Use of Selective Media to Recover *Salmonella* and *Vibrio cholerae* after Growth in Reconditioned Pork-Processing Wastewater†

KATHLEEN T. RAJKOWSKI* AND ROBERT L. DUDLEY

Microbial Food Safety Research Unit, U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, 600 East Mermaid Lane, Wyndmoor, Pennsylvania 19038, USA

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ABSTRACT

Selective plating media are used for the enumeration and isolation of bacterial pathogens from food and water samples. This study compared the quantitative recovery of *Salmonella* spp. and *Vibrio cholerae* grown in nutrient-limited, filter-sterilized, reconditioned wastewater over the temperature range of 4 to 45°C using nonselective and pathogen-specific selective media. Viable *Salmonella* were enumerated on tryptic soy agar (TSA) and XLT-4, and viable *V. cholerae* were enumerated on TSA and thiosulfate-citrate-bile-sucrose agar. There was a statistically significant ($P < 0.05$) higher recovery of both pathogens over the growth temperature range on TSA compared to the selective media. Trehalose, a stress-induced metabolite of *Salmonella*, was isolated from the cells grown in the reconditioned wastewater, whereas, the *V. cholerae* exhibited a change in cellular morphology from rod to coccoid shape. These results suggest that growth in nutrient-limited water injured or stressed the individual pathogens. Care should be used in choosing the procedure and plating medium for quantitative recovery of pathogens from such a nutrient-limiting environment.

The benefits of reclaimed wastewater usage are decreased water pollution and use costs, in addition to supplementing available water resources *(19)*. Applications of wastewater reuse include agricultural irrigation *(19)* and use by the food industry *(19, 25)*, especially the reconditioned process water from food-manufacturing operations *(25)*. The increased use of reclaimed water raises concerns about the occurrence and survival of pathogens in this environment *(26)*. For example, the survival and recovery of *Salmonella* spp. from surface water, weather pools, wastewater, sea water, and bottled water are well documented *(6, 18, 26)*. In addition, *Vibrio* spp. were reported to survive in drinking water, surface water, and ballast, bilge, and sewage *(4, 12)*.

The accurate identification and quantitative enumeration of these bacteria are essential to maintain public health. Yet stresses such as nutrient-limiting conditions *(4, 12, 18, 26)* may injure cells and may restrict the performance of traditional methods to identify these bacteria. In reviews on bacterial injury, Hurst *(14)* and Moriarty and Bell *(21)* reported that stress injury can occur when bacteria are grown in nutrient-limiting aquatic environments. Bissonnette et al. *(5)* and Hoadley and Cheng *(11)* reported that as a result of aquatic environmental stress, bacteria lost their ability to produce colonies on selective agars due to the inhibitory agents but produced colonies on nutritionally rich nonselective agars. Both suggest that a major proportion of the total population was physiologically injured as a result of exposure to the stresses of certain aquatic environments and emphasized that such damaged cells were not necessarily killed. However, the detection and enumeration of these injured survivors were impeded by the presence of inhibitory compounds in specific selective media *(5, 11)*. Under nutrient-limiting conditions as found in the water environment, *Salmonella (7, 9, 26)* and *Vibrio (12, 13)* are reported to lose the ability to form colonies during recovery on selective agars, and when these bacteria are grown under more severe (starvation) conditions, these injured or stressed cells were reported to enter a state of viable but nonculturable *(7, 15)*.

Injury is related to the severity of stress. Hoadley and Cheng *(11)* and Hurst *(14)* reported that the severity of the stress injury is related to the amount of actual cells recoverable using nonselective media. Injured cells may grow on nonselective media, but specific bacterial pathogens can only be quantified using a comparison of growth on selective media. The highly selective agar, XLT-4, and thiosulfate-citrate-bile-sucrose (TCBS) agar were developed for isolating salmonellae *(20)* and vibrios *(31)*, respectively. Information is limited as to the recoverability of injured *Salmonella* from a nutrient-deprived environment using XLT-4. West et al. *(31)* reported on the recovery rate of uninjured *Vibrio* using TCBS and tryptic soy agar (TSA) supplemented with 1% NaCl, but information is limited on the recovery rate of injured cells using TCBS. In addition to the recoverability of injured cells on selective media, Dupray et al. *(10)* reported that stressed *Salmonella* produced the intracellular osmoregulator trehalose under adverse growth conditions, which can be identified by nuclear mag-

* Author for correspondence. Tel: 215-233-6440; Fax: 215-233-6406; E-mail: krajkowski@arserrc.gov.

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netic resonance (NMR) analysis. Baker et al. *(4)*, Kjelleberg et al. *(15)*, and Novitsky and Morita *(22)* reported that nutrient-deprived *V. cholerae* undergo a visible cellular morphological change from rods to cocci under stress but are still culturable. In a previous study Rajkowski et al. *(26)* reported that both *Salmonella* and *Vibrio* grew under nutrient-limiting conditions in reconditioned plant wastewater with a coliform growth response >2. The objective of the present study was to determine if (i) there was a significant difference in recovery of stressed cells grown in nutrient-limiting reconditioned wastewater using the nonselective (TSA) and selective XLT-4 or TCBS agars, (ii) if *Salmonella* produced trehalose, and (iii) if *Vibrio* changed morphologically after growth under the nutrient-limiting conditions.

METHODS AND MATERIALS

Microorganisms. A three-strain mixture of either *Salmonella* sp. or *V. cholerae* was used for the growth studies. *Salmonella* Enteritidis was obtained from the Microbial Food Safety Research Unit's culture collection in this laboratory. *Salmonella* Typhimurium 798 and *Salmonella* Choleraesuis 38 were obtained from the U.S. Department of Agriculture National Animal Disease Center, Ames, Iowa. The stock culture of each serovar was maintained in brain-heart infusion broth (Difco Inc., Detroit, Mich.) and stored at 4°C. *V. cholerae* strains N16961-O1 Inaba-El Tor-toxagenic, O139 (non-O1)-1837, and O1-569B were obtained from the U.S. Food and Drug Administration, Washington, D.C. The stock cultures of each *Vibrio* strain were maintained in brain-heart infusion broth and stored at ambient temperature.

Preparation for the test inoculum of *Salmonella* or *Vibrio* was described earlier *(26)*. The final dilution of *Salmonella* or *Vibrio* was made in the test water to yield 10^3 to 10^4 CFU/ml.

Experimental design. On the day the study began, unchlorinated, reconditioned water was collected from a local pork-processing plant equipped with a wastewater treatment facility *(18)*. Water samples were shipped on ice to the Environmental Protection Agency laboratory, Cincinnati, Ohio for the coliform growth response and assimilable organic carbon bioassay studies *(3, 26)*. For use in the growth studies the unchlorinated reconditioned water was filter-sterilized using a 0.2-μm pore-size Nalgene filter (Nalge, Rochester, N.Y.). Sterility was confirmed by plating on TSA (Difco) before use.

Gradient growth temperature study. A liter of the filter-sterilized, unchorinated, reconditioned water (FUR) was inoculated with either the mixed culture of *Salmonella* or *Vibrio* to achieve a starting level of 10^3 to 10^4 CFU/ml. After mixing, 12 ml of the inoculated, unchlorinated, reuse water was distributed into a duplicate set of sterile L-shaped test tubes placed in the temperature gradient incubator (model TN-3F, Advantec, Toyo Roshi Inter., Co., Dublin, Calif.) containing 30 wells with the gradient set between 3.5 and 55.3°C. Growth of *Salmonella* was monitored at weekly intervals for up to 5 weeks by plating onto TSA and XLT-4 (Difco) agars and incubating the plates at 37 ± 1°C for 18 to 24 h before counting. *Vibrio* growth was monitored at intervals of 2, 6, 8, and 15 days by plating onto TSA and TCBS (Difco) agars and incubating the plates at 37 ± 1°C for 18 to 24 h before counting. The gradient temperature range of each well was verified at the end of the study using the thermocouple sensor attached to the gradient incubator. Two trials were performed for each organism.

Viability of the culture during the growth studies was determined by the LIVE/DEAD *Bac*Light Viability Kit (Molecular Probes, Inc. Eugene, Oreg.). The cells were concentrated by centrifugation at 2,000 × *g* for 15 min, resuspended in filtered-sterilized distilled water, and stained with the mixture of *Bac*Light nucleic acid stains. The stained cells were viewed under an Olympus BH2 Epifluorescence microscope (Olympus, Tokyo, Japan) fitted with a dichroic filter using blue fluorescence (λ = 490 nm).

NMR qualitative determination for trehalose from *Salmonella*. The *Salmonella* cells were extracted for trehalose by modifying the procedure of Dupray et al. *(10)*. At the end of the growth study, the *Salmonella* cells from the duplicate samples were combined to obtain a 3-ml sample. A control sample and the samples incubated at 28.4, 30, 32.1, 34.5, 37, and 39.4°C were extracted (only samples where a total volume of 3 ml was obtained). Instead of concentrating the cells by centrifugation *(10)*, the 3-ml samples were concentrated using a 0.2-μm pore-size Nalgene filter and rinsed twice with sterile deionized water, and the filtrates were discarded. The retentates were lysed with 2 ml of 10% trichloracetic acid (Fisher Scientific, Fair Lawn, N.J.) by placing the trichloracetic acid on the filter and filtering after 10 min contact time. A second extraction was done with a contact time of 5 min before filtering. The filtrate (total of 4 ml) was extracted with anhydrous ethyl ether (Mallinckrodt Chemical, Inc., Paris, Ky.) to remove the trichloracetic acid and the ether insolubles were evaporated to dryness. The *Salmonella* control consisted of the three-strain cocktail grown in brain-heart infusion broth at 37°C and processed as described above.

For NMR analysis the samples were prepared by dissolving the dry residue in 0.65 ml D_2O (Cambridge Isotope Laboratory, Andover, Mass.). Proton NMR spectra were obtained on a Varian Unity +400 NMR Spectrometer (Varian Associates, Palo Alto, Calif.) operating at 400 MHz. A presaturation pulse sequence was used to suppress the residual water peak in the spectrum. Typically 1,024 (9,600 data points) transients were collected for each spectrum. Each spectrum was collected at 30°C and the spectra were referenced using the water peak (4.73 ppm for trimethylsilane).

Electron microscopic examination of *Vibrio* cells. The *Vibrio* cells from the 32.9, 34.8, and 36.7°C were pooled and centrifuged at 2,500 × *g* to concentrate. Aliquots of 50 to 100 μl of the concentrated cells were adsorbed to the surface of freshly cleaned 12-mm-diameter glass coverslips. After drying, the coverslips were immersed in 2 ml of a 2% (vol/vol) glutaraldehyde–0.1 M imidizole buffer solution (pH 6.7) for 30 min followed by immersion in 2% osmium tetroxide–0.1 M imidazole buffer for 60 min and washed in distilled water for 30 min. After dehydrating in graded ethanol solutions, the coverslips were critically point dried. The samples were mounted on aluminum stubs using colloidal silver paste (Electron Microscopy Sciences, Ft. Washington, Pa.) and sputter coated with gold (LVC-76, Plasma Sciences, Inc., Lorton, Va.). Samples were viewed using a scanning electron microscope (model SEM-JSM 840A, JEOL, Peabody, Mass.), and digital images were collected using an Imix imaging workstation (Princeton Gamma-tech, Princeton, N.J.).

Statistical analysis. Analyses of variances were performed to determine the effect and interaction of media on bacterial growth. The analysis was performed for each time period separately. Calculations were performed using the general linear model procedure of the SAS/STAT software system *(28)*.

RESULTS

Gradient growth temperature study. Growth of the mixture of three *Salmonella* sp. occurred in the FUR water

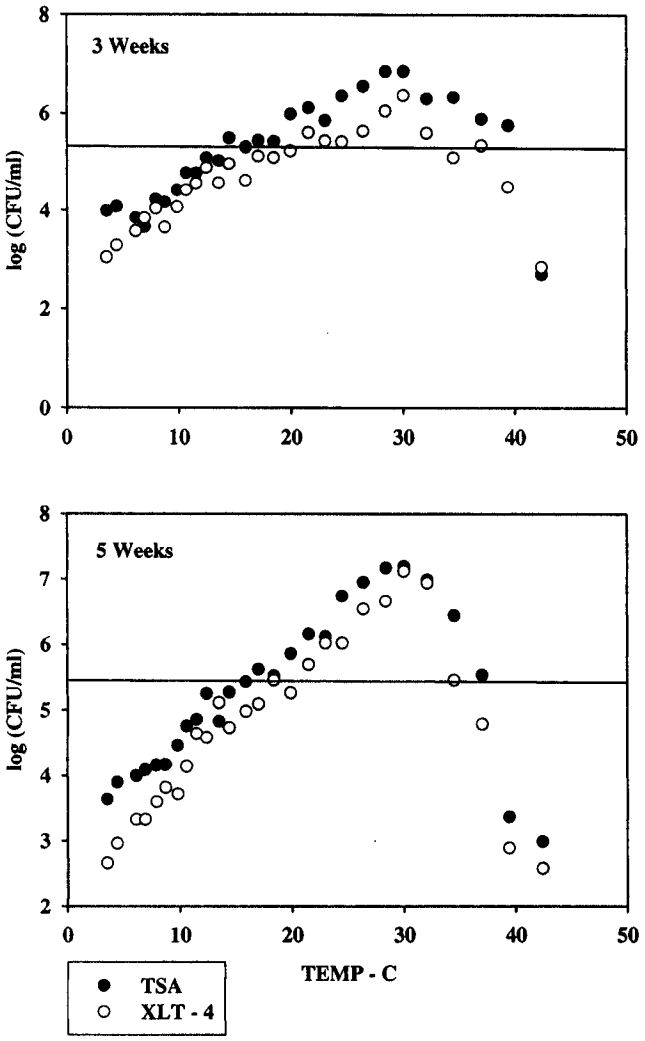

FIGURE 1. *Profiles of* Salmonella *serotypes after 3 and 5 weeks growth in filtered, prechlorinated, reconditioned wastewater over the temperature range of 3.5 to 42°C as recovered on TSA and XLT-4 agar.*

TABLE 1. *Percent difference between recovery of the three mixed serotypes of* Salmonella *on TSA and XLT-4 over temperature range of 4.4 to 46.4°C*

	Time in weeks				
°C	0	2	3	4	5
4.4	7.9	15.1[a]	19.6	21.4	24.1
5.1		11.4	16.5	18.1	22.5
6.1		9.2	7.3	16.4	16.8
6.9		13.5	4.7	13.8	12.0
7.9		12.7	4.5	9.0	8.2
8.7		15.4	12.5	11.1	10.8
9.8		16.2	7.9	11.1	7.2
10.6		13.2	7.5	6.7	2.5
11.5		3.8	4.6	4.4	5.8
12.4		11.6	4.1	3.9	2.7
13.5		2.1	9.3	4.5	2.1
14.4		3.1	9.6	2.5	5.5
15.9		1.5	13.2	4.3	6.3
17		5.3	5.9	1.5	3.0
18.4		3.8	6.1	3.7	4.7
19.9		4.2	12.5	4.8	2.9
21.5		2.1	8.3	2.9	2.3
23		1.3	7.2	1.4	1.4
24.5		6.9	14.8	4.6	3.0
26.4		5.8	14.0	0	4.2
28.4		5.4	11.8	2.6	0.7
30		6.7	6.7	3.6	3.5
32.1		20.2	11.1	17.1	5.3
34.5		29.4	19.4	25.0	15.3
37		—[b]	9.2	40.0	13.4
39.4		—[b]	22.1	20.6	14.2
42.4		3.2	5.3	11.4	13.7

[a] Statistically significant at $P > 0.05$ for all % differences.
[b] No growth on XLT-4 agar.

as measured by 1 log increase in CFU/ml enumerated on TSA was reported earlier *(26)*. The bioassay for the coliform growth response for the FUR used was 3.58 *(26)*. Cell counts for *Salmonella* grown in FUR after 3 and 5 weeks of incubation as enumerated on both TSA and XLT-4 are presented in Figure 1. The *y* axis is the actual cell count in CFU/ml and the *x* axis shows the gradient temperature range. The individual temperatures within the gradient incubator ranged from 4.4 to 46.4 ± 1°C and are listed in Table 1. The results above the solid line represent growth of the *Salmonella* in the FUR for the temperatures ranging from 14.4 to 39.4 ± 1°C. The number of *Salmonella* CFU/ml recovered was higher using TSA than the selective media, XLT-4. The differences in recovery for *Salmonella* are presented in Table 1. Statistical analysis showed that for all % differences there were higher recoveries on TSA than on XLT-4 ($P > 0.05$).

At the low temperature range (4.4 to 8.8°C) the cells just maintained their numbers and the difference in recovery is consistent. After 4 weeks at the higher temperature range (>32°C) differences in recovery begin to reflect the nonrecoverability on the selective agar. The differences in recovery on XLT-4 at both the high (>32°C) and low (4.4 to 8.8°C) temperature ranges were statistically higher ($P > 0.05$) when compared to the mid-growth range (9.8 to 30°C)

Growth of the three-strain cocktail of *V. cholerae* occurred over the temperature range of 10 to 37 ± 1°C as measured by 1 log increase in CFU/ml enumerated on TSA *(26)*. The bioassay for the coliform growth response for the FUR used was 2.07 *(26)*. Cell counts reported as CFU/ml (*y* axis) for the *V. cholerae* grown in FUR counted after 6 and 15 days of incubation are presented in Figure 2. Any point above the solid line represents growth of the *Vibrio*. The individual temperatures within the gradient incubator ranged from 4.7 to 51.1 ± 1°C (*x* axis in Fig. 2) and are listed in Table 2. The recovery on TSA in all cases was statistically significant ($P > 0.05$) and higher than the recovery on TCBS. For *V. cholerae* the % recovery differences are listed in Table 2.

Samples of *Salmonella* and *Vibrio* for those temperatures that did not produce colonies when plated on TSA (plate count <21 CFU/ml) were examined by the LIVE/DEAD *Bac*Light viability test kit. No viable cells were observed.

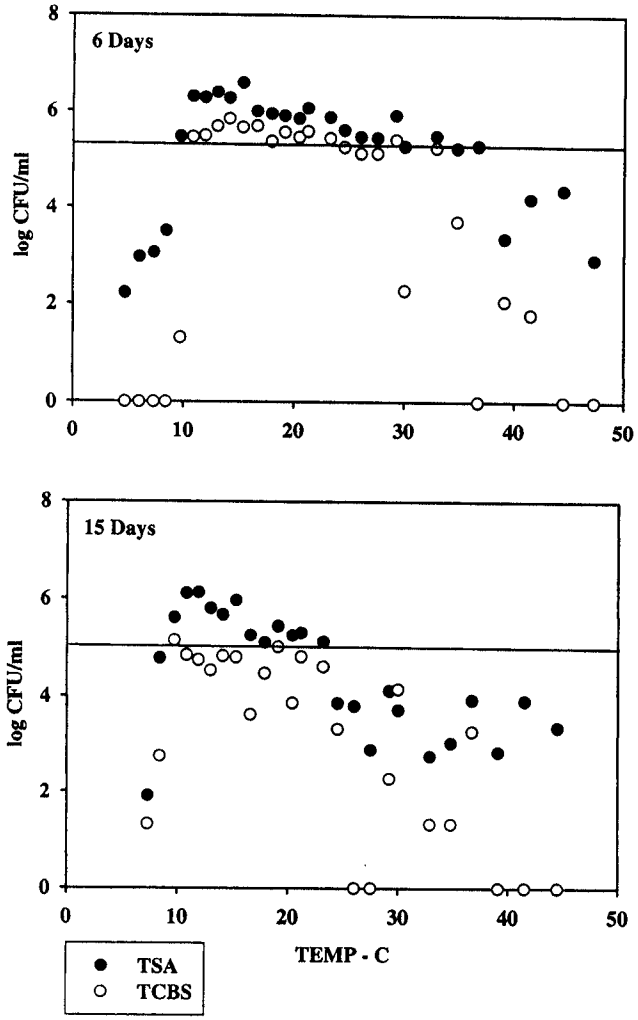

FIGURE 2. *Profiles of* V. cholerae *strains after 6 and 15 days growth in filtered, prechlorinated, reconditioned wastewater over the temperature range of 4.7 to 51.1 ± 1°C as recovered on TSA and TCBS agar.*

TABLE 2. *Percent difference between recovery of the three mixed strains of* Vibrio *on TSA and TCBS over temperature range of 4.7 to 51.1°C*

	Time in days				
°C	0	2	6	8	15
4.7	19.7	23.7[a]	—[b]	—	—
6		30.8	—	—	—
7.3		24.6	—	42.9	31.3
8.4		31.1	—	60.6	42.5
9.7		43.8	75.9	9.1	8.4
10.8		52.9	13.5	4.9	20.8
11.9		40.9	12.7	3.3	22.7
13		15.4	11.3	3.4	21.9
14.1		8.6	7.0	2.8	15.0
15.3		6.2	14.2	3.8	19.6
16.6		10.6	5.2	6.0	31.0
17.9		6.7	9.8	3.9	12.4
19.1		10.4	5.9	5.7	7.9
20.4		11.0	6.7	1.7	26.5
21.2		9.2	8.1	5.0	9.3
23.2		8.3	7.5	2.7	10.0
24.5		10.8	6.2	2.6	14.0
26		9.0	6.2	4.7	—
27.5		10.3	5.9	4.4	—
29.2		11.0	8.6	3.0	44.7
30		11.0	13.5	5.1	7.5
32.9		11.8	5.1	16.1	52.0
34.8		9.1	29.2	27.3	56.4
36.7		39.5	—	24.3	16.8
39.1		20.7	38.7	63.7	—
41.5		32.4	57.3	34.0	—
44.5		28.8	—	—	—
47.3		—	—	—	—
51.1		—	—	—	—

[a] Statistically significant at $P > 0.05$ for all % differences.
[b] — indicates no growth on TCBS agar.

Trehalose determination from *Salmonella.* Detectable signals for trehalose were obtained for the 39.4°C sample but not for the control (Fig. 3). The NMR spectra of extracts for all procedures tested showed dominant peaks corresponding to those for trehalose between 4 to 3.5 ppm, where ppm refers to the change in frequency relative to TMS. At the end of the growth study, the 39.4°C samples had decreasing cell counts on both the selective and nonselective media, and the presence of trehalose from these samples indicates injury to the cell. The samples incubated at 28.4, 30, 32.1, 34.5, and 37°C did not have detectable NMR signals for trehalose.

Electron microscopic examination of *Vibrio* cells. Using the scanning electron microscope, the control *V. cholerae* cells appeared as straight rods (Fig. 4, insert), whereas, the cells grown in FUR under nutrient-limited (starvation) conditions are no longer straight but appeared shorter and rounder with some being v-shaped, indicating stress to the cell (Fig. 4).

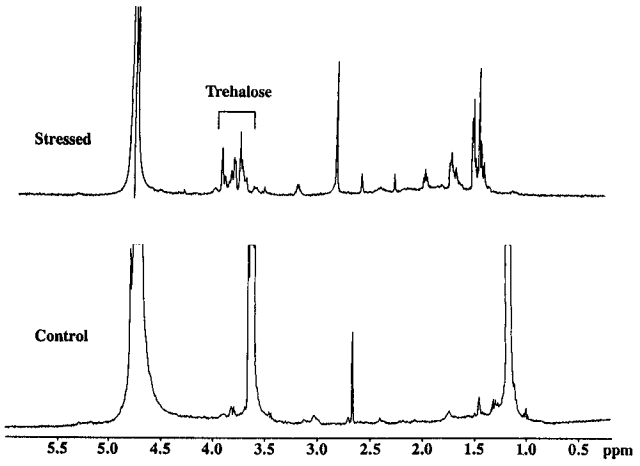

FIGURE 3. *Proton NMR spectra of a cell extract of the three-serotype mixture of* Salmonella *after 5 weeks growth in FUR. No trehalose peaks were present in extract from control cells. Trehalose peaks were present in extract of stressed cells.*

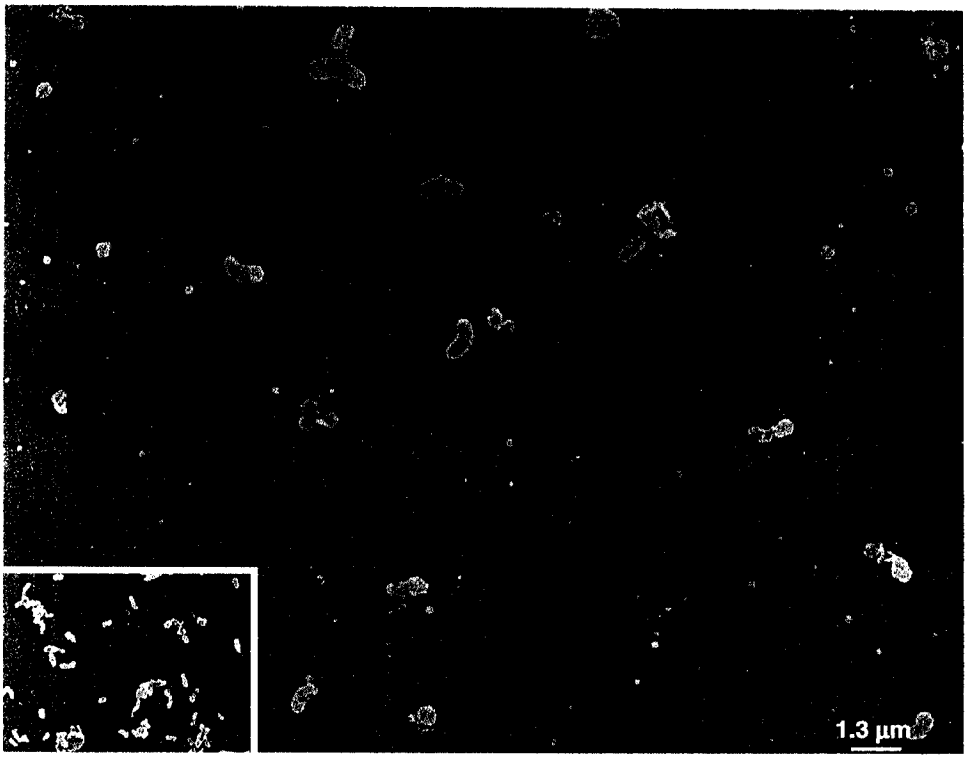

FIGURE 4. *Scanning electron micrographs of* V. cholerae. *Insert is one-fifth magnification and shows the control cells. Photomicrograph shows morphological changes in the cell structure.*

DISCUSSION

***Salmonella* recovery.** The *Salmonella* serotypes used in this study survived and grew in the FUR with a coliform growth response >2.0. Similar results of survival and growth in aquatic environments were observed for *Salmonella* Enteritidis *(7)* and *Salmonella* Typhimurium *(29)*. Even though survival and/or growth did occur in the nutrient-limiting environment of the waters, the results of the stress on the recoverability of the bacteria depend on the organism's culturability using selective and nonselective methods *(17, 27, 29)*. In his review of the various methods for detection of *Salmonella* in foods, Andrews *(2)* reported on the recovery of six selective plating media, including XLT-4 developed by Miller et al. *(21)*, which was used in this study. Overall Andrews *(2)* reported that XLT-4 had a better recovery rate than other selective agars. Yet, while we found that XLT-4 gave good recovery under the nutrient-limiting condition, recovery on XLT-4 was statistically significantly ($P > 0.05$) lower than on the nonselective agar. Roszak et al. *(27)* reported that for *Salmonella* spp. and Hoardly and Cheng *(11)* stated that for other indicator organisms, nonselective media were preferred over selective media to recover these bacteria from the water environment. McFeters *(17)* concluded that under laboratory conditions some bacteria grown and recovered from water containing limited nutrients were not recoverable on selective media. This decrease or inability to recover is attributed to the inhibitory ingredients of the selective media *(2, 5, 11)*. In addition, the decreased recovery is attributed to a stress response whereby the cells survive for long periods of time in the aquatic environments *(16, 26, 27)*. There are now reports on the stress response for *Salmonella* under starvation conditions *(10, 29)*.

In our study, trehalose was identified in the extracts of the *Salmonella* grown in the FUR, which indicates stress or injury from starvation. Dupray et al. *(10)* observed that there is intracellular synthesis of trehalose in *Salmonella* Manhattan as determined by NMR analysis of extracts after the cells were grown in wastewater. They report that the accumulation of trehalose is an osmoprotective compound produced by the cell as a survival mechanism. In our study, the identification of trehalose would explain the long-term survival of the *Salmonella* in FUR. It is implied *(10, 29)* that starvation stress is related to the production of the osmoprotectant through activation of *kat F*.

In addition to the synthesis of trehalose, there are reports that the viable-but-nonculturable state can occur in starvation-stressed *Salmonella* cells isolated from water *(27)*. In our study, we did not observe the state of viable but nonculturable. Microscopic examination after staining using the viability test kit showed that the cells were not viable. There are also reports by Baker et al. *(4)*, Lebaron and Joux *(16)*, and Thompson et al. *(30)*, that enrichment increases the recovery of the injured *Salmonella*, whereas, Chmielewski and Frank *(7)* and Roszak et al. *(27)* reported that the inability to recover with enrichment did not occur. In our study due to the limited initial volume, we were unable to perform any recovery procedure. This limited the study to just the quantitative difference between the media. Research is needed to determine the relationship between degree of stress and culturability on selective media, viable but nonculturable, and death.

***Vibrio* recovery.** The recovery of vibrios to prove their presence and survival in water environments is well documented *(4, 6, 23, 24)*. TCBS agar is the most widely used medium for recovery, but it does contain bile salts that can interfere with the outgrowth of slightly injured vibrios. Using *Vibrio* grown in broth culture, but not from cells grown in an aquatic environment, West et al. *(31)* reported on *Vibrio* recovery on TCBS from different manufacturers. Abbott et al. *(1)* compared TCBS and a nonbile-containing selective agar and claimed better recovery on the nonbile agar, but the disadvantage of using this agar was reduced colony size of *V. cholerae*.

In this study, we compared the recovery of *Vibrio* on TCBS and TSA after growth under starvation conditions and observed a statistically significant decrease ($P < 0.05$) in recovery on TCBS. This decrease occurred with increase in incubation time and at the high- and low-temperature gradient range. The *Vibrio* grew and survived at the lower temperature range. This observation of survival at the lower temperature in foods was reported by Corrales et al. *(8)*. Another survival mechanism that the vibrio uses under starvation stress conditions is morphological change in cellular shape from a straight rod to coccoid-shaped cells. Baker et al. *(4)*, Hood et al. *(12)*, and Östling et al. *(24)* reported on this change. In our study when the stressed cells were examined, we observed the change from rod- to coccoid-shaped with some bending to form v-shaped cells. In addition, Hood et al. *(12)* and Östling et al. *(24)* report that under starvation stress the vibrio's protein synthesis rate decreases. They *(12, 24)* proposed that certain lipids and carbohydrates may provide the endogenous energy sources needed for cell maintenance, and that under starvation stress the decrease in lipid and carbohydrate metabolism rate may affect cell wall and membrane integrity leading to the change in cell shape. Another mechanism for survival of vibrios in nutrient-limiting environments is the ability to enter the viable-but-nonculturable state *(14, 26)*. After microscopic examination of *Vibrio* cells using the stains provided in the viability test kit, we observed only dead cells, confirming our inability to enumerate cells. *V. cholerae* is reported to respond and grow with the addition of nutrients after entering the viable-but-nonculturable state due to starvation *(13, 27)*. Because of the limited volume used in the gradient incubator, we were unable to perform any enrichment methods to try to resuscitate the cells.

In conclusion, starvation-stressed *Salmonella* spp. and *V. cholerae* directly assayed on selective agar resulted in lower counts. Careful consideration should be taken in choosing the plating media for direct quantitative recovery of these pathogens from a nutrient-limiting environment without enrichment.

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