Combined Use of Cyanoditolyl Tetrazolium Staining and Flow Cytometry for Detection of Metabolically Active Bacteria in a Fed-batch Composting Process

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Microbial community dynamics with metabolically active bacteria during the start-up operation of a personal fed-batch composting (FBC) reactor were studied. The FBC reactor was loaded daily with household garbage for 2 months. Metabolically active bacteria were monitored by the redox-dye-staining method using 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), and the fluorescent formazans thus produced were detected by epifluorescence microscopy and flow cytometry (FCM). Microscopic CTC-positive (CTC+) counts accounted for 75–84% of the direct total count during the first week of operation and 19–35% thereafter. Slightly higher CTC+ counts were obtained by FCM. Culture-independent approaches by quinone profiling and denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA genes showed that a drastic population change from ubiquinone-containing members of the *Proteobacteria* to the *Actinobacteria* took place during the overall period of operation. The PCR-DGGE analysis of FCM-sorted CTC+ cells supported this observation but gave different major clones from those detected in the total community in some cases. These results suggest that metabolically active bacteria as measured by CTC staining are not always predominant in the FBC process.

Key words: fed-batch composting, tetrazolium reduction, flow cytometry, microbial community

The fed-batch composting (FBC) process not only has great promise for practical use in waste treatment technology but also provides a good model of microbial ecology in terms of microbial population dynamics during the biodegradation of solid organic substances. In general, this process uses relatively small-scale reactors which are loaded repeatedly with biowaste without removal of treated material during an appropriate period of operation. Therefore, FBC reactors have a lower and narrower range of temperature depending upon self-heating than the traditional single-batch composting system (24), which has three to four different thermal stages (11, 43). This characteristic feature of the FBC process may be responsible for particular microbial community dynamics different from those for the conventional composting system.

Previous studies on mesophilic FBC reactors for garbage treatment have shown that a population shift from members of the phylum *Proteobacteria* to those of the phylum *Actinobacteria* takes place during a start-up period of operation (17, 23, 25, 41). Moisture or water activity (a_w) may be one of the most important determinants of this population shift (25, 41). However, major questions to be answered are whether the predominant bacteria in the FBC process are always metabolically active and what kinds of microorganisms are actually responsible for the degradation of household organic waste. Although high culturability of microorganisms in FBC reactors provides circumstantial evidence for high proportions of viable bacteria (17, 26), there has been only fragmentary information about relationships between the predominant bacteria and metabolically active bacteria during the FBC treatment of garbage.

One of the most promising methods for the detection

of metabolically active microorganisms is the tetrazolium reduction assay using 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), which can quantify the activity of dehydrogenases. CTC is converted to a red fluorescent formazan as the result of reduction, and CTC-positive (CTC+) bacteria can be detected at a single cell resolution by epifluorescence microscopy (5, 7, 9, 14, 30–32, 37, 39, 44, 48), confocal laser scanning microscopy (12), and flow cytometry (FCM) (4, 8, 13, 21, 38, 40, 46). The main purpose of this study was therefore to elucidate relationships between the predominant bacteria and metabolically active bacteria in the FBC process by using the CTC staining technique, with both epifluorescence microscopy and FCM used for detection. Attempts to phylogenetically identify CTC+ bacteria by FCM sorting and denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA genes were also made.

Materials and Methods

Operation of reactors

A commercial personal composter, a SANYO model SMS-K2 (Sanyo Electric Co., Moriguchi, Japan), was used as the FBC reactor. The reactor has a working capacity of 33 L and contained 18 L of fresh wood chips (Sanyo) as the solid matrix at the start of operation. The organic waste used for composting was collected daily from a restaurant of Toyohashi University of Technology. Detailed information about the reactor and the biowaste has been given previously (23, 26). The organic wastes were cut into pieces of less than 3 cm square if needed, and added manually to the reactor. The reactor was operated for 2 months with a 24 h-fed-batch cycle at a waste-loading rate of 0.7 kg (wet wt) day⁻¹. Every hour in a batch cycle and just after waste addition, an impeller in the reactor automatically rotated to mix the solid waste-compost mixture (SCM) for 5 min, and this was the only way to aerate SCM. During the overall period of operation, the reactor was kept in an incubator room at 22°C and 50% humidity.

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Sample collection and physico-chemical analyses

SCM samples (5 g wet weight each) were collected from at least three different points of the core of the reactor at the end of each batch cycle on days 1, 3, 5, 7, 14, 21, 28, 42, and 56. Before sampling, the SCM was stirred and mixed well with a scoop. Collected samples were combined and screened by stainless steel sieve with a 5 mm mesh to remove large debris. Then, the samples were immediately subjected to physicochemical and microbiological analyses, whereas those for the quinone analysis were stored at -30°C until used. The mass reduction rate, moisture content, pH, core temperature, and conductivity were measured as described previously (41). Water activity was measured using an AW SPRINT HT-500 water activity analyzer (Novasina, Switzerland) according to the manufacturer's instructions.

Quinone analysis

Quinones were extracted from SCM samples with an organic solvent mixture and separated into menaquinone and ubiquinone fractions using Sep-Pak Vac silica gel cartridges (Waters, Milford, MA, USA). The quinone components of each fraction were quantified and identified by reverse-phase HPLC and photodiode array detection with external standards. Detailed information on these analytical procedures has been given previously (15, 16). Ubiquinones and menaquinones with n isoprene units in their side chain were abbreviated as Q- n and MK- n , respectively. Partially hydrogenated menaquinones were expressed as MK- $n(\text{H}_x)$, where x indicates the number of hydrogen atoms saturating the side chain.

Direct cell counting

For cell counting, 1 g (wet wt) of SCM sample was suspended in 9 mL of filter-sterilized 50 mM MOPS buffer (pH 6.5), sonicated for 90 sec (20 kHz; output power 50 W), and diluted with the buffer. Aliquots (10–50 μL) of these diluted samples were then used for direct cell counting. Direct total and viable bacterial counts were measured by epifluorescence microscopy with ethidium bromide staining (EtBr) or SYBR Green I and with a Molecular Probes LIVE/DEAD BacLight Viability kit (Invitrogen, Carlsbad, CA), respectively, as described (26, 47). Stained specimens were observed under an Olympus BX-50 epifluorescence microscope equipped with an Olympus model DP70 CCD camera. The number of stained cells was counted using the image analysis program WINROOF (Flovel, Tachikawa, Japan).

CTC+ cell counting

SCM samples were prepared as described above for total cell counting. Metabolically active cells were stained with the fluorescent redox dye CTC according to a previously described protocol (48) with minor modifications. The CTC reaction mixture contained 800 μL of SCM suspension in 50 mM MOPS buffer (pH 6.5), 1 mM CTC (Sigma-Aldrich, St. Louis, Mo), 25 mM Meldla's Blue (Dojindo Laboratories, Kamimashiki, Japan), 1 mM KCN, and 10 μL of a substrate mixture (5% peptone [w/v], 5% yeast extract [w/v] and 2% glucose [w/v]) in a total of 1.0 mL in 1.5-mL Eppendorf microtubes. The reaction mixture was incubated in a water bath at 30°C for 4 h with shaking. All these procedures were done in darkness. For microscopic detection, 5 μL of the reaction mixture was spotted on a slide glass, fixed by drying at 30°C for 5 min in darkness, and counter-stained for 5 min with 10 μL of a $1/10^4$ -diluted solution of SYBR Green I (Invitrogen). Among CTC+ bacteria, gram-positive bacteria were detected by post-extracting with acetone (47). CTC+ cell counts were obtained under an Olympus epifluorescence microscope as described for total cell counting.

Flow cytometry and cell sorting

The quantification and sorting of CTC-stained cells was performed using an EPICS ALTRA flow cytometer (Beckman-Coulter, Miami, FL) equipped with an air-cooled 15 mW argon laser operating at 488 nm. The emission of the two fluorochromes was recorded through specific band pass filters; 525 ± 15 nm for

SYBR Green I and 615 ± 20 nm for CTC formazan. An appropriate discriminator was set in the forward scatter mode to exclude small debris and cell fragments. Data on 10,000 cells were recorded and analyzed using EXPO 32 MultiComp software (Beckman-Coulter). In sorting, the number and amplitude of vibrations of the flow cell were 17.7 kHz and 30%, respectively, and a total of 40,000 cells possibly stained with CTC were collected. Epifluorescent microscopy showed that 76% and more of the cells thus collected under these sorting conditions were actually CTC+ cells.

16S rRNA gene-targeted PCR-DGGE

DNA was extracted from SCM samples by the method of Zhou *et al.* (49) with minor modifications as described previously (23). The variable region V3 of bacterial 16S rRNA genes, corresponding to positions 341–534 in the *Escherichia coli* 16S rRNA (3), was PCR-amplified using the forward primer 357f (5'-CCTACGGGAG-GCAGCAG-3') with a GC-clamp on the 5' terminus and the reverse primer 517r (5'-ATTACCGCGGCTGCTGG-3') as described by Muyzer *et al.* (22). Amplification was performed using an AmpliTaq Gold Taq DNA polymerase kit (Applied Biosystems, Foster City, CA) and a Takara Thermal Cycler (Takara Bio, Otsu, Japan). The PCR profile was 10 min for activating the polymerase at 94°C and then 25 or 35 cycles of 1 min at 94°C , 1 min at 53°C , and 1 min at 72°C , and finally 5 min of extension at 72°C . The PCR products were checked by agarose gel electrophoresis and staining with ethidium bromide. The amplicons were purified with a MicroSpin S-HR400 Column (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's recommendations. DGGE was performed in a 10% (w/v) polyacrylamide gel at 200 V for 3.5 h using a Bio-Rad DCode™ system (Bio-Rad, Hercules, CA). The gel gradient used for separation was 40–60%. After electrophoresis, the gel was stained with SYBR Green I for 30 min following the manufacturer's instructions and observed with a TOYOBO FAS-III min+ DS-30 transilluminator (Toyobo, Tokyo, Japan).

Sequencing and phylogenetic analysis

Major DGGE fragments were cut from the gel, purified using a GeneClean Spin kit (Bio 101, Vista, CA), and subcloned with a pTBlue Perfectly Blunt cloning kit (Novagen, Madison, WI) according to the manufacturer's instructions. Transformation of *E. coli* competent cells was carried out by following a standard manual of molecular cloning (36). Plasmid DNA was isolated and purified by using Wizard Minipreps (Promega) as instructed by the manufacturer. The nucleotide sequence of DNA was determined with a cycle sequencing kit and an automated DNA sequencer as described previously (18). We sequenced at least 3 clones per DGGE fragment, and confirmed all these clones to have the same sequence. Sequence data were compiled with the GENETYX-MAC program (Genetyx, Tokyo, Japan) and subjected to a BLAST homology search (1) and the matching of sequences in the Ribosomal Database Project (RDP) II (6) to ascertain the phylogenetic position of isolates. The multiple alignment of sequence data, calculation of the corrected evolutionary distance (20), and construction of a neighbor-joining phylogenetic tree (35) were performed using the CLUSTAL W program ver. 1.83 (42). The topology of the tree was evaluated by bootstrapping with 1,000 trials (10).

Nucleotide database accession numbers

The 16S rRNA gene sequences reported in this study have been deposited under DDBJ accession numbers AB474788 to AB474793.

Results

Changes in physico-chemical parameters

The mass reduction rate during the overall period of FBC operation was 94%, indicating a good waste-reduction efficiency. The core temperature of the reactor varied between

33 and 43°C due to self-heating (Fig. 1a). The pH of SCM decreased to 5.7 at the early stage and then increased gradually during the overall period, reaching around 9.5 after 2 months of operation (Fig. 1b). The moisture content was over 50% for 10 days from the start of waste delivery but became stable at 30 to 40% during the latter half of operation (Fig. 1c). The a_w decreased linearly with time and reached a value of between 0.94 and 0.95 at the end of operation (Fig. 1d). In view of the changes in temperature, moisture, and pH, the reactor could be regarded as reaching the acclimated phase after 1 month of operation. These physicochemical changes are typical of the mesophilic FBC process during the start-up period as described previously (24, 41).

Succession of total, viable, and CTC+ counts

The total bacterial count increased sharply from the start of waste loading and reached an order of 10^{11} cells g⁻¹ (dry wt) of SCM on day 7 of operation (Table 1). Thereafter, the total count became stable at between 2.9×10^{11} and 5.0×10^{11} g⁻¹ (dry wt). The direct viable count measured with a LIVE/DEAD BacLight kit accounted for 75–89% of the total count during the first week and 49–66% of the total count thereafter.

The CTC+ count as measured by epifluorescent microscopy was 75–84% of the total count during the first week of operation but dropped sharply to 19–35% under steady-state conditions (on days 28–56) (Table 1). A decrease in the proportion of CTC+ populations with operation time was also found by FCM (Table 1 and Fig. 1). FCM measurements gave higher CTC+ counts than did the microscopic analysis. This was due to the overestimated CTC+ counts obtained by FCM, as microscopic checking of FCM-sorted CTC+ cells revealed that 76–92% of all the cells collected were actually CTC+. There was a highly positive correlation between the microscopic count and the corrected FCM count ($r^2=0.9476$).

Most of the CTC+ cells detected during the first week (>70%) were decolorized by treatment with acetone (data not shown). On the other hand, only 12–30% of the CTC+ cells found at the steady-state stage were decolorized by acetone treatment. This suggested that most of the metabolically active bacteria at the steady-state stage were gram positive,

Fig. 1. Changes in physico-chemical parameters during fed-batch composting of household organic waste. (a) core temperature; (b) pH; (c) moisture content; (d) water activity (a_w).

as it has been shown that CTC+ gram-positive bacteria are not decolorized with acetone (47).

Succession of quinone profiles

The total quinone content of SCM increased sharply during the first 2 weeks of operation, and thereafter became stable at between 170 and 240 nmol g⁻¹ (dry wt) (Table 1). Assuming that 1 nmol of total respiratory quinones corresponds to 2.1×10^9 cells of bacteria in soil and compost (16), the total counts predicted based on the quinone contents are 3.6 – 5.0×10^{11} cells g⁻¹ under steady-state conditions. These values are similar to the direct total counts obtained by epifluorescent microscopy. Early in the FBC process (on days 1–14), ubiquinones predominated (59–85 mol% of the total content). With time, however, the amount of ubiquinones decreased gradually with a concomitant increase in the amount of partially saturated menaquinones. At the end of operation, ubiquinones and partially saturated menaquinones accounted for 14 and 57 mol% of the total content, respec-

Table 1. Comparative direct total, viable, and CTC+ counts of bacteria and quinone contents in the FBC reactor

Sample on day:	Direct total count ($\times 10^{11}$ cells g ⁻¹ dry wt) ^a	Direct viable count (% of total) ^b	CTC+ count (% of total)			Quinone content		
			Epifluorescence microscopy ^c	FCM	Total content (nmol g ⁻¹ dry wt)	Mol%		
						Q-n	MK-n	MK-n(H _x)
1	0.085	nd ^d	nd	nd	nd	nd	nd	nd
3	0.25	75±5	75±3	nd	11	85	14	1
5	0.69	88±2	84±3	nd	40	70	28	2
7	1.0	89±3	82±2	88 (81) ^e	69	68	28	4
14	2.9	66±5	40±3	69 (53)	170	59	30	11
28	5.0	57±6	35±5	68 (52)	240	36	34	30
42	3.9	52±4	26±4	46 (37)	230	21	31	48
56	4.0	49±4	19±6	34 (26)	240	14	29	57

^a SYBR Green I count by epifluorescence microscopy

^b LIVE/DEAD BacLight count (average±standard deviation of three determinations).

^c Average±standard deviation of three determinations.

^d nd, not determined.

^e Figures in parentheses indicate corrected values based on the microscopic analysis of FCM-sorted cells.

Fig. 2. FCM analyses of CTC+ bacteria in the reactor on days 7 (a), 14 (b), and 42 (c). Assemblies of particles surrounded by lines I and II indicate cells stained only with SYBR Green I and CTC+ cells, respectively, and figures in parentheses denote the number of cells measured. Cells in area II were sorted and subjected to PCR-DGGE analyses as the CTC+ fraction.

tively. The most abundant quinone species detected under steady-state conditions were MK-8(H₄) (25 mol%). Other quinone homologs accounting for more than 5 mol% at the end of operation were Q-10 (11%), MK-7 (11%), MK-8 (9%), MK-9(H₄) (6%), MK-9(H₂) (5%), and MK-9(H₈) (5%). These data indicate that a population shift from ubiquinone-containing members of the *Proteobacteria* (i.e., *Alpha*-, *Beta*-, and *Gammaproteobacteria*) to the *Actinobacteria* took place during the FBC operation, well consistent with previous reports (17, 23, 25, 41).

PCR-DGGE analyses

CTC+ cells collected from the FBC reactor by FCM sorting were subjected to 16S rRNA gene-targeted PCR-DGGE analyses in comparison with the total bacterial cells. Although amplification with 25 cycles rather than 35 cycles might be better to detect DGGE clones representing the bacterial composition, the former PCR resulted in the production of faint PCR signals and markedly fewer numbers of DGGE bands. Therefore, only results of PCR with 35 cycles are shown herein. As shown in Fig. 3, a drastic change in DGGE patterns of both total and CTC+ populations was observed during the first 3 weeks of operation. Thereafter, the PCR-DGGE pattern became relatively stable in the two fractions. It is worth noting that the total and CTC+ cell fractions occasionally gave different PCR-DGGE patterns from each other,

as was the case on days 3, 14, and 56 (see also Fig. 4).

A total of 20 major DGGE fragments were cut from the gel, subcloned, and then sequenced. The results of a BLAST homology search and a RDP-II sequence match analysis of the 16S rRNA gene sequences determined are shown in Table 2. Also, a neighbor-joining phylogenetic tree for these clones and their phylogenetic relatives retrieved from databases is shown in Fig. 4. The DGGE clones analyzed were classified into 9 different phylogenetic groups that corresponded to established species or uncultured bacteria of the phyla *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* (the classes *Alphaproteobacteria* and *Gammaproteobacteria*). These results showed that the proteobacterial clones were abundant early in the process, whereas actinobacterial clones were detected at the steady-state stage (on day 21 and thereafter). This community change was similarly found in both CTC+ and total population fractions.

Discussion

The detection of viable and metabolically active populations in microbial communities in terms of quantity and quality is an important task in research on microbial ecology. Approaches to this research include microradioautography-fluorescence in situ hybridization (29, 45), stable isotope probing (27, 28, 34), and staining with viability-probing fluorescent dyes such as CTC (19). Since the CTC staining method is rapid and simple to perform and requires no expensive instruments or a restricted experimental area, it is widely used for the detection of metabolically active microorganisms at a single-cell resolution. However, the method itself gives no information about the quality of target organisms in terms of phylogeny and taxonomy, although a modified version with acetone treatment can roughly estimate the proportion of gram-positive and gram-negative bacteria. To address this issue, we combined the use of CTC staining, FCM, and cell sorting followed by PCR-DGGE as reported herein. Before this study, there had been few reports on the phylogenetic characterization of CTC+ populations as estimated with a combination of FCM sorting and PCR-DGGE profiling (2). To our knowledge, the present study is the first to apply this approach to compost microbial communities.

This study revealed that the proportion of CTC+ populations to the total bacterial population was high early in the FBC process but decreased with time. This decrease is not due to technical reasons but reflects an actual reduction in numbers of metabolically active bacteria, because the proportion of the total viable count as measured with the LIVE/DEAD BacLight kit decreased with operation time. Previous studies have shown that the culturability of microorganisms in FBC reactors at the steady-state stage is high, as the plate count of aerobic heterotrophic bacteria accounts for approximately 50% of the direct total count (26, 41). On comparing this with the direct viable count and the CTC+ count recorded in this study, it is likely that most of the viable bacteria present in the FBC reactor under steady-state conditions are cultivable on ordinary culture media, although why the CTC+ count might be occasionally lower than the plate count is not known. One major determinant of the increase in the proportion of metabolically inactive or dead bacteria at the

Fig. 3. 16S rRNA-targeted PCR-DGGE analysis of the total bacterial community (lane A) and the CTC+ bacterial community (lane B) in the FBC reactor. A negative image of DGGE patterns (detected by staining with SYBR Green I) on days 1 to 56 is shown. The days on which SCM samples were taken are shown on the top of the DGGE image. The DGGE bands shown by arrows with figures are those subjected to sequence analyses.

Fig. 4. Neighbor-joining distance matrix tree of 16S rRNA gene sequences of the DGGE clones detected in the FBC reactor and their phylogenetic relatives. The accession numbers for the sequences are shown behind organism names. *Acidobacterium capsulatum* strain 161^T (D26171) was used as an outgroup to root the tree. Scale bar=5% nucleotide substitution rate (K_{nuc}). Nodes showing a bootstrap value of more than 80% (1,000 resamplings) are shown by solid circles. The days on which the DGGE clones were detected are shown on the right side: a, detected in the total bacterial fraction; b, detected only in the CTC+ fraction; +, detected in both fractions; -, not detected.

Table 2. Phylogenetic identification of PCR-DGGE clones from the reactor

DGGE band	Clone name	BLAST match		RDP-II match with type strain		Phylum/class
		Organism (accession number)	% Similarity	Organism (accession number)	% Similarity	
1	A56	Uncultured compost bacterium 570-OTU2 (AJ297627)	100	<i>Globicatella sulfidifaciens</i> LMG 18844 ^T (AJ297627)	100	<i>Firmicutes</i>
2/3	B1-3	Uncultured alphaproteobacterium LTUA07014 (AY144208)	100	<i>Kaistia adipata</i> Chj404 ^T (AY039817)	91.7	<i>Alphaproteobacteria</i>
4	AB7	<i>Xanthomonas codiae</i> LMG 8678 ^T (Y10765)	100	<i>Xanthomonas codiae</i> LMG 8678 ^T (Y10765)	100	<i>Gammaproteobacteria</i>
5	A3	<i>Acinetobacter</i> sp. ATCC 17922 (Z93436)	99.5	<i>Acinetobacter calcoaceticus</i> ATCC 23055 ^T (Z93434)	97.5	<i>Gammaproteobacteria</i>
6/7/8	AB14-28	Uncultured bacterium WET-E-A15 (EF659256)	100	<i>Tenacibaculum skagerrakense</i> D30 ^T (AF469612)	88.4	<i>Bacteroidetes</i>
9/10/11/12	B5-56	<i>Rhizobium</i> sp. AC100 (AB069724)	100	<i>Sinorhizobium fredii</i> ATCC 35423 ^T (X67231)	99.4	<i>Alphaproteobacteria</i>
13/14/15	A21-56	<i>Knoellia sinensis</i> SAFR-013 (AY167851)	100	<i>Sterptomyces macrosporus</i> DSM 41449 ^T (Z68099)	99.4	<i>Actinobacteria</i>
16/17	B21-42	Uncultured bacterium Niitsu31-44 (AB188009)	98.4	<i>Sphingobacterium daejeonense</i> TR6-04 ^T (AB249372)	87.8	<i>Bacteroidetes</i>
18/19/20	AB21-56	<i>Gordonia terrae</i> ATCC 25594 ^T (X81922)	100	<i>Gordonia terrae</i> ATCC 25594 ^T (X81922)	100	<i>Actinobacteria</i>

fully acclimated stage may be the availability of water.

Consistent with previous papers (17, 23, 25, 41), this study has shown that a drastic population shift from ubiquinone-containing *Proteobacteria* (especially *Alpha*- and *Gammaproteobacteria*) to the *Actinobacteria* takes place during the start-up operation of the mesophilic FBC process. Takebayashi *et al.* (41) have reported that a_w is one of the most important determinants of this population change. They reported that the isolates of *Actinobacteria* were much more tolerant of low levels of a_w than those of *Proteobacteria*, and *Firmicutes* isolates exhibited intermediate tolerance (41). In this study, quinone profiling and PCR-DGGE profiling of the total and CTC+ populations demonstrated a population change concomitant with a decrease in a_w during the overall period of operation. These observations suggest that the phylogenetic composition of metabolically active bacteria in the FBC process also changes from the *Proteobacteria* to *Actinobacteria* during the overall period of operation. In the conventional batch composting process, actinobacterial populations become more abundant during the cooling and maturation phase (11, 33, 43), in which the availability of certain nutrients (e.g., lignin and humic acid) may be an important determinant of population dynamics. The potential significance of such specific nutrients in addition to a_w as factors affecting population changes in the FBC should be elucidated in further study.

It is worth noting that the CTC+ and total populations occasionally gave different PCR-DGGE patterns to each other. This suggests that metabolically active bacteria as measured by CTC staining are not always predominant in the FBC process, although the overall population shift from ubiquinone-containing *Proteobacteria* to the *Actinobacteria* may take place not only in the total community but also in CTC+ populations. Since the FBC reactor is seeded periodically with large populations of microorganisms on the loading of fresh biowaste, it may be that these exogenous microbes temporally grow faster or have higher levels of activity than the predominant bacteria in the reactor. For example, this may be the case in the detection of an

alphaproteobacterial clone (B5-56) on day 56 (Figs. 3 and 4). In view that Q-10, a quinone species specific to the *Alphaproteobacteria* and some fungi, still accounted for more than 10% of the total quinone content at the end of FBC operation, however, one can not exclude the possibility that these ubiquinone-containing microorganisms play primary roles in the degradation of biowaste under steady-state conditions.

In conclusion, the combined use of CTC staining and FCM sorting followed by PCR-DGGE profiling or other molecular approaches has great promise for the rapid detection and phylogenetic identification of metabolically active bacteria in a microbial community. The overall procedure of this method can be completed within a couple of days. Substrate-specific CTC staining and FCM sorting may give more useful information about relationships between the kind of microorganisms involved and substrate utilization patterns. The method presented here can be applied not only to studies of compost microbial communities but also to research on microbial ecology in various environments.

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