identify which organelle had the decolorization activities against the dye (Table 6). The cytoplasm had higher specific

Table 5. Degradation activity on bromophenol blue by cell culture filtrates and cell-free extracts of *Clostridium perfringens* AB&J.

Cell compartments	Decolorization activity (units)	Protein concentration (mg ml^{-1})	Specific activity (units mg^{-1})
Cell filtrates	0.00	0.02	0.00
Cell-free extracts	10.11	5.10	1.98

Table 6. Comparison of decolorization activities on bromophenol blue by cell fractions of *Clostridium perfringens* AB&J.

Cell fractions	Decolorization activity (units)	Protein concentration (mg ml^{-1})	Specific activity (units mg^{-1})
Periplasm	0.36	3.81	0.09
Cytoplasm	11.72	30.85	0.38
Cytoplasmic membrane	0.25	4.84	0.05

activity (0.38 units mg^{-1}) than cytoplasmic membrane (0.05 units mg^{-1}) and periplasm fraction (0.09 units mg^{-1}). Periplasm fraction showed 0.09 units mg^{-1} specific activity, but protein contents and enzymatic decolorization activity were markedly lower than others. It seemed that the periplasm fraction was contaminated with proteins from the cytoplasm fraction, when the cell was treated with EDTA. Cytoplasmic membrane fraction exhibiting low decolorization activity was also suspected to be contaminated during ultra-centrifugation. Yatome *et al.* [35] reported that when the supernatant of *Norcardia corallina* was treated with triphenylmethane dye, the decolorization activity appeared. *Pseudomonas pseudomallei* [33] degraded the triphenylmethane group of dyes, however, the location of enzymes responsible for degradation was not mentioned. Jeong *et al.* [12] showed that supernatants of *Enterobacter cloacae* decolorized triphenylmethane dye. McDonald *et al.* [17] reported that triphenylmethane demethylase was present in microsome, and Yatome *et al.* [34] provided evidence that Gram-positive bacteria have triphenylmethane demethylase, whereas Gram-negative bacteria have not. In the case of *B. subtilis* that is Gram-positive, demethylase is in the cytoplasm. In the present study, Gram-positive *C. perfringens* AB&J was shown to have decolorization activity of the triphenylmethane group of dyes and demethylase to degrade bromophenol blue was found to be in the cytoplasmic compartment.

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