solution was added, and the sawdust was agitated for 1 minute. An aliquot of sawdust was sampled and the target microorganism extracted (see below). The temperature of the sawdust was maintained in an incubator. The concentration of microorganisms in the sawdust was measured by extraction at several time periods. Each experiment was replicated twice.

In order to measure the concentration of *E. coli* in the composting toilet sawdust, microorganisms were extracted from the sawdust using a 3% (w/v) beef extraction solution (Otaki *et al.*, 2002). The beef extract (MP Biomedicals, Japan) was dissolved in deionized water, adjusted to pH9.5 with NaOH, and then sterilized. A weighed sample of sawdust (0.4 g) was added to a 20-mL volume of extraction solution and agitated for 3 minutes to extract the microorganisms. It was reported that the percentage recoveries of *E. coli* obtained using this method were 70 to 100% (Otaki *et al.*, 2002). In the present study, the recoveries of *E. coli* were confirmed by measuring the concentrations of *E. coli* in the stock solution and initial concentration in sawdust at 0 hr in every experiment. They were approximately 70% and stable in every experiment.

A phosphate buffer solution was used to dilute the extract to a suitable concentration for measuring microorganisms. For PCR and PMA cross-linking (see below), 10-fold diluted extraction solutions were used as samples in order to reduce the pH effect and materials from the extraction solutions.

Measurement of *E. coli* using three kinds of media

The concentrations of *E. coli* in the samples were measured in triplicate by the double agar layer method using TSA (Difco) and DESO (Desoxycholate Agar, Eiken Chemical Co., Japan), and C-EC (Compact Dry EC, Nissui Pharmaceutical Co., Japan). Compact Dry EC contained X-Gluc and Magenta-GAL.

Measurement of *E. coli* using PCR

Two hundred μL of samples with or without PMA cross-linking (see below) were centrifuged at 14,000 rpm for 5 min, and the supernatants were changed to PBS before DNA extraction. Deoxyribonucleic acid was extracted using a QIAamp DNA mini kit (Qiagen, USA). For real-time PCR, the 25-μL reaction mixture contained 5 μL of sample, 12.5 μL of 2×TaqMan Gene Expression Master Mix (Applied Biosystems, USA), forward and reverse primer (with a final concentration of 1 μM, Invitrogen), TaqMan MGB Probe with a final concentration of 200 nM and distilled water. The PCR cycling conditions were 2 min at 50°C and 10 min at 95°C, and 40 cycles of 15 sec at 95°C and 1 min at 60°C using an ABI 7300 Real-Time PCR System (Applied Biosystems). The PCR target region was the *tnaA* gene started at 464, which generated specific amplicons of 136 base pairs. The primer and the probe were 5'-GGG GCG GTG ACG CAG-3' (forward), 5'-CCT GGT GAG TCG GAA TGG TG-3' (reverse), and 5'-FAM-CGA TGA TGC GCG GCG-MGB-3', respectively (Bernasconi *et al.*, 2007). Every sample was measured in triplicate.

Investigation of the PMA cross-linking condition

Propidium monoazide was dissolved in distilled water at 10 mg/mL as the stock solution, and stored at –20°C in the dark. The stock solution was appropriately diluted for PMA cross-linking in every experiment. The PMA solution was added to the sample

and mixed. This was incubated in the dark, and then exposed to light using a 650 W halogen light source (tungsten halogen lamps 650 W, 100 V; Selecon Pacific MSR575W, Philips, Netherlands). The sample tubes were laid horizontally in cool water to avoid excessive heating.

At first, we investigated the appropriate PMA cross-linking condition (concentration of PMA solution, incubation time and light exposure time) for both PMA-PCR and PMA-TSA. In this study, the maximum concentrations of *E. coli* samples for PMA cross-linking were approximately 10^5 CFU/mL. Therefore, we confirmed that the condition made links between PMA and DNA, and that no DNA was detected by PCR. For the confirmation, the naked DNA of 10^5 CFU/mL *E. coli* (in PBS) extracted using a QIAamp DNA mini kit (Qiagen) was measured by PCR after PMA cross-linking. In addition, in order to confirm that the condition did not inactivate intact *E. coli*, 1.1×10^5 CFU/mL and 9.5×10^2 CFU/mL of *E. coli* after PMA cross-linking were measured by the double agar layer method using TSA (Difco).

Investigation of PMA cross-linking for *E. coli* after UV treatment

Escherichia coli after UV treatment can photoreactivate with exposure to visible light. A wavelength around 380 nm was reported to cause photoreactivation (Takebe, 1983; Kamiko and Ohgaki, 1989), and the wavelength range of the halogen light used in this study covered it. Figure 1 shows the transparent spectrum of yellow cellophane (110500, TOYO Co., Japan). The yellow cellophane absorbs wavelengths around 380 nm and is considered to inhibit photoreactivation. Therefore, tubes containing UV-treated (see *UV treatment*) samples were covered with this yellow cellophane and exposed to light. In order to confirm the effect, the *E. coli* concentrations of samples with or without yellow cellophane were measured by the double agar layer method using TSA (Difco). However, there is a possibility that the yellow cellophane may inhibit PMA cross-linking. Therefore, PMA cross-linking with yellow cellophane was carried out for DNA extracted from 200 μL of 10^5 CFU/mL *E. coli* solution.

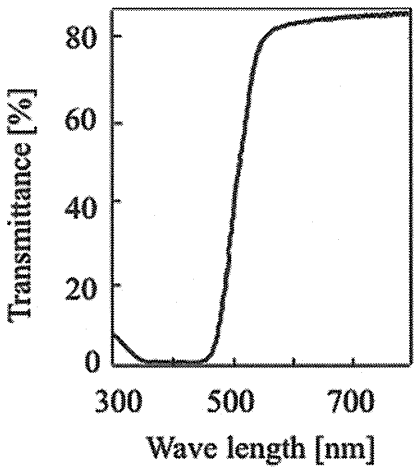

Fig. 1 - Transparent spectrum of yellow cellophane.

Estimation of the damage to *E. coli*

We simultaneously used six different methods, three different kinds of media, TSA media with PMA cross-linking (PMA-TSA), PCR, and PCR with PMA cross-linking (PMA-PCR).

According to the detection principles of the three kinds of media, which were TSA, DESO and C-EC, damage to *E. coli* can be assumed (Kubo and Otaki, 2005, 2006; Wang and Otaki, 2007; Mizozoe *et al.*, 2010; Kazama and Otaki, 2011, 2012). *Escherichia coli* is capable of metabolizing proteins and growth can be detected by TSA. Therefore, when *E. coli* could not be detected by TSA, it was assumed that its nucleic acid and/or metabolic function were damaged. Desoxycholate Agar, a selective agar, can detect *E. coli* that can grow by metabolizing lactose in the presence of desoxycholic acid. Gram-positive bacteria are unable to grow in the presence of desoxycholic acid because they lack an outer membrane and their growth is inhibited by its surface-active effects. Therefore, when *E. coli* cannot be detected by DESO, this indicates that its outer membrane and/or nucleic acid and/or metabolic function have been damaged. Compact Dry EC, also a selective agar, can detect *E. coli* that can produce ß-glucuronidase, which is the enzyme involved in the metabolism of peptone, pyruvic acid, and lactose. Therefore, when *E. coli* cannot be detected by C-EC, it is assumed that its enzyme activity and/or nucleic acid and/or metabolic function have been damaged. By comparing the detected concentration on each medium, the damage to *E. coli* could be estimated, as shown in Table 1.

In this research, PMA-TSA was used as a newly developed method to confirm the outer membrane damage. When *E. coli* cannot be detected by PMA-TSA, it is assumed that its outer membrane and/or nucleic acid and/or metabolic function have been damaged. Therefore, if *E. coli* can be detected by TSA, but not by PMA-TSA, it indicates that only its outer membrane has been damaged.

However, investigation by the three kinds of media and PMA-TSA could not distinguish whether the DNA was damaged. Therefore, the distinction of DNA damage was investigated by PCR. Polymerase chain reaction was also used with PMA-PCR to confirm whether the outer membranes were damaged or not. By comparing the detected concentration by each method, the damage to *E. coli* could be estimated, as shown in Table 2.

Table 1 - Estimated parts of *E. coli* damaged according to the detection differences among the three kinds of media (Kazama and Otaki, 2012).

Detections by media*				Estimated parts
TSA	DESO	C-EC		which were damaged
×	×	×	→	Nucleic acid and/or metabolism (and also enzyme activity and outer membrane)
○	○	×	→	Enzyme activity
○	×	○	→	Outer membrane
○	×	×	→	Enzyme activity and outer membrane

*○, detected; ×, not detected.

Table 2 - Estimated parts of *E. coli* damaged according to the detection differences among the methods (Kazama and Otaki, 2012).

Detection by methods*			Estimated parts which were damaged
TSA	PMA-TSA		
×	×	→	Nucleic acid and/or metabolism (and also enzyme activity and outer membrane)
○	×	→	Outer membrane

Detection by methods*			Estimated parts which were damaged
PCR	PMA-PCR		
×	×	→	Nucleic acid (and also outer membrane)
○	×	→	Outer membrane

*○, detected; ×, not detected.

RESULTS AND DISCUSSION

Investigation of the PMA cross-linking condition

As a result of our investigation, it was proper to add 1 μL of 1 mg/mL PMA solution to 200 μL of sample, incubate the sample mixed PMA for 3 min in the dark, and then expose it to a light source at a distance of 15 cm for 5 min.

DNA extracted from 10^5 CFU/mL *E. coli* was not detected by PCR after PMA cross-linking, and the concentrations of intact *E. coli* after PMA cross-linking were not significantly decreased (initial concentration: 1.1×10^5 CFU/mL or 9.5×10^2 CFU/mL, concentration after PMA cross-linking: 1.1×10^5 CFU/mL or 8.6×10^2 CFU/mL). Therefore, it was assumed that this PMA cross-linking condition was sufficient to inactivate *E. coli* with compromised membranes, but not *E. coli* with intact membranes.

PMA cross-linking for *E. coli* after UV treatment

According to the above result, UV-treated *E. coli* with or without yellow cellophane were placed about 15 cm from the light source and exposed to light for 5 min. They photoreactivated in the condition without yellow cellophane (Fig. 2(a)), but did not photoreactivate with it (Fig. 2(b)).

For the inhibition of yellow cellophane for PMA cross-linking, 2.5% of *E. coli* (10^5 CFU/mL) could be detected by PCR after PMA cross-linking with yellow cellophane. It was considered that the wavelength of 464 nm was the most effective for PMA-cross linking (Biotium Inc., 2010), and this yellow cellophane absorbed it. The light exposure time was set longer (7 or 10 min), but it was not effective for PMA cross-linking. Therefore, the same PMA cross-linking condition (retention time: 3 min, light exposure: 5 min) but with yellow cellophane was used for both PMA-TSA and PMA-PCR after UV treatment. If UV treatment causes outer membrane damage, we can see an approximate 2-log reduction using this condition.

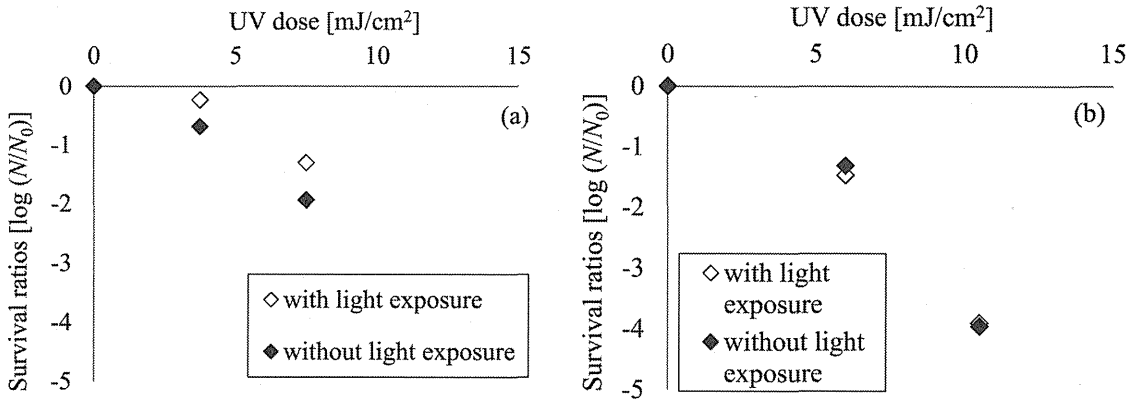

Fig. 2 - Change of the survival ratios of *E. coli* treated by UV with or without light exposure.
(a) without yellow cellophane; (b) with yellow cellophane.

Results of the disinfection experiments

Figure 3 shows changes in the survival ratios of *E. coli* by UV treatment, Cl treatment, and sawdust treatment as determined by three kinds of media, PMA-TSA, PCR and PMA-PCR. For the results of Cl treatment, the Ct (chlorine concentration × exposure time) values were different in every experiment and therefore all data were plotted.

Damage to E. coli by UV treatment

In Fig. 3(a), no significant differences were observed in the inactivation rate by any of the three media. Therefore, inactivation was assumed to be mainly due to damage to nucleic acids and/or metabolic functions because *E. coli* could not be detected by any of the three media, as shown in Table 1. If nucleic acids were damaged, *E. coli* could not be detected by both PCR and PMA-PCR, as shown in Table 2. However, no significant decrease was observed with the results under this UV dose condition. Therefore, it could not clarify that *E. coli* caused damage to DNA, and not to the outer membrane. When comparing the inactivation rates by TSA and PMA-TSA, no significant differences were observed. This indicated that *E. coli* did not damage the outer membrane. And these methods could evaluate the inactivation rate by UV treatment, though PCR and PMA-PCR could not.

Damage to E. coli by chlorine treatment

In Fig. 3(b), the inactivation rate by TSA was lower than the rates for the other two media, which indicated that chlorine treatment caused more significant damage to enzyme activity and/or outer membrane than to nucleic acids and/or metabolic functions, as shown in Table 1. Differences were also observed when the inactivation rate by TSA was compared to that by PMA-TSA. Therefore, chlorine treatment resulted primarily in damage to the outer membrane as shown in Table 2. *Escherichia coli* with damage to the outer membrane had the potential to grow because they could be detected by TSA. However, no significant difference was observed between the inactivation rate less than C × T 1.8 by PCR and the inactivation rate by PMA-PCR, which indicated that *E. coli* had no outer membrane damage. It was considered that because chlorine damage to the outer membrane was slight (Cho *et al.*, 2010), cell penetration of PMA was insufficient

to inhibit amplification of the target gene. However, PMA could bind to other gene areas and inhibit *E. coli* from growing on TSA. Then, a difference was observed between the inactivation rate by TSA and that by PMA-TSA. In short, using PCR and PMA-PCR could show outer membrane damage, but the inactivation rates could not be evaluated. By using TSA and PMA-TSA, it was considered possible to distinguish *E. coli* with damaged to the outer membrane sensitively and quantitatively.

Damage to E. coli by sawdust treatment

According to the results, at 37°C with 50% water content (Fig. 3(c-1)), inactivation was assumed to be mainly due to damage to metabolic functions and/or nucleic acids. At 50°C with 50% water content (Fig. 3(c-2)), the increase in temperature was assumed to cause damage to metabolic functions and/or nucleic acids because the inactivation rate by each medium was significantly higher compared with the rate at 37°C with 50% water content. However, the inactivation rate by TSA was lower than the rates for the other two media and PMA-TSA, and a difference was also observed when the inactivation rate by PCR was compared with that by PMA-PCR, which indicated that a temperature increase caused more significant damage to the outer membrane than to the metabolic functions. In short, high temperature conditions resulted primarily in damage to the outer membrane, and *E. coli* had the potential to grow.

In addition, for *E. coli* with only outer membrane damage (not slight), the difference in the survival ratio between TSA and DESO should be similar to that between PCR and PMA-PCR, as the difference between TSA and DESO was similar to that between

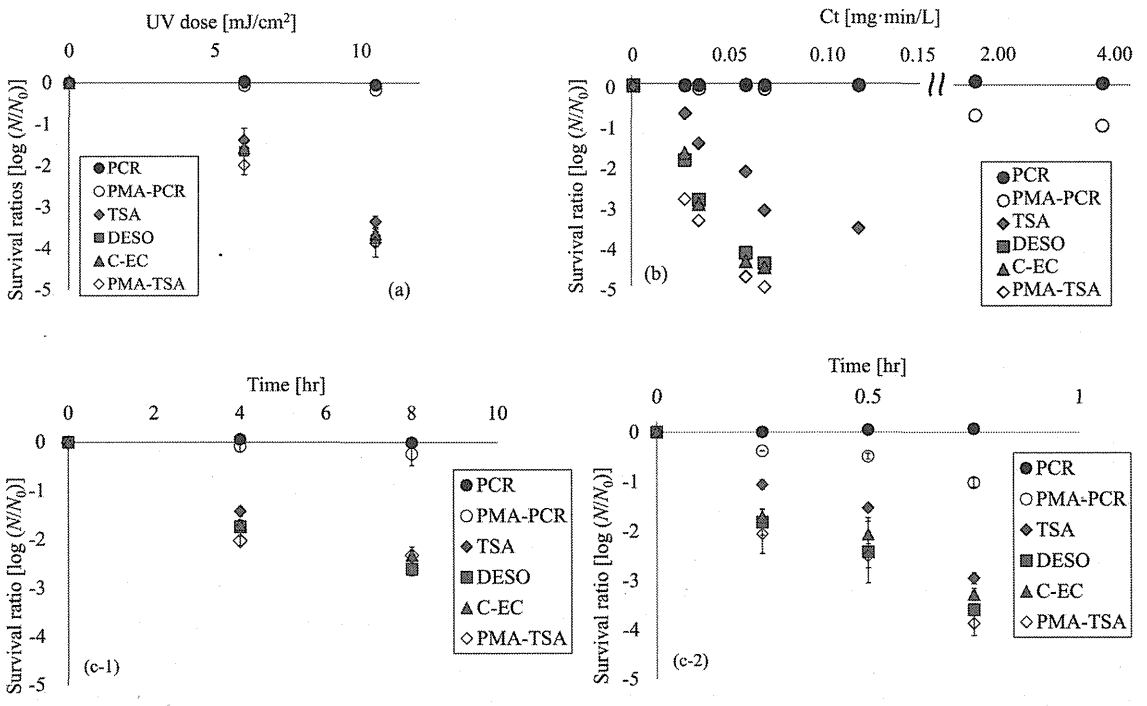

Fig. 3 - Change in the survival ratios of *E. coli* by UV, Cl and sawdust treatment.
(a) UV treatment; (b) Cl treatment; (c-1) sawdust treatment at 37°C with 50% water content; (c-2) sawdust treatment at 50°C with 50% water content.
*Error bars show 95% confidence.

PMA-TSA and TSA. These differences were compared using the results in Fig. 3(c-2). The difference between TSA and DESO was calculated by the inactivation rate constant of DESO divided by that of TSA. The difference between PCR and PMA-PCR employed the inactivation rate constant of PMA-PCR because the survival ratio of PCR did not change. They were respectively 1.34 and 1.25, and very close values. This result indicated that these methods could distinguish damage (not slight) to the outer membrane of *E. coli*.

CONCLUSIONS

The inactivation mechanisms of *E. coli* by UV treatment, chlorination and sawdust treatment were investigated, and the results were compared with other detection methods. As for the results, the damaged parts of *E. coli* could be estimated approximately by three different media, and *E. coli* with damage to each part could be detected quantitatively. However, this method could not distinguish the outer membrane damage from enzyme activity damage. The method comparing PCR to PMA-PCR might reveal whether the outer membrane of *E. coli* was damaged, however, it was difficult to evaluate the kinetics of the damage reaction rate. The disinfection effect should be assessed quantitatively because *E. coli* with outer membrane damage has the potential to grow. This research investigated a newly developed method using PMA combined with TSA to distinguish the damage to the outer membrane of *E. coli*. The results by TSA and PMA-TSA indicate that UV treatment did not cause *E. coli* outer membrane damage, while chlorination and high temperature sawdust treatment did. This method could distinguish outer membrane damaged *E. coli* that has the potential to grow, and evaluate the inactivation rate by PMA cross-linking using 1 μg of PMA for 10^4 CFU of *E. coli*. In addition, by using yellow cellophane it was considered that *E. coli* with outer membrane damage could be distinguished even though *E. coli* that can photoreactivate existed together. The method comparing TSA to PMA-TSA could distinguish *E. coli* with damage to the outer membrane sensitively and quantitatively.

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