MICROBIAL APPROACHES TO CHARACTERIZATION OF COMPOSTING PROCESSES

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■ Passage in 1979 of the Resource Conservation and Recovery Act (RCRA) and the Clean Water Act by Congress resulted in a mandate to control and regulate the use of solid wastes on the land (Federal Register, 1979). The more recent landfill crisis followed by the ocean dumping ban and tightening of air emission standards has significantly influenced solid waste practice in the direction of bio-degradation via composting.

A rapid increase in composting technologies concomitant with increased environmental concern regarding the fate of bacteria, particularly pathogens, means that new opportunities and dangers exist with regard to microbial disposition of solid waste. The impact of new compost technologies on microorganism selection is not known and uncertainty exists as to survival mechanisms of potential pathogens initially present in waste.

New microbial methodologies are needed to characterize bacteria involved in composting, and to determine the influence of compost technology on bacterial species selection, especially pathogens.

The public attitude towards biological treatment and recycling of waste is generally positive, but concerns regarding the icrobiological disposition and impact of these wastes are being raised (Clark et.al., 1983; Farrell, 1993). Implicit in EPA rules governing dispersal of solid wastes is the requirement that a significant reduction of bacterial pathogens will take place prior to land application (Federal Register, 1991). However, monitoring of actual bacterial densities has until recently not been required (Federal Register, 1992). With the new required vigilance in monitoring of *Salmonella* and Fecal coliform prior to land application of solid waste, we expect a sharp rise in reported pathogen values and with it regulatory and litigating actions on the part of EPA and public (Goldstein, 1994).

It is therefore likely that there will be an increased demand for improved microbiological services and for products which aid control of solid waste microbial populations.

Modern phenotypic procedures permit the rapid and cost-effective identification of bacteria. These new methodologies have to our knowledge never been applied to compost microbiology. Unfortunately, new methods in environmental microbiology have to compete with traditional methodologies. There is a pressing need to focus modern methods on developing a microbial ecology (Stahl, 1993) which should be helpful for composters.

Until now, the identities of the bacterial species involved in the composting of wastes has not been well studied, probably because of lack of methods. The development of DNA-probe methods (Curiale, 1990) and phenotypic techniques (Stager & Davis, 1992; Krieger, 1992) present tools to identify at the genus and species level the bacteria important to composting various wastes.

Current composting practice relies on indigenous microorganisms to complete the needed biochemical transformations to achieve a finished or stable product. Stability as defined by low CO_2 respiration and lack of continued self-heating is often not achieved. Many compost samples surveyed by our laboratory show relative immaturity and moderate phytotoxicities. It is not clear whether microbial pathways are not completed or the correct bacteria for compost stabilization are not present. Lacking knowledge on microflora, it may be difficult for engineered compost technologies to achieve desired levels of control.

This situation of uncertainty in composting would be acceptable if it were not for at least two problems: the negative impact of unripe compost on seedling growth and the adaptabil-

ity of opportunistic pathogens to become heat resistant and to function as dominant decomposers in compost systems. In this paper, we concern ourselves with the latter subject.

Isolation and identification of prominent organisms

A sample was taken during the composting process and 4 grams suspended in 40 ml of sterile distilled water. The sample was vortexed and allowed to soak for about 1hr to remove the bacteria adhering to the compost. Using BUGM (Biolog) of trypticase soy agar, 0.1ml of this suspension was smeared over an agar plate with a glass rod and the plate incubated at 53°C or 37°C. A sample was picked from this growth with a sterile loop, streaked, and grown at 37°C. Individual colonies were picked and restreaked. The pure clone was then restreaked for identification with a computerized Microlog identification system.

The *E. coli* and *Salmonella* gene probe kits prepared by Gene-Trak (Framingham, MA) were used for detection of these bacteria in compost material. Methods for isolation of these bacteria were as stated in the gene probe kit protocols. These methods require viable bacteria to give positive results. It was previously observed that even though *E. coli B* was inoculated into a municipal wastewater sludge at about 10^7 cells/gr of fresh solids, these methods produced negative results immediately after inoculation. Yet, in these same samples *E. coli* was readily detected after 5 days of composting. Thus, the protocol was altered to include a 24hr. incubation in nutrient broth with 0.5% lactose. With this method the *E. coli* was easily detected in samples immediately after inoculation.

Isolation of thermophilic mutants of *E. coli B, S. typhimurium Q* and *Pseudomonas aeruginosa* PA01was undertaken. The isolation and demonstration of the origin of these thermotolerant (48°C) and mesothermophilic (54°C) mutant strains has been previously described (Droffner & Yamamoto, 1991a, 1991b).

Compost samples were drawn from a cure pile of a source separated compost pilot project in Fairfield, Connecticut. Compost sampling began on day 15, after semi-in vessel processing. The production of this compost has been described elsewhere (Beyea, et al. 1992). Samples of biosolids and food scraps were blended with sawdust and woodchips+leaves, respectively, to achieve desired C:N ratios. This mixture was moistened with a mixed liquid culture of *E. coli* and *Salmonella typhimurium Q* to obtain approximately 10^7 cells/gr. of total

Table 1. Mutants of various bacterial species and the indicated temperatures of growth above the parental strain limit

Strain Isolated [a]	Maximum temperature of growth, C	Detection by Gene Probe (+) or (-)
Salmonella typhimurium Q (parent)	42°	+
Salmonella typhimurium Q ttl [b]	48°	+
Salmonella typhimurium Q ttl, mth [c]	54°	+
Escherichia coli B (parent)	44°	+
Escherichia coli B ttl	48°	+
Escherichia coli B ttl, mth	54°	?
Pseudomonas aeruginosa PAO1 (parent)	42°	NA [d]
Pseudomonas aeruginosa PAO1 ttl	48°	NA
Pseudomonas aeruginosa PAO1 ttl, mth	54°	NA

[a] For methods of isolation, see Droffner & Yamamoto (1991a, 1991b).
[b] ttl, thermotolerant- growth to 48° C
[c] mth, mesothermophilic- growth to 54° C
[d] NA = not analyzed

sample. Subsequently, 4-liter samples of each were transferred to 4.5-liter laboratory self-heating compost vessels for the duration of the study. The vessels permitted sub-sampling for bacterial analysis at the times indicated.

Results and Discussion

Preliminary data collected by our laboratory suggests microorganisms initially present in wastes dominate the composting process. Furthermore, this data also indicates that under certain conditions, thermo-tolerance spreads rapidly to organisms not believed to be heat resistant. The dissemination of thermal tolerance may take place through similar, complicated mechanisms as are responsible for the increase among bacteria of antibiotic resistance (Davies, 1994; Begley, 1994). Conversely, it may be the result of mutations enabling growth at elevated temperatures (Droffner and Yamamoto, 1985; 1991a; 1991b). In Table 1 we show mutants of various bacterial species and the indicated temperatures of growth above the upper limit for the parental strain. The data show that three gram negative bacteria are capable of mutating to grow at least as high as 54^{o}C. We also have good evidence that *Salmonella* will mutate to grow at 68^{o}C (Brinton & Droffner, *unpublished data*).

It has been generally assumed from previous work that thermophilic temperatures reached during composting are sufficient to destroy pathogens (Wiley, 1962; Wiley & Westerberg, 1969). We have demonstrated, however, that the genera *Escherichia, Salmonella* and *Pseudomonas* all have the capacity to produce mutants able to grow at elevated temperatures (Droffner et. al., 1991a, 1991b). We have isolated an *E. coli* mutant capable of growing at 65^{o}C (Droffner & Brinton, 1994). These genera all carry a genetic operon, *cel*, for the degradation of cellobiose, which is expressed only at 48^{o}C and above suggesting a genetic basis for surviving at elevated temperatures (Droffner, et.al. 1992a).

Survival of potential pathogens in composts may be partly dependent on physical conditions. For example, pathogens may reside in cooler zones of windrows or as clumps along edges of static piles (Cloutier and Brinton, *unpublished data)*. We compare survival in outdoor composting versus in-vessel laboratory systems. In outdoor composting (Table 2) and laboratory in-vessel trials (Table 3), we show that both *E. coli*. and *Salmonella* survive similarly where 60^{o}C temperatures have been recorded for long periods of time. This data suggests that opportunistic pathogens adapt to become thermoresistant strains in composting.

Table 2. Survival of *E. coli* and *Salmonella* during outdoor composting and curing of source separated municipal solid wastes.

Compost Age (days)	Temperature of compost at time of sampling - C	MUG test [a]	DNA-Probe [b] E. coli/Shigella	DNA- Probe *Salmonella* [c]
15	59	+	na [d]	+
22	60	+	na	+
29	62	+	na	+
44	62	+	na	+
56	40	+	+	–
90	40	–	–	–

[a] Tested for *E. coli* in a lauryl sulfate tryptone broth containing 4-Methylumbelliferyl-B-D-Glucoronide (MUG) for the presence of glucoronidase
[b] Gene probe kit for *E. coli/Shigella*, Gene-Trak, Framingham, MA
[c] Gene probe kit for *Salmonella*, Gene-Trak, Framingham, MA
[d] na = not analyzed

A critical question is whether surviving mutants actually are pathogenic. Another important question is whether it is possible to shift the populations to effective and safe organisms. Clearly, if heat is not the mechanism which removes pathogens, then other routes like bacterial competition must be given more attention.

Our observations using phenotypic methods (Stager & Davis, 1992) to identify bacteria have shown that in thermophilic composts where the starting material originates from human waste many known potentially pathogenic species can be found to be predominant. Included are species in the families *Enterobacteraceae* and *Pseudomonadaceae* including *Escherichia coli, Serratia marcesens, Citrobacter freundii, Klebsiella pneumoniae, Pseudomonas aeruginosa, Pseudomonas pseudoalcaligenes, Pseudomonas alcaligenes, Alcaligenes fecalis* and *Acinetobacter genospecies 15*. An *E. coli* which is capable of growing at temperatures of at least 65C was isolated and its identity proven by phenotypic and DNA-probe methods. The trait proved to be unstable, and was lost in cold storage (Droffner & Brinton, 1994).

We have observed *Pseudomonas aeruginosa* as the most prevalent organism in a thermophilic food waste composting operation whereas other Pseudomonads not demonstrated to be pathogenic to humans are found in the thermophilic manure composts (Brinton & Droffner, *unpublished data*). Gram positive bacteria found in thermophilic compost are *Staphylococcus cohnii, Staphylococcus sciuri, Staphylococcus hominis, Enterococcus gallinarum, Bacillus thuringiensis/cereus, Bacillus sphaericus* and *Bacillus brevis*. The majority of these isolates will form mats of growth between 53-60 C, but formation of individual colonies is rare at these elevated temperatures.

It should be noted that some of these surviving organisms have been found to be very important for phyllospheric disease control with plants (Ketterer & Schwager, 1992). This sug-

Table 3. Survival of *E. coli B* and *Salmonella typhimurium Q* inoculated at 10^7 /gr in laboratory in-vessel composting of food waste and municipal biosolids, as determined by DNA probes.

Waste Sample Analyzed	Temperature	*E. coli / Shigella* METHOD I [a]	*E. coli / Shigella* METHOD II [b]	*Salmonella* [c]
Food waste Control	22°C	–	na	–
Food compost day 0 [d]	22°C	+	na	+
Food Compost day 7	5 days at 60-70°C	+	na	+
Food Compost day 11	9 days at 60-70°C	+	na	–
Food Compost day 20	8 days at 50-60°C	–	na	–
Control- uncomposted sludge	22°C	–	–	+
Sludge compost day 0 [d]	22°C	–	+	+
Sludge compost day 7	5 days @ 60–70°C	+	na	+
Sludge compost day 11	9 days @ 60°C	+	na	+
Sludge compost day 20	8days @ 60° down to 30° C	–	–	+

[a] Method I: E. coli selected for by growing directly on lauryl tryptose broth
[b] Method II: E. coli selected for by growing in nutrient broth with 0.5% lactose for 24hrs followed by lauryl tryptose broth
[c] Salmonella was grown in nutrient broth with 0.5% lactose then selected for with tetrathionate broth or selenite cysteine broth and grown in GN (HAJNA) broth
[d] Compost was inoculated with about 10^7 /g E. coli and 10^7 /g Salmonella typhimurium Q
(+) = positive; (–) = negative; na = not analyze

gests that methods can and should be developed to control microbial infections using other bacteria, once the various organisms are identified, and provided they are not found to be human pathogens.

The potential pathogenicity of strains isolated from composts has never to our knowledge been assessed. With *Pseudomonas aeruginosa*, for example, the presence of toxin A can be measured. Furthermore, with *Enterobacteraceae*, genes for adhesion and invasion of mammalian cells can be measured to assess pathogenicity.

An example of the potential value of applying new phenotypic methods is in potato farming. Significant new concerns have been raised by Maine growers about the proliferation of new virulent strains of *Phytophora infestans* resistant to many modern fungicides. New technologies are needed for combative efforts. *Pseudomonas* spp. have been found to act as biological control agents against pathogenic fungi which are common in field crops (Carruthers, et.al. 1994). Our data from phenotypic screening show these bacteria are dominant in certain types of composts. Thus, it may be possible to develop effective biological strategies for the control of *Phytophora infestans*. This would be a boon to farmers and support the growing interest in sustainable, biologically-oriented agriculture.

The Need to Identify Bacteria Performing the Composting

Previously, data has been collected suggesting that bacterial pathogens are not capable of surviving the composting process (Wiley, 1962; Wiley & Westerberg, 1969; U.S. EPA, 1985; US EPA, 1988). However, our work shows that *E. coli* and *Salmonella* can survive beyond PFRP up to 50 days in compost piles with 60^{o}C recorded temperatures. Furthermore, *E. coli* can become one of the most prominent organisms during composting. Moreover, especially in food waste composts, *Pseudomonas aeruginosa* can also become a dominant organism (Brinton & Droffner, *unpublished data).*

We have observed that *E. coli* can sometimes not be demonstrated in secondary wastewater biosolids. However, they will overcome the inhibitory effects, presumably caused by surfactants, and subsequently grow during composting (Droffner & Brinton, 1994). In view of this, some consideration is needed to evaluate what "negative" results mean. When pathogens are not found, it does not necessarily mean they are absent. While some studies have concluded that pathogen re-growth can occur (Burge et.al., 1987) the possibility that the organisms may have been present all along, but not detected must be evaluated (Droffner & Brinton, 1992).

Our data strongly indicates that studies are needed to identify the bacteria performing the composting. Furthermore, it would be helpful to know if bacterial types are controlled or selected for by the form of technology used for composting. Before more technology development in composting takes place requiring large capital expenditures, it would be prudent to determine what the predominant bacteria degrading selected wastes are and whether composting can result in proliferation of potential human or plant pathogens. If proliferation exists, then new measures to assure the safety of the product and to bring about suitable control need to be developed. Alternatively, the identification of bacteria which prevent the growth of plant and human pathogens should be extremely useful to composting technology. We expect that collection of information of this kind will be of great value to the general public and will be useful in developing superior technologies and diagnostic products.

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