Recovery and Survival of *Escherichia coli* O157:H7 in Reconditioned Pork-Processing Wastewater†

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MS 98-244: Received 17 September 1998/Accepted 17 February 1999

ABSTRACT

The pathogen *Escherichia coli* O157:H7 has been recovered from various water sources and food samples. The growth potential of this bacterium in nutrient-limited, reconditioned wastewater from a pork-processing plant was determined over a temperature range of 4 to 46°C. Even though the biological oxygen demand of the wastewater was <2 mg/liter, results of bioassays for assimilable organic carbon and the coliform growth response of the water suggested that sufficient nutrients were present to support limited bacterial growth. A three-strain mixture of *E. coli* O157:H7 grew over the temperature range of 10.2 to 29.4°C. Bioassays appear to be a good indicator of the ability of this wastewater to support growth of this pathogen. Statistically higher levels of bacterial growth ($P < 0.05$) were detected on a nonselective medium (tryptic soy agar) than on a selective medium (sorbitol–MacConkey agar), suggesting that stress or injury of the bacterium occurs when the organism is exposed to the nutrient-limited conditions of the wastewater. These results indicate that *E. coli* O157:H7 can survive and grow in this particular nutrient-limited wastewater, suggesting a potential hazard if this water becomes contaminated with this pathogen.

Reclamation and reuse of wastewater in agricultural processes are used as a means of extending water supplies and decreasing water fees. Reconditioned wastewater applications range from crop, pasture, and recreational irrigation to supplementing potable water supplies in food-processing plants *(6, 28)*. There is no national standard regulating these applications *(25)*. The use of reconditioned water in U.S. food-processing plants is approved by governmental agencies, such as the U.S. Department of Agriculture, on a process-by-process basis *(25, 28)*, whereas irrigation use is approved on a state-by-state basis *(6, 28)*. Rose *(25)*, in a review of the U.S. Department of Agriculture policy and conditions to be met for use of recycled water in meat and poultry plants, lists areas in the process where recycled and reuse water is approved for use. Other food-processing areas in which reuse is allowed include egg, butter, and vegetable washing and aquaculture operations *(19)*.

To ensure the safety of reconditioned water, a residual chlorine level should be maintained *(19)*. Palumbo et al. *(18)* reported that *Aeromonas hydrophila*, a waterborne pathogen, could grow in pork plant reconditioned water after the residual chlorine was inactivated by thiosulfate. Rajkowski et al. *(21)* reported that unchlorinated pork plant reconditioned water could support the growth of *Salmonella* spp. and *Vibrio cholerae* spp. The possible contamination of reconditioned water by *Escherichia coli* O157:H7 and its use for agricultural purposes poses a potential for disease outbreaks *(3)*. In their summary of waterborne disease outbreaks during 1989 through 1990, Herwaldt et al. *(9)* reported that an *E. coli* O157:H7 outbreak associated with drinking water was responsible for four deaths. Rice et al. *(23)* reported that *E. coli* O157:H7 from this outbreak could survive in the unchlorinated drinking water up to 70 days at 5°C and had a more rapid die-off at 20°C. Other *E. coli* O157:H7 isolates were shown to also survive in many water types *(30)*. In view of the occurrence and survival of *E. coli* O157:H7 in drinking water and other aquatic environments and the resulting public health concerns, we investigated the growth and survival of *E. coli* O157:H7 in reconditioned wastewater. We investigated the recovery of this bacterium from the unchlorinated, reconditioned wastewater using both selective and nonselective agar media over a temperature range of 4.2 to 45.6 ± 1°C. The relationship of growth to water quality, as measured by bioassays of coliform growth response (CGR) and assimilable organic carbon (AOC), was examined.

MATERIALS AND METHODS

Growth determination. A three-strain mixture of *E. coli* O157:H7 was used for the growth studies. Strains 933, 45753-35, and A9218-C1 were obtained from the in-house culture collection of the Microbial Food Safety Research Units. Stock cultures of each strain were maintained in brain–heart infusion broth (Difco Laboratories, Detroit, Mich.), stored at 4°C, and transferred monthly.

The three strains were cultured individually overnight by transferring 0.1 ml of each culture to 50 ml of brain–heart infusion broth contained in a 250-ml Erlenmeyer flask, placed on a rotary shaker (model 35620, Lab-Line Instrumentation, Inc., Melrose Park, Ill.), and shaken at 150 rpm at 37°C. The overnight cultures were centrifuged at 3,300 × *g* for 15 min to concentrate the cells, and the supernatant fluid was decanted and discarded. To remove

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† Mention of brand or firm name does not constitute an endorsement.

TABLE 1. *Analysis of CGR and AOC in unchlorinated, reconditioned water*

Water sample	AOC (μg of carbon equivalents/liter)			CGR [log(N_5/N_0)]	BOD[c] (mg/liter)
	P-17[a]	NOX[b]	Total		
1	10	362	372	2.09	<2
2[d]	88	376	464	2.87	<2
3	58	351	409	2.74	<2

[a] *Pseudomonas fluorescens* P-17.
[b] *Spirillum* NOX.
[c] BOD, biological oxygen demand (determined by the company's processing plant).
[d] Representative results are presented for this water sample.

nutrients, cells were washed once and resuspended in sterile deionized water. The three strains of *E. coli* O157:H7 were combined and diluted in sterile deionized water to yield a concentration of 10^5 to 10^6 CFU/ml. A final dilution to yield 10^4 to 10^5 CFU/ml was made in the reconditioned wastewater sample.

Bioassay determinations. *Enterobacter cloacae* was used for the CGR bioassay. *Spirillum* NOX and *Pseudomonas fluorescens* P-17 were used for the AOC bioassay *(1)*. The inoculum was prepared as described by Rice et al. *(24)*.

Gradient growth temperature study. On the day of the study, unchlorinated, reconditioned wastewater was obtained from a local pork-processing plant with a wastewater treatment facility *(16, 21)*. The unchlorinated, reconditioned wastewater was transported immediately to the laboratory, where it was filter-sterilized using a 0.2-μm-pore Nalgene filter (Nalge, Rochester, N.Y.) and kept refrigerated until used in the growth studies. Separate water samples, taken at the same time, were sent chilled by overnight mail to the U.S. Environmental Protection Agency laboratory in Cincinnati, Ohio, for CGR and AOC bioassays. Biological oxygen demand results were provided by the company (Hatfield, Inc., Hatfield, Pa.) and were obtained according to prescribed methods *(1)*.

One liter of filtered, unchlorinated, reconditioned wastewater (FUR) was inoculated with the three strains of *E. coli* O157:H7 to achieve a starting level of 10^3 to 10^4 CFU/ml. After mixing, 12 ml of the inoculated FUR was transferred by pipette into two sets of sterile, L-shaped test tubes and then placed in the temperature gradient incubator, which contained 30 slots (model TN-3F, Advantec, Toyo Roshi International, Dublin, Calif.), with the gradient set between 3.5 and 55.3°C. Growth of *E. coli* O157:H7 was determined by surface-plating the duplicate samples from each L-shaped tube on tryptic soy agar (TSA; Difco) and sorbitol–MacConkey agar (s-MAC; Difco). Results from plating were averaged. Two trials were performed. In addition, recovery plating was performed on TSA and s-MAC, and the plates were incubated for 2 h at 37°C and then overlaid with s-MAC and TSA, respectively. All plates were incubated at 37°C for 18 to 24 h before counting. The gradient temperature range for each sample well was verified at the end of the study using the thermocouple sensor attached to the gradient incubator.

At the end of the growth study, the viability of the cultures was determined by using the LIVE/DEAD BacLight Viability Kit (Molecular Probes, Inc., Eugene, Oreg.) as described earlier *(21)*.

Statistical analysis. Analysis of variance was performed to determine the effect and interaction of media on bacterial growth. The analysis was performed separately for each time period. Calculations were performed using the general linear model procedure of the SAS/STAT software system *(26)*.

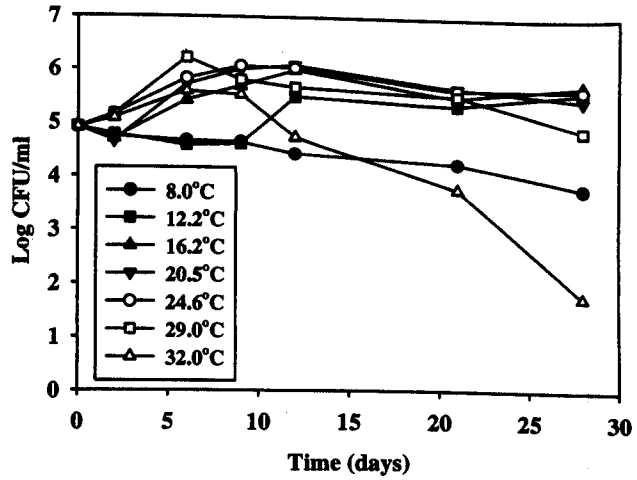

FIGURE 1. *Growth and survival of the three-strain* E. coli *O157:H7 mixture in filtered, reconditioned wastewater sample 2 held at temperatures of 8.0 to 32°C and counted on TSA.*

RESULTS

Results for CGR and AOC for the FUR wastewaters taken at different times and used in this study are presented in Table 1. FUR water sample 1 did not support the growth of the *E. coli* O157:H7 mixture. Cell counts dropped sharply from the initial inoculum level after 2 days of incubation at 4.9 and 22.7°C, whereas no cells were recovered after 2 days of incubation at temperatures above 23.7°C.

FUR water samples 2 and 3 supported growth of the three-strain mixture of *E. coli* O157:H7, as determined by an increase of 1 full log unit in the counts on TSA. The profiles of growth and survival between 4.2 and 45.6 ± 1°C in the FUR water were collected. The data for both trials were similar, and the results obtained with FUR water 2 are presented in Table 1. Representative growth TSA profiles are presented in Figure 1. The *E. coli* O157:H7 mixture remained viable and could be recovered for up to 28 days, but no growth occurred at temperatures between 4.2 and 9.1 ± 1°C and at 30.4 ± 1°C. Die-off occurred at temperatures above 32°C after 9 days. An increase of 1 log unit occurred between 10.3 and 29 ± 1°C. The cell population reached maximum density for the various temperate ranges at different times: 6 days for 27.5 to 29 ± 1°C, 9 days for 18.4 to 26 ± 1°C, 13 days for 12.2 to 17.4 ± 1°C, and 28 days for 10.3 to 11.2 ± 1°C.

Recovery of *E. coli* O157:H7 using the nonselective agar (TSA), selective agar (s-MAC), TSA with an overlay of s-MAC, and s-MAC with an overlay of TSA was compared. Representative recovery profiles for growth after 13 days for TSA and s-MAC are presented in Figure 2. The difference in recovery ranged from 0 to 42.2%. Statistical analysis showed that there was higher recovery using TSA over s-MAC ($P > 0.05$). Representative profiles comparing recovery between TSA and TSA plus s-MAC (Fig. 3A) and between s-MAC and s-MAC plus TSA (Fig. 3B) showed

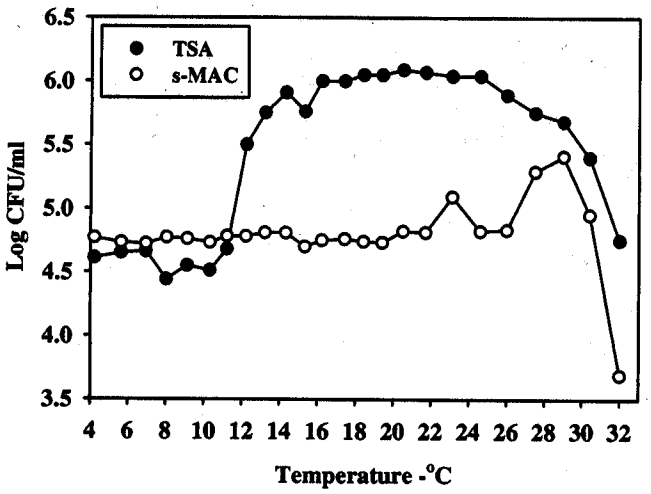

FIGURE 2. *Counts of the three-strain* E. coli *O157:H7 mixture after 13 days of growth in filtered reconditioned wastewater sample 2 held at temperatures of 4.2 to 32°C and recovered on TSA and s-MAC agars.*

no difference. There was no statistical difference in recovery.

FUR wastewaters in which no viable cells of *E. coli* O157:H7 were detected (minimum level of detection was <21 CFU/ml) were examined by using the BacLight kit. No viable cells were observed, indicating that the nonculturable cells were not viable.

DISCUSSION

Coliform bacteria, specifically *E. coli,* are the most frequently reported bacteria in water *(3).* These bacteria are used as indicators of water quality and pathogens contained in the water *(3).* Coliform bacteria and *E. coli* can survive and grow in drinking water *(5, 11, 13).* Rice et al. *(23)* reported the survival of *E. coli* O157:H7, which is associated with hemorrhagic colitis, in drinking water at a low temperature of 5°C with rapid die-off at 20°C using s-MAC as the recovery medium. Wang and Doyle *(30)* also reported survival at 8°C. Their findings of survival at low temperatures under nutrient-limiting conditions are consistent with the findings of this study. *E. coli* O157:H7 survived longer and reached maximum growth later in the FUR water at the lower temperature studied.

Survival of *E. coli,* coliform bacteria, and more recently *E. coli* O157:H7 *(30)* in environmental waters has been reported *(1, 12, 15, 29).* Using FUR water, another water source that can be used for irrigation and certain food plant processes, we were able to show that FUR water supports survival and growth of *E. coli* O157:H7. Previously, Rajkowski et al. *(21)* studied the growth of *Salmonella* spp. using a water sample with a CGR level of 2.09 and an AOC level of 372. The same water sample used in that study was used in this study and was labeled water sample 1. This water sample supported only survival of the *E. coli* O157:H7 mixture in the lower temperature range. Rice et al. *(24)* proposed that the water would support microbial growth when the CGR was ≥1, and LeChevallier et al. *(11)* reported that an AOC level of >100 μg/liter had 82% more

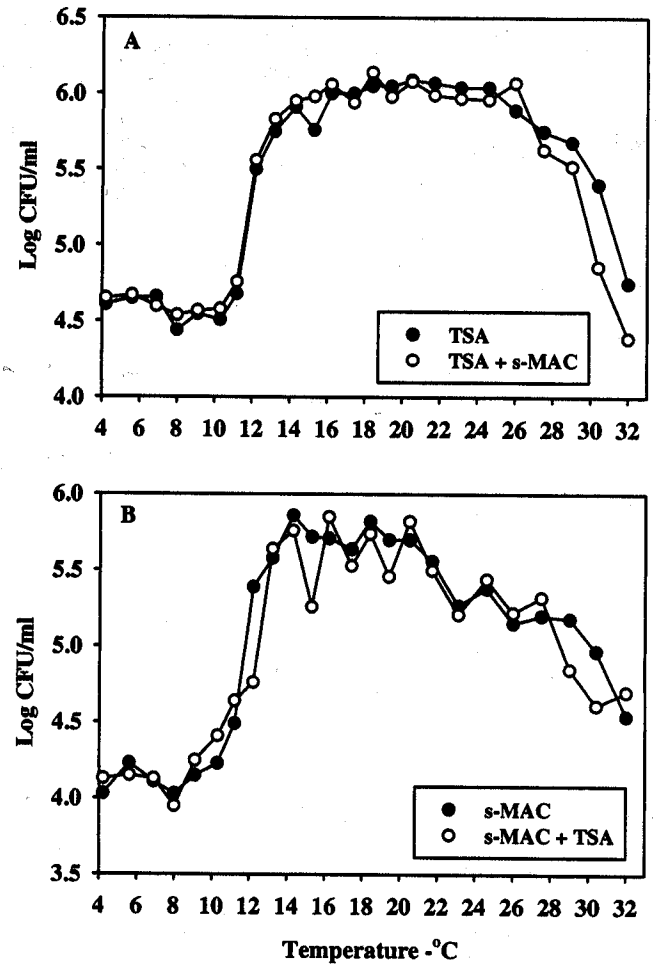

FIGURE 3. *Counts of the three-strain* E. coli *O157:H7 mixture after 13 days growth in filtered reconditioned wastewater sample 2 held at temperatures of 4.2 to 32°C as recovered on (A) TSA and TSA overlaid with s-MAC and (B) s-MAC and s-MAC overlaid with TSA.*

positive coliform samples. When the CGR was >2.7 and the AOC level was >409 μg/liter of the FUR water, the *E. coli* O157:H7 mixture grew. Lim and Flint *(12)* also reported increased survival time of *E. coli* in lake water (nutrient-limiting) with the addition of nutrients from a synthetic sewage source. Rice et al. *(24)* proposed that the bioassay for CGR and AOC is a better estimate of a water's ability to support the growth of pathogens.

E. coli O157:H7, retrieved from environmental water samples, were reported as injured cells because of nutrient deprivation *(10, 13, 14, 31).* When injured cells are isolated using selective agars, the bacteria are under additional stress. In this study, we obtained a statistically significant ($P > 0.05$) increase in recovery using TSA over s-MAC, which indicated that starvation stress could occur. Rajkowski and Dudley *(20)* reported that when *Salmonella* spp. and *Vibrio cholerae* were grown in FUR water, there was a statistical difference ($P > 0.05$) between recovery on TSA over the selective medium used for these pathogens. They also reported that the *Salmonella* spp. and *Vibrio cholerae* exhibited evidence of starvation stress *(20).* It was reported that for *E. coli* K-12 (a nonpathogenic strain) un-

der carbon-deficient conditions, the cells increase peptidase activity as a survival mechanism *(22)*. Hengge-Aronis *(8)* reported that for *E. coli* K-12 under starvation stress, the stationary-phase cell may develop resistance to additional stress factors. Gauthier and Clément *(7)* reported that short periods of starvation in oligotrophic waters may increase survival in human stomach acid, thereby possibly increasing the likelihood of disease in the human host. Some have suggested that the loss of the ability of starved *E. coli* to be recovered by plating may be due to the cell either not being viable or being viable and entering the nonculturable state *(8, 17, 27, 30)*. In our study, when no *E. coli* O157:H7 cells were recovered by plating on TSA, the cells were dead, as shown by BacLight, which measures cell membrane integrity. Loss of viability associated with the loss of membrane integrity indicated that the cells were indeed dead. Bogosian et al. *(4)* determined that when *E. coli* K-12 counts declined, the decline was due to death. Additional research is needed to identify whether *E. coli* can become viable but nonculturable in the nutrient-limiting water environment.

In conclusion, *E. coli* O157:H7 survived and grew in unchlorinated, reconditioned wastewater. Recovery was lower when the selective agar was used, suggesting starvation stress injury. Therefore, the residual chlorine levels required to prevent survival and growth of *E. coli* O157:H7 in water reconditioned for use in food-processing plants must be maintained.

ACKNOWLEDGMENTS

The author gratefully acknowledges the cooperation and assistance of personnel at Hatfield, Inc., who provided the reconditioned wastewater and biological oxygen demand results; John Phillips (Consulting Statistician, U.S. Department of Agriculture, Agricultural Research Service, North Atlantic Area), who conducted the statistical analysis; and James Patsy, who provided technical support.

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