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Metabolic Activity of Pathogenic Bacteria during Semicontinuous Anaerobic Digestion

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In natural environments such as anaerobic digesters, bacteria are frequently subjected to the stress of nutrient fluxes because of the continual changes in the flow of nutrients, and to survive, they must be capable of adapting readily to nutrient changes. In this study, the metabolic activities of *Escherichia coli*, *Salmonella typhimurium*, *Yersinia enterocolitica*, *Listeria monocytogenes*, and *Campylobacter jejuni* were studied within culture bags (Versapor-200 filters, 0.22-μm pore size) in laboratory anaerobic digesters. The metabolic activity of these bacteria was indicated by their adenylate energy charge (EC) ratios and their ability to incorporate [^{3}H]thymidine, which was related to the respective changes in viable numbers within the culture bags during anaerobic digestion. Fluctuations in the adenylate EC ratios, the uptake of [^{3}H]thymidine, and the viable numbers of *E. coli*, *S. typhimurium*, *Y. enterocolitica*, and *L. monocytogenes* cells were probably due to constant changes in the amount of available nutrients within the anaerobic digesters. The viability of *S. typhimurium* increased quickly after a fresh supply of nutrients was added to the system as indicated by the uptake of [^{3}H]thymidine and an increase in the adenylate EC ratios. The viable numbers of *E. coli*, *S. typhimurium*, *Y. enterocolitica*, and *L. monocytogenes* organisms declined rapidly from 10^7 to 10^8 CFU/ml to 10^3 to 10^4 CFU/ml and remained at this level for an indefinite period. The decimal reduction time calculated during the period of exponential decline ranged from 0.8 to 1.2 days for these bacteria. *C. jejuni* had the greatest mean decimal reduction time value (3.6 days). This bacterium had adenylate EC ratios of less than 0.5 during anaerobic digestion, although the adenylate nucleotide concentrations in the cells were much greater than those in the other enteric cells. The results show that the enteric bacteria investigated probably exist in transient states between different stages of growth because of fluctuating nutrient levels during anaerobic digestion.

In natural environments, bacteria are frequently subjected to the stress of nutrient fluxes which may be due to either the partial or the complete absence of substrates. Fluctuations in nutrient availability may lead to a transition between balanced growth and either unbalanced or complete cessation of growth. This is known as the starvation-survival state, which has been defined as "the physiological state of bacteria due to an insufficient level of nutrients to supply energy for growth and reproduction" (19). Bacteria have evolved different starvation-survival mechanisms which allow them to survive within low-nutrient environments (6, 18, 20, 26). Enteric bacteria such as *Escherichia coli*, *Salmonella enteritidis*, and *Campylobacter jejuni* have a continual supply of nutrients within the mammalian gut. However, it has been reported that these bacteria have a viable but nonculturable state under stressed conditions which enables them to survive in low-nutrient environments such as soil and water (11, 25, 27, 31).

Bacteria can survive starvation if they maintain their cellular processes with energy obtained from endogenous substrates such as storage materials, RNA, and protein (14). Cells containing storage granules are reported to be more viable after a period of starvation than those which have none (7, 14). *E. coli* and *Salmonella typhimurium* utilize glycogen preferentially before degrading RNA or protein under starvation conditions. It has been reported that DNA per biomass increases in *Arthrobacter* spp. under starvation conditions since energy is obtained from the endogenous degradation of cellular components (23). The energetic status of bacteria is indicated by their adenylate energy charge (EC) ratio, which is determined by the response to the EC of enzymes catalyzing ATP-regenerating and ATP-utilizing reactions.

Anaerobic digesters are transient-state environments because of continual changes in the flow of nutrients. To function within such an environment, bacteria must be capable of adapting readily to nutrient changes. In an earlier study it was reported that after a rapid decline in viable numbers, from 10^7 to 10^8 CFU/ml to 10^3 to 10^4 CFU/ml within 6 to 8 days, a period followed in which there was no further $\log_{10}$ reduction in the viable numbers of *E. coli*, *S. typhimurium*, *Yersinia enterocolitica*, and *Listeria monocytogenes* organisms (13). Viable populations (10^3 to 10^4 CFU/ml) of these pathogenic bacteria could be maintained indefinitely during semicontinuous anaerobic digestion. Mesophilic anaerobic digestion had little effect in reducing the viable numbers of *C. jejuni* organisms.

The aim of this study was to determine the physiological state of *E. coli*, *S. typhimurium*, *Y. enterocolitica*, *L. monocytogenes*, and *C. jejuni* after their viable populations had appeared to decline to a steady viable number during semicontinuous anaerobic digestion. Bacterial cultures were suspended in 0.22-μm-pore-size filter culture bags within the anaerobic digesters. Samples were removed, and adenylate EC ratios and the incorporation of [^{3}H]thymidine into a trichloroacetic acid (TCA)-insoluble fraction by metabolically active cells were related to respective changes in viable numbers of pathogenic

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bacteria. Short-term changes are expected in the bacterial populations during semicontinuous anaerobic digestion, particularly after the digesters are fed. This was examined by monitoring the diurnal changes in the physiological state of *S. typhimurium* during anaerobic digestion.

MATERIALS AND METHODS

Bacterial strains. *S. typhimurium* 0035 (human fecal isolate), *Y. enterocolitica* CP4 (bovine waste isolate), and *L. monocytogenes* LM1 (human fecal isolate) were obtained from P. N. Levett. *C. jejuni* 4451, isolated from poultry litter, was obtained from B. Macfarland, Veterinary Research Laboratories, Belfast, United Kingdom. *E. coli* K-12 *strA* was constructed by P1cm transduction from *E. coli* K-12 DP1059 *strA* (obtained from D. Pinney, London, United Kingdom) into *E. coli* K-12 wild type (NCIMB 10083) (17). Stock cultures of all bacterial strains were freeze-dried in horse serum (Sigma) containing inositol (7% [vol/vol]).

Media. The growth conditions and media used in this study have been described previously (13).

Anaerobic digesters and culture bags. Laboratory anaerobic digesters were constructed with 1.0-liter culture vessels (Quickfit) fitted with a multisocket flange lid containing five ports sealed with silicone bungs. They were inoculated with sludge (200 ml) obtained from a full-scale anaerobic digester and were subsequently fed daily at a 25-day hydraulic retention time. Biogas produced during anaerobic digestion passed through a tube connected to a bottle (1.0 liter) containing $NaH_2PO_4 \cdot 2H_2O$ (2.0 M) acidified (pH 2.0) with concentrated H_2SO_4. Biogas production was directly related to the amount of $NaH_2PO_4 \cdot 2H_2O$ solution displaced per day. The digesters were mixed by being shaken each day and maintained at 35°C.

The metabolic activity of the pathogenic bacteria in pure culture was studied within culture bags in the laboratory anaerobic digesters. The culture bags (30 ml) were made from Versapor-200 filters (0.2-μm pore size; Gelman Sciences) which were double heat sealed and also sealed with lasso tape. Polytetrafluorethylene tubing passed from each culture bag through a silcone bung and subsequently through the ports in the lids of the digesters to a peristaltic pump (Schuco, London, United Kingdom). Conditions within the anaerobic digester had no apparent effect on the culture bag's integrity after 1 month.

Substrate. Beef cattle slurry was the substrate for the anaerobic digesters. The cattle were fed on grain and silage from October to May, and during the summer, they were put to pasture. Slurry collected from the farm was stored at 4°C and subsequently diluted to 5 to 10% total solids (TS) prior to feeding of the digesters. pH was in the range of 7.0 to 7.4. All slurry fed to the digesters was pasteurized at 60°C for 30 min (13).

Analytical methods. The performance of the laboratory anaerobic digesters was assessed daily by monitoring biogas production and percentage of methane present. Headspace samples (10 ml) were analyzed with a Shimadzu GC-9A gas chromatograph fitted with a gas sampling port and aligned to a Chromatopac GR-3A data processor. The column was a 2.0-m double-wound glass column packed with Chromosorb 106 (Phase Separations Ltd.). During analysis, the carrier gas (N_2) flow rate was 50 ml/min, and the column temperature was 30°C. Each sample was analyzed in triplicate, and the percentage of methane present was determined from a methane calibration curve. Biogas production varied between 510 and 620 ml/day, which contained 42 to 50% methane.

TS and volatile solids of the slurry and anaerobic digester samples were determined according to the standard methods for wastewater analysis (1). TS and volatile solids of the anaerobic digesters ranged from 4.1 to 5.6% and 22 to 31%, respectively. pH and redox potential (E_h) measurements of slurry and anaerobically digested liquid samples were determined with a pH meter (Philips model PW 9418) with an Ag-AgCl microelectrode and a platinum microelectrode, respectively. The pH meter was calibrated with two standard buffer solutions of pH 4.0 and 7.0 at 20°C. The E_h electrode was calibrated with a ferrous-ferric solution (15). The pH of the anaerobic digesters was 7.6, and E_h ranged from −320 to −350 mV.

Adenylate nucleotide measurements. Samples (5.0 ml) of mid-log and stationary-phase cells of *E. coli*, *S. typhimurium*, *Y. enterocolitica*, and *L. monocytogenes* were centrifuged (2,500 relative centrifugal force; MSE MicroCentaur Centrifuge; MSE, Manor Royal, Crawley, United Kingdom) for 5 min, resuspended in 1.0 ml Ringer's solution (25% [vol/vol]; Oxoid Ltd.), and filtered (Whatman type B, 1.0 μm). Stationary-phase cells of *C. jejuni* and bacterial samples from the culture bags were treated similarly. Adenylate nucleotides were extracted from the filters in Tris-EDTA buffer (pH 7.5; Sigma) and stored at −20°C (8).

The adenylate nucleotides present in the Tris-EDTA buffer extracts were determined in triplicate as described previously (12). Each Tris-EDTA buffer extract (200 μl) was gently mixed with the assay reagents and enzymes in a photometer tube and inserted into the counting chamber of an ATP photometer (Lumac Cell Tester 1030). The luciferin-luciferase reagent (Sigma) was automatically injected into the photometer tube, and the peak light emission was displayed as relative light units (RLU) after 10 s. Background emission from the luciferin-luciferase reagent and Tris-EDTA blanks was also recorded. ATP, ADP, and AMP (Sigma) calibration curves were prepared by plotting standard concentrations (1 to 5 μM/ml) against RLU.

The net RLU of each sample was determined by subtracting the Tris-EDTA buffer blank and the luciferin-luciferase reagent blank from that of each sample. The concentration of ATP in each extract was directly related to the mean net RLU value. The ADP concentration of each extract was determined by subtracting the mean ($n = 3$) RLU of ATP from that of the ATP-ADP samples. Similarly, the AMP content of each extract was determined by subtracting the mean RLU of ATP+ADP from that of the ATP+ADP+AMP samples. The concentrations of ATP, ADP, and AMP present in each sample were determined by relating the mean RLU ($n = 3$) of each extract to the respective adenylate nucleotide concentration on the calibration curve. The adenylate EC ratios of the pathogens were calculated (2).

Incorporation of [^{3}H]thymidine. [^{3}H]thymidine incorporation by bacterial cells was determined as previously described (9). Each sample (600 μl) was incubated with [^{3}H]thymidine (21 Ci/mmol; Amersham). At time zero, 100-μl subsamples were pipetted in triplicate onto glass fiber filters (Whatman type C) fitted in a filter manifold (Millipore). An equal volume of ice-cold 10% (wt/vol) TCA (BDH, Poole, United Kingdom) was added and left for 5 min. TCA-insoluble material was collected by vacuum filtration and rinsed with ice-cold 5% TCA (wt/vol). The remaining subsamples (100 μl) were extracted after 10 min of incubation by using 10% and 5% TCA as described above, and the filters were left to dry. [^{3}H]thymidine incorporated by the bacteria was measured in 3.0 ml of PCS (Amersham) scintillant and counted for 5 min on a Packard Tri-Carb liquid scintillation spectrometer. A quench curve was determined by the channel ratio method in which

chloroform (Aldrich Chemical Co.) was used as a quenching agent (5). The incorporation of [^{3}H]thymidine by each sample at 0 h (T_0) was subtracted from that after 10 min (T_{10}). The mean disintegrations per minute and standard deviations of triplicate samples were determined.

Experimental procedure. *E. coli* and *S. typhimurium* were grown aerobically in nutrient broth (Oxoid) at 37°C. *Y. enterocolitica* was grown under similar conditions at 30°C, and *L. monocytogenes* was grown in brain heart infusion broth at 37°C, whilst *C. jejuni* was grown under microaerophilic conditions in cooked meat broth (Oxoid) at 42°C. The metabolic activity of each bacterium was examined in triplicate anaerobic digesters.

A maximum of four culture bags were present within each anaerobic digester. Early-stationary-phase cultures of *E. coli*, *S. typhimurium*, *Y. enterocolitica*, *L. monocytogenes*, and *C. jejuni* were pumped into culture bags within the anaerobic digesters. One culture bag in each digester was inoculated with *S. typhimurium*, which acted as a control inoculum. The decimal reduction time (T_{90}) defined as the time taken for viable counts of a population to decrease by 1 logarithmic unit ($\log_{10}$) was calculated during the exponential decline of each bacterial population from a linear regression equation (13, 28, 30). Mean T_{90} values and standard deviations for each bacterium were calculated from triplicate cultures. T_{90} values among replicates were compared by a one-way analysis of variance, and the significance of these variations was assessed by the F test (13, 30). No significant differences were found among the T_{90} values of *S. typhimurium* in the different anaerobic digesters. This would imply that the slurry fed, and the conditions within each digester, had a similar effect upon pathogen survival. The bacterial cultures were sampled at 1-, 4-, and 8-h intervals each day after the anaerobic digesters were fed. The viable bacteria were enumerated on their respective agars (13). After the initial rapid decline in viable numbers was observed (days 0 to 7), daily changes in the adenylate EC ratios and the incorporation of [^{3}H]thymidine were examined in the 4-h samples of *E. coli*, *S. typhimurium*, *Y. enterocolitica*, *L. monocytogenes*, and *C. jejuni*. Differences among the mean T_{90} values of these enteric bacteria were assessed by comparing their regression coefficients by an unplanned comparison test (GT-2 method). Upper and lower comparison intervals were calculated for each regression coefficient by Gabriel's approximate method. Two survival curves were significantly different if their 95% comparison intervals did not overlap (13, 30).

The effect of slurry additions on the viability of *S. typhimurium* was examined in both fed and unfed anaerobic digesters. Stationary-phase cells of *S. typhimurium* were pumped into three culture bags within two laboratory anaerobic digesters. On day 10, the EC ratios and the incorporation of [^{3}H]thymidine by *S. typhimurium* were examined in the fed and unfed anaerobic digesters.

RESULTS

Decimal reduction times. The decline in viable numbers of *S. typhimurium* 0035 organisms as shown in Fig. 1 is typical also of the decline in viable numbers of *E. coli* K-12 10083 *strA*, *Y. enterocolitica* CP4, and *L. monocytogenes* LM1 cells during semicontinuous anaerobic digestion. This was also reflected by the fact that there were no significant differences among the mean T_{90} values of these bacteria, which ranged between 0.8 and 1.2 days (Table 1). After a period (6 to 8 days) of rapid decline in viable numbers, a point was reached at which the rate of decline was zero and residual viable populations of approximately 10^4 CFU of *S. typhimurium* per ml were maintained within the culture bags during semicontinuous anaerobic digestion (Fig. 1). Similarly, viable populations of approximately 10^3 CFU of *E. coli*, *Y. enterocolitica*, and *L. monocytogenes* per ml were maintained within the culture bags. In contrast, the viable numbers of *C. jejuni* declined by less than 2 $\log_{10}$ units within a similar period, and thereafter, the viable numbers remained at approximately 10^6 CFU/ml within the culture bags during semicontinuous anaerobic digestion (Fig. 1). *C. jejuni* had a significantly greater mean T_{90} value (3.6 days) than *S. typhimurium*, *Y. enterocolitica*, or *L. monocytogenes* (Table 1).

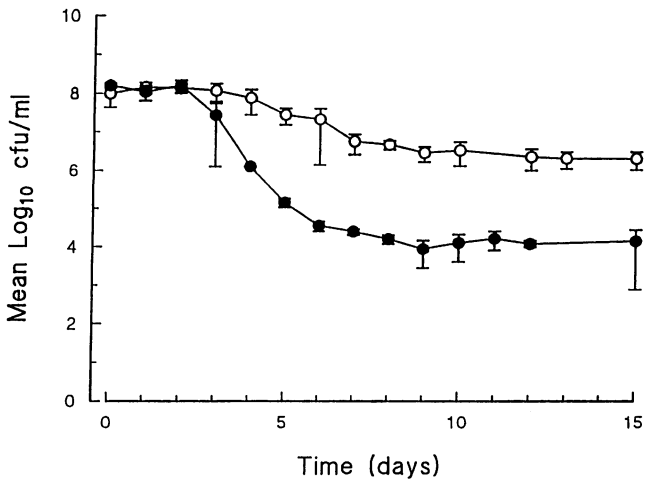

FIG. 1. Reduction in viable numbers of *C. jejuni* (○) and *S. typhimurium* (●) during semicontinuous anaerobic digestion. Each point is the mean of three replicates, and standard deviation bars are shown.

Metabolic activity of the pathogenic bacteria. The endogenous metabolism of bacteria declines rapidly when growth is reduced as indicated by the adenylate EC ratio in this study (Table 2). However, DNA synthesis continued for a period. On day 8 and thereafter, the viable populations of *E. coli*, *S. typhimurium*, *Y. enterocolitica*, and *L. monocytogenes* remained at approximately 10^3 to 10^4 CFU/ml within the culture bags. These bacteria also appeared to have similar metabolic activities during semicontinuous anaerobic digestion. The viable numbers of *E. coli* cells increased slightly on day 8 during the 4-h period after the anaerobic digesters were fed (Table 2). During this period, their metabolic activity was comparable to that of stationary-phase cells (Table 3). The viable numbers of *E. coli* cells did not appear to change between days 9 and 11 although the mean adenylate EC ratio indicated a loss in viability (Table 2). On day 12, the viable numbers of *E. coli* cells increased slightly, and this corresponded to a mean adenylate EC ratio of 0.6. On day 9, the mean adenylate EC

TABLE 1. Mean decimal reduction times ($\bar{x}T_{90}$) of the pathogenic bacteria within culture bags during semicontinuous anaerobic digestion

Organism	Mean T_{90} (days)	u[a]	l[a]
Escherichia coli K-12 10083 *strA*	1.2	1.7	0.9
Salmonella typhimurium 0035	1.1	1.6	0.9
Yersinia enterocolitica CP4	0.9	1.3	0.4
Listeria monocytogenes LM1	0.8	1.4	0.6
Campylobacter jejuni 4451	3.6	25.0	1.6

[a] u and l, upper and lower limits of the mean T_{90}, respectively.

TABLE 2. Viability of pathogenic bacteria within culture bags during semicontinuous anaerobic digestion

Organism	Time (days)	Mean viable no. (10^3 CFU/ml) (± SD)	Mean EC ratio (± SD)	[^{3}H]thymidine mean dpm (± SD)
Escherichia coli	8	9.1 (3.3)	0.52 (0.35)	170 (82)
	9	3.5 (2.8)	0.43 (0.37)	214 (16)
	10	5.2 (2.3)	0.30 (0.28)	138 (41)
	11	3.4 (1.3)	0.21 (0.11)	392 (95)
	12	3.8 (0.5)	0.60 (0.51)	230 (110)
Salmonella typhimurium	8	5.5 (4.5)	0.46 (0.35)	333 (31)
	9	6.2 (5.7)	0.54 (0.40)	217 (59)
	11	4.6 (3.4)	0.12 (0.15)	423 (43)
Yersinia enterocolitica	8	8.8 (5.3)	0.32 (0.10)	143 (35)
	9	7.4 (9.3)	0.43 (0.23)	232 (102)
	10	3.6 (3.3)	0.44 (0.25)	282 (114)
	11	9.9 (5.2)	0.40 (0.15)	164 (58)
Listeria monocytogenes	8	8.8 (5.3)	0.67 (0.20)	244 (38)
	9	1.9 (1.8)	0.48 (0.23)	205 (90)
	10	1.4 (0.5)	0.53 (0.22)	270 (15)
	11	1.1 (0.4)	0.64 (0.28)	214 (13)
Campylobacter jejuni	9	1.5 (1.4)[a]	0.29 (0.07)	ND[b]
	10	1.3 (0.7)[a]	0.21 (0.07)	ND
	13	2.0 (0.9)[a]	0.35 (0.10)	ND
	14	1.9 (0.9)[a]	0.19 (0.01)	ND

[a] 10^6 CFU/ml.
[b] ND, not detected.

ratio and the quantity of [^{3}H]thymidine incorporated by *S. typhimurium* cultures were comparable to those of their stationary-phase cells (Table 3). The viable numbers of *S. typhimurium* organisms also declined between days 9 and 11, and this was reflected by a low mean EC ratio on day 11 although [^{3}H]thymidine was incorporated by the cells (Table 2). Bacterial samples taken quickly after the digesters were fed may have shown higher EC ratios than those reported. The large standard deviations for the adenylate EC ratios of *E. coli*, *S. typhimurium*, *Y. enterocolitica*, *L. monocytogenes*, and *C. jejuni* are probably due to variations in the replicate samples from culture bags within different anaerobic digesters.

The viable numbers of *Y. enterocolitica* cells increased within the culture bags in the 1-h samples after the anaerobic digesters were fed but had declined in the 4-h samples during days 8 to 11. Thus, the adenylate EC ratios of *Y. enterocolitica* were constantly low (<0.5) in this study although the cells did incorporate [^{3}H]thymidine (Table 2). Stationary-phase cells of *Y. enterocolitica* also had a low EC ratio (0.33), but they were capable of incorporating [^{3}H]thymidine in amounts similar to those of those cells within the culture bags sampled 4 h after the digesters were fed (Tables 2 and 3).

TABLE 3. Viability of the pathogenic bacteria during mid-log and stationary growth phases

Organism	Mid-log cells		Stationary-phase cells	
	EC ratio	[^{3}H]thymidine (dpm)	EC ratio	[^{3}H]thymidine (dpm)
Escherichia coli	0.70	20,390	0.50	680
Salmonella typhimurium	0.70	20,737	0.51	204
Yersinia enterocolitica	0.86	8,147	0.33	170
Listeria monocytogenes	0.86	21,888	0.52	890
Campylobacter jejuni	NT[a]	NT[a]	0.50	ND[b]

[a] NT, not tested.
[b] ND, not detected.

The mean adenylate EC ratios of *L. monocytogenes* ranged from 0.48 to 0.67 between days 8 and 11 (Table 2). On day 8, the cells had a mean EC ratio of 0.67, and approximately 8.8×10^3 CFU/ml were present within the culture bags. On day 9, the viable numbers of *L. monocytogenes* organisms declined to 1.9×10^3 CFU/ml, which correlated with a decline of the mean EC ratio to 0.48 (Table 2). The metabolic activity of *L. monocytogenes* within the culture bags as indicated by the adenylate EC ratios and the uptake of [^{3}H]thymidine was similar to that of stationary-phase cells (Table 3).

C. jejuni was maintained at approximately 10^6 CFU/ml within the culture bags (Fig. 1). The mean adenylate EC ratios ranged from 0.2 to 0.4 between days 9 and 14 (Table 2), which were slightly less than that obtained for stationary-phase cells (Table 3). However, the levels of the adenylate nucleotides in *C. jejuni* cells were much greater than those present in the other enteric bacteria examined in this study. The concentrations of AMP, ADP, and ATP were 1.55, 2.3, and 1.5 μM/ml, respectively, in stationary-phase cells of *C. jejuni*, and in samples obtained from culture bags the concentrations were 1 to 5.5, 1 to 8, and 0.5 to 3 μM/ml, respectively. *E. coli* cells sampled from the culture bags contained 0.25 to 2 μM, 0.2 to 0.6 μM, and 0.2 to 1.4 μM AMP, ADP, and ATP per ml, respectively, and this was representative of the adenylate nucleotide concentrations found in cells of *S. typhimurium*, *Y. enterocolitica*, and *L. monocytogenes*. Both stationary-phase cells of *C. jejuni* and cells sampled from the culture bags were found to be unable to incorporate [^{3}H]thymidine (Tables 2 and 3).

Daily changes in the metabolic activity of *S. typhimurium*. Viable numbers of *S. typhimurium* cells declined rapidly up to day 8 within culture bags present in two anaerobic digesters. After this period, the numbers remained at approximately 10^3 CFU/ml. The mean T_{90} values were 1.3 and 1.5 days. Both digesters were treated similarly up to day 10. Biogas production by both digesters was 520 to 540 ml/day and the methane content was 49%. pH of both digesters was 7.6, and the E_h was −330 mV. TS and volatile solids were 4.5 to 5.8% and 20 to 22%, respectively, in the two anaerobic digesters. On day 10, fluctuations in the viable numbers and metabolic activity of *S. typhimurium* were examined in which one digester was fed and the other was not.

The changes in viable numbers of *S. typhimurium* organisms in both fed and unfed anaerobic digesters during day 10 are shown in Table 4. Two hours after feeding the digester, the viable numbers of *S. typhimurium* increased. This corresponded with an increase in the adenylate EC ratio from 0.12 to 0.76, and a small amount of [^{3}H]thymidine was incorporated by the cells (Table 4). The viable numbers declined between 2 and 4 h, and this corresponded with a decline in the EC ratio to 0.28 in the 4-h samples. However, at 24 h, the EC ratio had increased to 0.57, [^{3}H]thymidine continued to be incorporated by the cells, and the viable numbers in the fed digester had increased slightly (Table 4). Viable numbers of *S. typhimurium* cells within the unfed anaerobic digester declined during day 10, although by 24 h a slight increase was observed. The adenylate EC ratio of these cultures remained below 0.5, and no [^{3}H]thymidine was incorporated by the cells during day 10 (Table 4). AMP concentrations (0.5 to 1.3 μM/ml) of the cells present in the unfed digesters were very high in comparison with either ADP (0.33 to 0.38 μM/ml) or ATP (0.2 to 0.4 μM/ml) levels. Viable numbers of *S. typhimurium* cells increased in both digesters after they were fed on day 11.

TABLE 4. The effect of nutrient supply on the metabolic activity of *S. typhimurium* during anaerobic digestion

Anaerobic digester	Time (h)	Mean viable no. (10^3 CFU/ml) (± SD)	Mean EC ratio (± SD)	[^{3}H]thymidine mean dpm (± SD)
Fed	0	10.0 (7.3)	0.12 (0.03)	ND[a]
	2	13.0 (11)	0.76 (0.32)	45 (18)
	4	3.8 (0.8)	0.28 (0.23)	61 (17)
	24	4.0 (0.7)	0.57 (0.41)	156 (38)
Unfed	0	19.0 (2.7)	0.43 (0.23)	ND
	2	7.5 (3.0)	0.31 (0.19)	ND
	4	7.4 (3.0)	0.18 (0.14)	ND
	24	8.5 (1.7)	0.35 (0.15)	ND

[a] ND, not detected.

DISCUSSION

The rate at which the viable numbers of the enteric bacteria declined during semicontinuous anaerobic digestion appeared to be dependent upon the bacterial species and the availability of nutrients within the system. *E. coli*, *S. typhimurium*, *Y. enterocolitica*, and *L. monocytogenes* are all capable of utilizing carbohydrates and therefore must compete with the other nonmethanogenic bacteria for these nutrients. Hence, the rapid decline in viable numbers after inoculation may have been due to an inadequate supply of available nutrients to support viable populations of 10^7 CFU/ml, although sufficient nutrients were present to support viable culturable populations of approximately 10^3 CFU/ml. In contrast to the other bacteria, *C. jejuni* utilizes amino acids and vitamins which are released during the breakdown of proteinaceous material and dead cells (29). Thus, there may be less competition for these substrates during anaerobic digestion, and this may help to explain why higher viable numbers (10^6 CFU/ml) of *C. jejuni* were present.

The mean T_{90} values of both *E. coli* and *S. typhimurium* in this study are comparable to those in other reports in which these bacteria had T_{90} values of 1 to 2 days and 2 to 4 days, respectively, during mesophilic anaerobic digestion (13, 21, 22, 28). In an earlier study, it was reported that in a mixed system *Y. enterocolitica*, *L. monocytogenes*, and *C. jejuni* had mean T_{90} values of 2.5, 35.7, and >71 days, respectively. In this study, the mean T_{90} values of these bacteria were significantly lower within the culture bags than within the mixed system (13). A similar effect was reported in which *E. coli* and *S. typhimurium* survived for longer periods when added to slurry than within nylon bags during full-scale anaerobic digestion (22). This effect may be due to a lower supply of nutrients available to the bacteria within the culture bags compared with the level of nutrients available to the cells in direct contact with the organic material within the anaerobic digester. Bacteria adhering to organic material are suitably positioned to utilize the nutrients released during its breakdown, and this would increase their viability (3).

During semicontinuous anaerobic digestion, daily changes were observed in the viable numbers and metabolic activity of *E. coli*, *S. typhimurium*, *Y. enterocolitica*, and *L. monocytogenes*. These changes were probably related to variations in the amount of available nutrients present in the slurry which was fed to the anaerobic digesters each day. When the anaerobic digesters were not fed, the adenylate EC ratios of *S. typhimurium* were less than 0.5, and the cells were unable to incorporate any [^{3}H]thymidine. This would suggest that the cells present were not metabolically active. In contrast, 2 h after the semicontinuous anaerobic digesters were fed, an increase in viable numbers of *S. typhimurium* organisms was observed. The adenylate EC ratio was 0.71, although an EC ratio of 0.8 is indicative of growth. It is possible that the EC ratio was 0.8 or higher during the 0- to 2-h period between feeding the digester and sampling. A small amount of [^{3}H]thymidine was incorporated by *S. typhimurium* cells fed on day 10, which probably reflects DNA replication, so it would appear that the bacteria respond quickly to an increase in nutrients. However, the viable numbers of *S. typhimurium* organisms at 24 h on day 10 were considerably less than that at 0 h. This may have been due to a lower level of available nutrients in the slurry fed to the digesters on day 10. In addition, other nonmethanogenic bacteria and the methanogenic bacteria also respond quickly to fresh nutrients as a large increase in gas production was observed after feeding mesophilic anaerobic digesters (16). This is due to an increase in the fermentation of substrates resulting in an increase in hydrogen, carbon dioxide, and acetate concentrations from which methane is subsequently produced.

Changes in the amount of [^{3}H]thymidine incorporated by cells, and the adenylate EC ratios of *E. coli*, *S. typhimurium*, *Y. enterocolitica*, and *L. monocytogenes* from day to day, probably reflect changes in the levels of available nutrients within the anaerobic digesters. Under growth conditions, the adenylate EC ratio of *E. coli* is 0.8 (4). However, during stationary or starved conditions this ratio decreases to 0.5 or less, respectively. In this study, the metabolic activity of *E. coli*, *S. typhimurium*, *Y. enterocolitica*, and *L. monocytogenes* during anaerobic digestion was similar to that of their respective stationary-phase cells. The adenylate EC ratios of these bacteria fluctuated above and below 0.5 from day to day. In contrast, the EC ratios of *C. jejuni* remained below 0.5, although the cellular adenylate nucleotide concentrations were greater than those in the other enteric bacteria. The higher T_{90} value of *C. jejuni* and its low EC ratio along with its inability to incorporate [^{3}H]thymidine indicate physiological characteristics that differ from those of the other enteric bacteria studied under anaerobic digestion conditions. The other enteric bacteria examined all incorporated [^{3}H]thymidine after their viable populations remained at 10^3 or 10^4 CFU/ml. It is likely that, in the presence of available nutrients, DNA replication is initiated. Bacterial cells within the culture bags may have fluctuated between a viable and culturable state and a viable but nonculturable state. *E. coli*, *S. typhimurium*, and *C. jejuni* cells can transform themselves from a viable culturable state to a viable nonculturable state when exposed to nutrient-limited environments (10, 24, 25, 27).

It can be concluded that enteric bacteria are maintained at a constant population level after a period during semicontinuous anaerobic digestion. Bacterial cells within this environment probably exist in transient states between different stages of growth because of fluctuations in nutrient levels. Growth occurred quickly after a fresh supply of nutrients was added to the anaerobic digesters as indicated by the uptake of [^{3}H]thymidine and the increase in adenylate EC ratios.

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