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Characteristics of Mesophilic Bacteria Isolated during Thermophilic Composting of Sewage Sludge

KIYOHIKO NAKASAKI, MASAYUKI SASAKI, MAKOTO SHODA,* AND HIROSHI KUBOTA

Research Laboratory of Resources Utilization, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama, Japan

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The degree of inactivation by UV irradiation was different between vegetative cells and spores of bacteria isolated from sewage sludge composting at 60°C. By using this property, a method to estimate the spore ratio of a mixture of vegetative cells and spores was presented. This UV irradiation method was applied to the estimation of the spore ratio of sewage sludge compost samples collected at several stages of composting. The spore ratio of mesophilic bacteria in the samples obtained at the thermophilic stage of 60°C was 40% at most. The vegetative form of mesophilic bacteria showed a thermotolerance property at 60°C by forming colonies but showed no respiratory activity at that temperature.

Composting is the process of degradation of complex and heterogeneous organic material by a mixture of microorganisms: bacteria, actinomycetes, and fungi, both mesophilic and thermophilic. We demonstrated the succession of microorganisms, both mesophilic and thermophilic, during the composting process of sewage sludge and investigated the contribution of these microorganisms to CO_2 evolution caused by degradation of organic matter in the sludge (4). During the experiments, a large number of mesophilic bacteria were isolated even at the thermophilic stage of 60°C. The role and the existing form of the mesophilic bacteria at the thermophilic stage of 60°C are of primary interest.

In this work, we introduced a simple UV irradiation method to estimate the spore ratio of bacteria in composting. The thermotolerance property and the activity of CO_2 evolution of mesophilic bacteria isolated from the composts were also investigated at 60°C.

MATERIALS AND METHODS

Composting. Dewatered sewage sludge cake from a municipal wastewater treatment plant was composted in a laboratory-scale composting reactor in which temperature was controlled by varying the air flow rate at 60°C as long as possible. The sludge was a mixture of raw settled sludge and excess sludge, containing approximately 60% water, 7.5% slaked lime, and 2.5% ferric chloride as dewatering agents. As the initial pH of the sludge was around 11, CO_2 gas was supplied to neutralize the material and to shorten the lag period of the reaction induced by such high pH. The cake, which was ground and sieved to <5 mesh, was mixed with composted product as a seed in various ratios. The CO_2 evolution rate was measured continuously during the composting process. Samplings of compost material were conducted several times during the composting. Details of the composting operation and preparation of composting materials are described in the accompanying paper (4).

Isolation of dominant bacteria in compost samples. Isolation of bacteria, both mesophilic and thermophilic, was conducted by using Trypticase soy agar (BBL Microbiology Systems) medium. The medium composition is as follows (grams per liter of distilled water): Trypticase peptone, 17; phytone peptone, 3; NaCl, 5; K_2HPO_4, 2.5; glucose, 2.5; agar, 20 (pH 7.3). Ten-gram (wet weight) compost samples were suspended in 90 ml of sterile water and homogenized at 10,000 rpm for 10 min with a homogenizer. By serial dilution in sterile water, the suspension was spread onto the medium plates. Then the plates were incubated at 30°C for 3 days or at 60°C for 2 days. The isolated bacteria were purified on the same agar plates and some characteristics of the bacteria were checked. The spore formation of the isolated bacteria was tested on manganese-fortified Trypticase soy agar plates (10 mg/liter as $MnSO_4 \cdot 4H_2O$).

Preparation of cells for UV radiation. (i) Isolated bacteria. Vegetative cells of the bacteria isolated from the compost samples were obtained from colonies formed on Trypticase soy agar plates. Spores were formed on Trypticase soy agar plates fortified with manganese, 10 mg/liter, as $MnSO_4 \cdot 4H_2O$ after 3 days of incubation at 30°C. Microscopic examination showed the population to be almost 99% spores. No purification of spores was done.

The mixtures of the vegetative cells and spores were prepared by adjusting the incubation time on manganese-supplemented Trypticase soy agar plates. A higher spore ratio was observed at the longer incubation time. The morphological difference between the spore and vegetative cells was always confirmed by means of microscopic observation. The vegetative cells or spores thus collected were suspended in sterile water and spread on Trypticase soy agar plates.

(ii) Compost samples. Compost samples were mixed with sterile water and homogenized with a homogenizer for 10 min at 10,000 rpm. The suspension was diluted with sterile water and spread on Trypticase soy agar plates.

UV radiation. The prepared plates described above were exposed to UV radiation with a 10-W UV lamp at a distance of 30 cm for a certain period of time, which was varied from 0 to 120 s. After each irradiation time, the plates were incubated for 3 days at 30°C for mesophilic bacteria. The average numbers of colonies formed on the three plates were counted as a viable cell number. The viable cell number/initial viable cell number ratios were plotted aginst the UV irradiation time, and the death rate curves of bacteria were obtained.

Thermotolerance test of mesophilic bacteria at 60°C. A thermotolerance test was carried out at 60°C, which was the operational temperature of composting. The isolated bacte-

* Corresponding author.

TABLE 1. Some morphological and growth characteristics of bacterial strains isolated from lime sludge composts

Isolated bacteria	Gram stain	Spore formation	Morphology	Growth at:	
				30°C	60°C
BL1	+	+	Rod	+	−
BL2	+	+	Rod	+	−
BL3	+	+	Rod	+	−
BL4	+	−	Sphere	+	−
BH1	+	+	Rod	−	+

ria (BL1, BL2, and BL3) harvested from the medium plates were suspended in sterile water and spread on the agar plates. Some plates were placed in a 60°C incubator and others were placed in a 30°C incubator for 6 to 18 h. When the colonies formed on the plates became slightly visible after 6 to 18 h of incubation at 30°C, these plates were transferred to a 60°C incubator in which the plates were placed for 12, 24, and 48 h. Then the plates were moved back to the 30°C incubator and incubated for more than 3 days. When the colonies increased in size to 2 to 4 mm in diameter, they were counted as viable cells. As a reference, *Escherichia coli* IAM1239 was grown on the same agar medium.

A thermotolerance test of the isolated bacteria in a liquid medium was also carried out. The colonies of strains were suspended in Trypticase soy liquid medium and incubated at 60°C. The cell suspension sampled from the liquid medium was spread on the agar plates, and the number of colonies formed at 30°C was counted.

Respiratory activity test of mesophilic bacteria. The respiratory activity of the isolated mesophilic bacteria which showed thermotolerance at 60°C was measured at both 30°C and 60°C. Two plastic boxes (nominal volume, 400 ml) with tightly sealed lids were prepared. Oxygen electrodes were inserted in the lids. An agar plate on which the isolated bacteria, BL1, BL2, and BL3, were grown at 30°C for 48 h was placed in each box. Then the box was placed in a 30 or 60°C incubator and the O_2 concentration in the box was measured. After continuous measurement for 12 h, the cells were harvested from each agar plate by gentle scraping and suspended in sterile water. The suspension was spread on the fresh agar plates and the viable cell numbers were determined after incubation at 30°C.

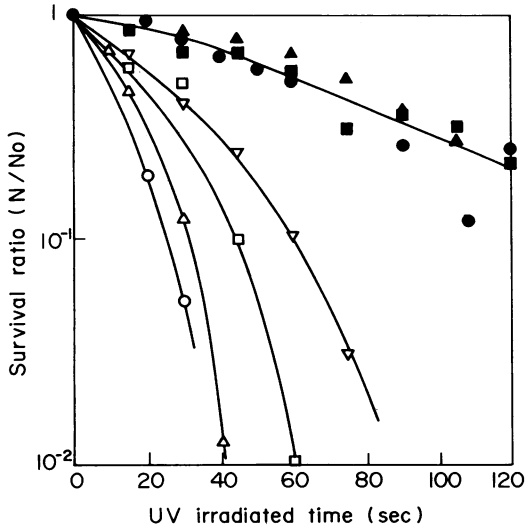

FIG. 1. Death rate curves by UV irradiation of vegetative cells and spores of bacteria isolated from sewage sludge composts. Symbols: ○, BL1 (vegetative); ●, BL1 (spore); △, BL2 (vegetative); ▲, BL2 (spore); □, BL3 (vegetative); ■, BL3 (spore); ▽, BL4 (vegetative). N_0, Initial number of cells or spores; N, number of cells or spores at UV radiation time *t*.

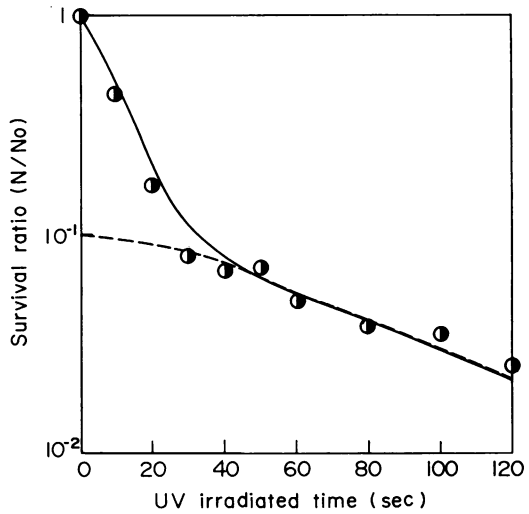

FIG. 2. Death rate curve of a mixture of vegetative cells and spores of strain BL1. N_0, Initial number of cells; N, number of cells at UV radiation time *t*.

RESULTS

Death rate curves of isolated mesophilic bacteria. Some morphological and growth characteristics of the isolated

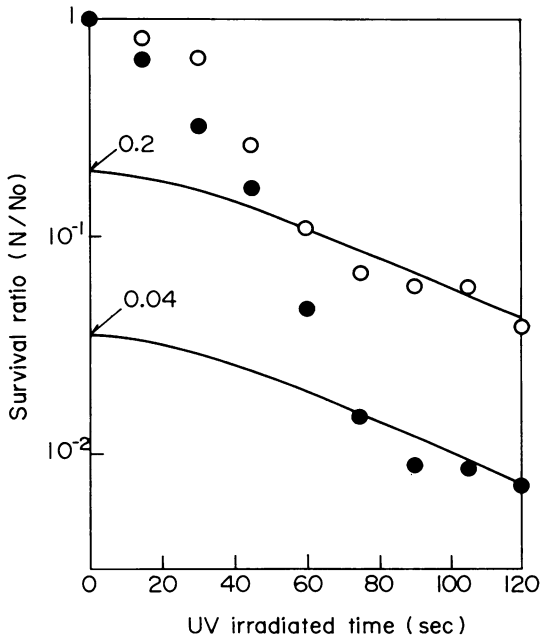

FIG. 3. Death rate curves of mesophilic bacteria in compost samples collected at different times during run A. Symbols: ●, sampled at 36 h (temperature, 43°C); ○, sampled at 64 h (60°C). N_0, Initial number of colonies formed on the plates; N, number of colonies at UV radiation time *t*.

bacteria are shown in Table 1. Although no identification was made, strains BL1, BL2, and BL3 were all gram-positive and sporeforming, with colonies having different appearances. BL4 was isolated only rarely at the start of composting, but became dominant during the period of temperature increase and disappeared at 60°C. BH1 cells were the sole dominant thermophilic bacteria throughout the runs. Death rate curves for these isolated mesophilic strains under UV radiation, in both vegetative cells and spores, are shown in Fig. 1. The degree of inactivation by UV radiation was remarkably different for vegetative cells and spores.

Estimation of spore ratio. The death rate curve under UV irradiation for a mixture of vegetative cells and spores of BL1 is shown in Fig. 2. The initial sharp decline in cell number is due to the death of vegetative cells and the slope at the later period is due to the death of spores. The spore ratio, defined as spore number/number of cells appearing initially on agar plates, can be obtained by extrapolating the death rate curve at the later period to the ordinate. The line of the death rate curve of spores was drawn in the same slope as that of pure spores in Fig. 1. The spore ratio obtained was 10% (Fig. 2), whereas microscopic observation of the same sample was about 6%. The reason for this may be that the dead cells cannot be distinguished from live ones by microscopic observation. Further investigation to distinguish live cells from dead cells was not done.

The death rate curves of mesophilic bacteria in two compost samples collected at different stages of the composting are shown in Fig. 3. The slopes of Fig. 2 and 3 were drawn the same. The spore ratios of mesophilic bacteria thus estimated at different stages of the composting are summarized in Table 2. Each run differs in the mixing ratio of raw

TABLE 2. Estimated spore ratios of mesophilic bacteria in composts during composting in four runs

Run	Seed/raw sludge ratio (%)	Sampling time (h)	Temp (°C)	Spore ratio (%)	Cell no. × 10^8 (cells/g [dry wt] of compost)
A	0	3	18	9	
		36	43	4	
		40	50	16	16
		64	60	20	6
		88	60	10	22
		128	36	7	
B	10	2	20	29	
		39	43	1	
		47	60	27	26
		72	60	22	18
		96	57	28	15
		120	33	28	
C	24	3	19	32	
		23	43	10	
		30	60	40	24
		49	60	25	33
		74	60	39	22
		118	29	39	
D	133	3	15	7	
		21	47	8	
		48	60	44	3
		71	60	27	25
		93	60	24	30
		136	41	40	

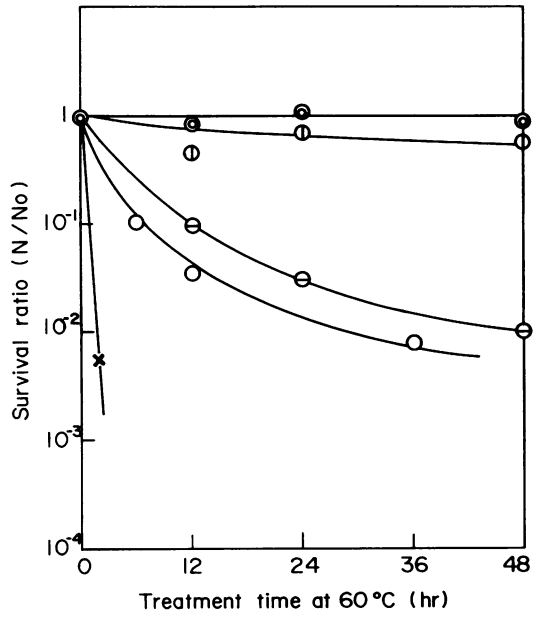

FIG. 4. Comparison of thermotolerance to 60°C of strains BL1 and *E. coli*. Symbols: BL1—○, treated in liquid medium at 60°C; ⊖, treated on agar plates at 60°C immediately after the cells were spread on the plates; ⊕, treated on agar plates at 60°C after growth at 30°C for 6 h; ◎, treated on agar plates at 60°C after growth at 30°C for 12 h; *E. coli*—×, treated on agar plates at 60°C after growth at 30°C for 10 h. N_0, Initial number of colonies, N, number of colonies at time *t*.

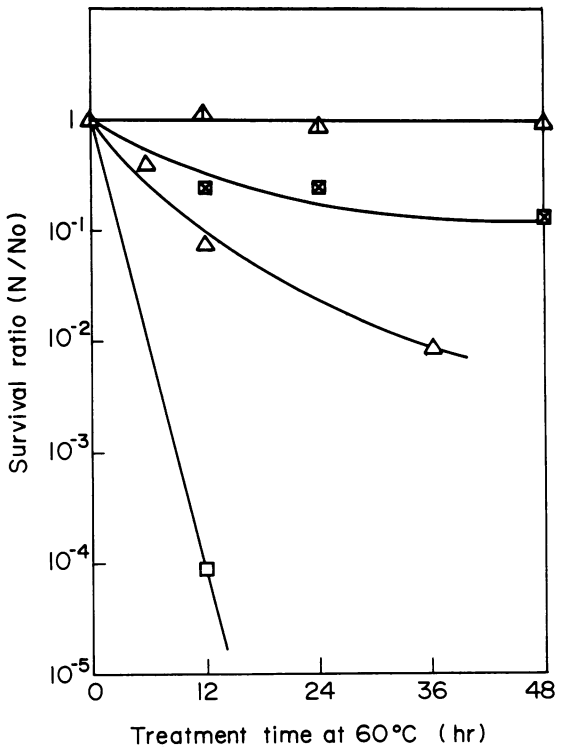

FIG. 5. Comparison of thermotolerance to 60°C of strains BL2 and BL3. Symbols: BL2—△, treated in liquid medium at 60°C; ▲, treated on agar plates at 60°C after growth at 30°C for 12 h; BL3—□, treated in liquid medium at 60°C; ⊠, treated on agar plates at 60°C after growth at 30°C for 18 h. N_0, Initial number of colonies; N, number of colonies at time *t*.

sludge and seed. The spore ratio of mesophilic bacteria was 40% at most. This means that most of the mesophilic bacteria in the compost materials were in the vegetative form under the thermophilic conditions of composting.

Features of the mesophilic bacteria under thermophilic conditions. The result of a thermotolerance test at 60°C for strain BL1 is shown in Fig. 4. Compared with the sharp thermal inactivation of *E. coli*, strain BL1 was more thermotolerant to 60°C. The cells treated in the liquid medium were at a lower level of thermotolerance than those cultivated on the agar plates. Once the cells grew at 30°C for more than 12 h, almost all of the colonies appearing on the agar plates resumed growth after 60°C treatment. A similar tendency was observed for strains BL2 and BL3 (Fig. 5), whereas BL4 did not survive at 60°C.

Respiratory activity of mesophilic bacteria. A linear decrease in O_2 concentration was observed at 30°C for more than 12 h in the plastic box with the agar plate in which mesophilic bacteria were incubated. The total viable cell number on the plate after the test was on the order of 10^9. The average oxygen consumption rate (moles of O_2 per cell per hour) was about 10^{-14}, which was the same as observed for other bacteria (1). No consumption of O_2 was detected for cells tested in a similar test at 60°C. The viable cell number was on the order of 10^8, and microscopic observation showed that the cells grown at 60°C were all in the vegetative form.

DISCUSSION

It is well known that X-ray or gamma ray irradiation is lethal to bacterial cells. The spores of bacteria have been reported to be the most resistant to irradiation when relative radiation sensitivities of microorganisms are compared (2). The effects of microwaves and X rays on bacterial spores have been investigated (3, 5). However, no attempt had been made so far to use UV rays as a means to estimate the spore ratio in a mixture of vegetative cells and spores. We tried to use gamma rays or heat to estimate the spore ratio but consistent results were not obtained. The use of UV, which is handled more easily than X rays or gamma rays, has been found to be effective for estimation of bacterial spores.

The death rate curves for some vegetative cells and spores shown in Fig. 1 were somewhat curvilinear. As the same tendency was reported for vegetative cells of *Micrococcus* sp. and spores of *Clostridium* sp. with gamma radiation (2), this tendency can be considered to be inherent in the bacterial species rather than due to the methods used.

The UV irradiation method was used to obtain the spore ratio of isolated mesophilic actinomycetes. However, there was no significant difference between the death rate curves of vegetative cells and spores. The reason is not clear but the result indicated that the sensitivity of spores of actinomycetes to UV is rather weak and comparable to that of vegetative cells. The number of mesophilic actinomycetes in the compost material was $<10^2$ per gram (dry solid) of compost, as shown in the accompanying paper (4); thus, the contribution of the mesophilic actinomycetes can be discounted.

A large number of mesophilic bacteria were isolated at the thermophilic stage of 60°C and more than 60% of them were in a vegetative state (Table 2). The results of the thermotolerance test showed that they can survive at 60°C by forming colonies in the compost material, although they cannot be directly visualized. Once the visible colonies had formed on the agar plates, a higher thermotolerance was seen (Fig. 4 and 5). These strains lost their activities faster in liquid medium than on agar plates. Since no O_2 consumption was observed at 60°C, mesophilic bacteria were found to contribute almost nothing to the degradation of organic matter in composting.

LITERATURE CITED

1. **Brock, T. D.** 1966. Principles of microbial ecology. Prentice-Hall Inc., Englewood Cliffs, N.J.
2. **Goldblith, S. A.** 1971. The inhibition and destruction of the microbial cell by radiations, p. 285–305. *In* W. B. Hugo (ed.), Inhibition and destruction of microbial cells. Academic Press, Inc., New York.
3. **Goldblith, S. A., and D. I. C. Wang.** 1967. Effect of microwaves on *Escherichia coli* and *Bacillus subtilis*. Appl. Microbiol. **15**:1371–1375.
4. **Nakasaki, K., M. Sasaki, M. Shoda, and H. Kubota.** 1985. Change in microbial numbers during thermophilic composting of sewage sludge with reference to CO_2 evolution rate. Appl. Environ. Microbiol. **49**:37–41.
5. **Zamenhof, S., H. Bursztyn, T. K. R. Reddy, and P. Zamenhof.** 1965. Genetic factors in radiation resistance of *Bacillus subtilis*. J. Bacteriol. **90**:108–115.