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Growth History Influences Starvation-Induced Expression of *uspA, grpE,* and *rpoS* and Subsequent Cryotolerance in *Escherichia coli* O157:H7

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ABSTRACT

In this study, we investigated the effect of starvation on cryotolerance of *Escherichia coli* O157:H7 grown in tryptic soy broth (TSB) and Luria-Bertani broth (LB). Starved cells (cells suspended in water at 37°C for 6 h) and control cells (cells in TSB or LB) were frozen at −18°C for up to 240 h in their respective growth media. The *E. coli* grown in TSB showed a greater starvation effect (the difference in percent survival of starved and control cells) and cryotolerance. The starved *E. coli* grown in TSB showed a 30% increase in their ability to survive frozen storage for 24 h at −18°C. The corresponding increase in survival for LB-grown *E. coli* was only 3.8%. Cryotolerance induced by starvation of TSB- and LB-grown *E. coli* was correlated with the expression of genes involved in general stress response pathways, such as *uspA, grpE,* and *rpoS.* The expression of *uspA, grpE,* and *rpoS* was quantified by measuring the green fluorescence generated from autofluorescent *E. coli* harboring p*uspA*::*gfp,* p*grpE*::*gfp,* and p*rpoS*::*gfp* gene fusions. The results obtained in this study indicate that *uspA, grpE,* and *rpoS* were induced on starvation when *E. coli* was grown in TSB, and their expression correlated well with subsequent induction of cryotolerance developed at −18°C. In contrast, cells grown in LB and subsequently exposed to starvation conditions showed no increase in expression of *uspA, grpE,* or *rpoS,* and, as expected, these cells did not exhibit increased cryotolerance at −18°C. Knowledge of molecular mechanisms involved in cross-protection might make it possible to devise strategies to limit their effects and lead to ways to predict the survival of foodborne pathogens in stressful environments.

Escherichia coli O157:H7 causes severe illness, including bloody diarrhea and renal failure *(19).* Foods of animal origin and many animals, including dairy cattle, deer, and sheep, are major reservoirs of the organism. Consequently, food manufacturers need effective means to process their products safely, yet economically. Efforts are continuing to develop models of pathogen survival during processing. However, their reliability is dependent on a thorough knowledge of the factors involved in bacterial cell death or survival *(2, 15, 20).* The composition of growth medium, growth phase, growth temperature, and prior exposure of bacterial cells to sublethal stresses has been reported to influence level of gene expression and subsequent survival *(1, 9, 11, 23).* In the developing field of predictive food microbiology, such effects are important.

Previous work has shown that the stress imposed by starvation altered the ability of *E. coli* O157:H7 to survive subsequent heat treatments *(23)* and that this thermal tolerance correlated with induction of the heat shock proteins UspA and GrpE. The results indicated that UspA plays an important role in starvation-induced thermal tolerance at 37°C, but at lower temperatures, GrpE might have a greater influence on this response. Similarly, osmotic shock caused a significant induction of the *uspA* gene and of *rpoS* (RNA polymerase sigma factor) at 37°C and room temperature, whereas osmotic shock at 5°C did not induce these genes *(6).*

It might be possible to develop mechanistic models describing the survival of bacteria in stressful environments by gaining knowledge of responses at the molecular level. To do this, information must be accumulated to determine how previous growth history can influence gene expression. Thus, the objective of this study was to investigate the effect of growth media on starvation-induced cryotolerance of *Escherichia coli* O157:H7 and its correlation with expression of heat shock genes *uspA* and *grpE* and the general stress response gene *rpoS.*

MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* O157:H7 (Ent C9490) *(14)* used in this study was isolated from a patient infected by the consumption of undercooked meat. The construction of plasmids p*uspA*::gfp_{uv}, p*grpE*::gfp_{uv}, and pBS-rrnBTrpo-SpUV containing, respectively, a fusion of *uspA, grpE,* and *rpoS* promoter region to the reporter gfp_{uv} gene was previously described *(5, 23).* The *promoterless*::gfp_{uv} plasmid in *E. coli* O157:H7 was used as a negative control.

Growth conditions. Frozen stocks maintained at −80°C were streaked on tryptic soy agar (TSA; BD Diagnostic Systems, Sparks, Md.) and LB agar (LBA; BD Diagnostic Systems) containing ampicillin at 50 μg/ml for p*uspA*::gfp_{uv} and p*grpE*::gfp_{uv}, or 100 μg/ml for p*rpoS*::gfp_{uv}, and incubated at 37°C. A single colony of *E. coli* was inoculated in 5 ml of both tryptic soy broth (TSB; pancreatic digest of 17 g/liter casein and enzymatic digest

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TABLE 1. *Primers used in this study*

Primer	Sequence (5′–3′)	Amplified region
UspAR	ACAATCAGCATATCAACGTG	*uspA* (437 bp)
UspAL	TGGCTTATAAACACATTCTCA	
TufAR	CTTAGCCAGTACCTGACCAC	*tufA* (700 bp)
TufAL	CATCAACACTTCTCACGTTG	

of 3 g/liter soybean meal, 2.5 g/liter dextrose, 5 g/liter sodium chloride, 2.5 g/liter dipotassium phosphate; BD Diagnostic Systems) and Luria-Bertani broth (LB; pancreatic digest of 10 g/liter casein, 5 g/liter yeast extract, 10 g/liter sodium chloride; BD Diagnostic Systems) containing 50 μg/ml ampicillin for p*uspA*::*gfp*$_{uv}$ and p*grpE*::*gfp*$_{uv}$ and 100 μg/ml for p*rpoS*::*gfp*$_{uv}$. The cultures were grown at 37°C for 16 to 20 h. Each culture was inoculated (10 μl) into 100 ml of TSB or LB in a 500-ml flask and grown at 37°C with shaking at 200 rpm for 16 h.

Starvation of bacteria. To simulate starvation conditions, *E. coli* cells were harvested by centrifugation at 3,840 × *g* for 10 min at 4°C and washed twice with sterile distilled water. The pellet was resuspended in pretempered distilled water at 37°C in a 500-ml flask. Bacteria suspended in water are considered starved, and those grown in TSB or LB served as controls. The control and experimental flasks were incubated at 37°C for 6 h with 200 rpm shaking *(23).*

Cryotolerance studies. After 6 h of starvation at 37°C, 1 × 10^6 cells (1 ml in 1.5-ml tubes; 1:1,000 dilution) were frozen in their respective growth media at −18°C for 24, 72, 120, and 240 h. After storage at −18°C, control and starved cells were thawed at room temperature and the viable count was estimated by surface plating 50 μl of the appropriate dilution prepared in 0.1% peptone water onto either TSA or LBA, followed by incubation of the plates at 37°C. Results were expressed in terms of percent survival and starvation effect (%) *(9).*

$$\text{Starvation effect} = \%\ \text{survival of starved cells} - \%\ \text{survival of control cells}$$

Sample treatment for measuring fluorescence. One milliliter of sample was taken after every 2-h interval for green fluorescence estimation and viable count. For green fluorescence measurement, approximately 10^9 cells (undiluted) were centrifuged at 16,000 × *g* for 3 min. The pellet was washed in 1 ml of distilled water twice and resuspended in distilled water. The fluorescence of each sample was measured with a Victor^2 1420 multilabel counter (Wallac Oy, Turku, Finland) at an excitation wavelength of 395 nm and an emission wavelength of 510 nm. Two hundred microliters of sample was added to one well of a 96-well black enzyme-linked immunosorbent assay plate, and the fluorescence intensity was monitored. Sampled bacteria were serially diluted with 0.1% peptone water and plated on TSA or LB to determine viable count as described earlier.

The data are reported as specific fluorescence intensity (SFI), defined as the fluorescence obtained from 10^9 CFU/ml *(6)* and change in fluorescence intensity, which is calculated as follows:

$$\text{Change in fluorescence intensity} = (\text{fluorescence for starved cells} - \text{fluorescence for control cells})_{t6} - (\text{fluorescence for starved cells} - \text{fluorescence for control cells})_{t0}$$

RT-PCR. Four 1-ml volumes of cells (10^7 CFU/ml, 1:100 dilution) were centrifuged at 16,000 × *g* for 3 min to pellet the cells. RNA was subsequently extracted with Trizol (Invitrogen, Gaithersburg, Md.) according to the manufacturer's direction. Reverse transcription (RT)–PCR was carried out as described earlier *(12)* with the following modifications. DNase treatment of the RNA sample was carried out at 37°C for 30 min. Extracted mRNA was serially diluted in molecular-grade water (undiluted and diluted 1:10, 1:100, 1:1,000, 1:10,000, 1:100,000) before RT-PCR to compare band patterns from control and starved cells. The PCR mix consisted of 45 μl of PCR Platinum Supermix (Invitrogen), 4 μl of a mixture of forward and reverse primers (2 pmol for each primer) (Table 1), and 2 μl of cDNA. PCR was conducted in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Foster City, Calif.). The amplification process consisted of 1 cycle of 94°C for 3 min; 35 cycles of 94°C for 45 sec, 55°C for 30 sec, 72°C for 45 sec; and a final extension at 72°C for 7 min. After amplification, the tubes were cooled to 4°C. Amplification products were separated on 1% agarose gels and visualized with a Gel Doc system (Bio-Rad Laboratories, Hercules, Calif.).

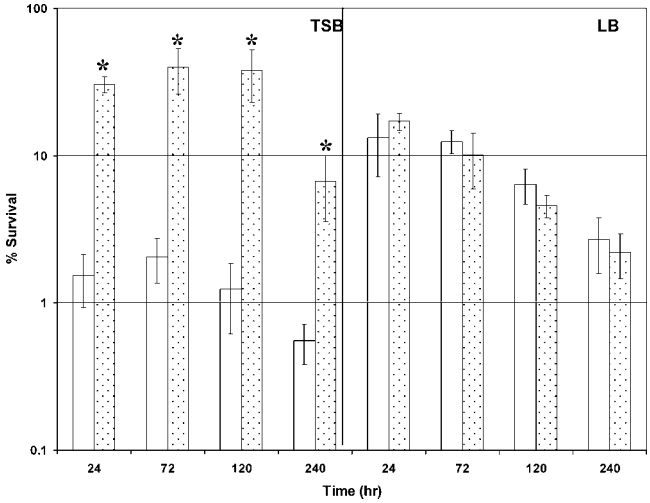

FIGURE 1. *Effect of 6 h of starvation on cryotolerance of TSB-grown and LB-grown* E. coli *O157:H7 at −18°C. Control (□), starved (⬚). Bars marked * showed significant increase (*$P < 0.01$*) in survival from the control.*

Statistical analysis. Experiments were carried out in triplicate, and the average values of the triplicate measurements are reported in ''Results.'' The statistical significance of differences among treatments was determined by Student's *t* test and was considered statistically significant at $P < 0.01$.

RESULTS

Effect of growth medium on starvation-induced cryotolerance of *E. coli* O157:H7. When *E. coli* grown in TSB was starved in distilled water at 37°C for 6 h before freezing, the ability to withstand subsequent freezing at −18°C was significantly improved compared with the control culture (Fig. 1). On the contrary, LB-grown *E. coli* did not show increased cryotolerance on starvation. The cryotolerance of *E. coli* increased from 1.5 to 30.6% after 24 h storage at −18°C when cells were grown in TSB and starved for 6 h at 37°C. The cryotolerance of LB-grown *E. coli* was much lower; 13.2 and 17.09% for control and starved *E. coli*, respectively.

Correlation between starvation-mediated cryotolerance and expression of general stress response genes in *E. coli* O157:H7. The change in cryotolerance determined

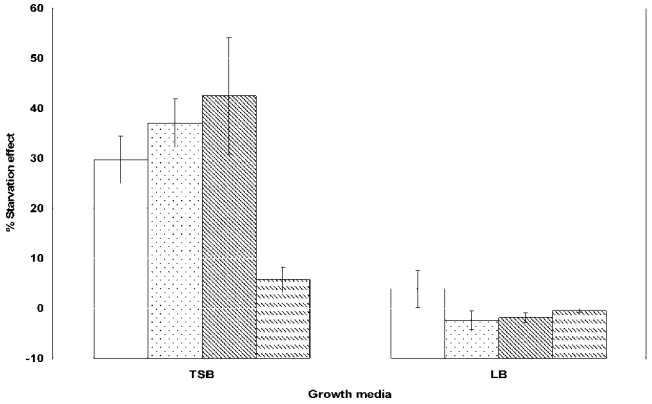

FIGURE 2. *Starvation effect after 24 (□), 72 (▨), 120 (▧), and 240 h (▩) of storage at −18°C of TSB-grown and LB-grown* E. coli *O157:H7. Starvation effect = % survival of starved cells − % survival of control cells.*

in terms of starvation effect (%; Fig. 2) of TSB- and LB-grown *E. coli* was compared with gene expression (Fig. 3) levels on starvation. The starvation effect was greatest for *E. coli* O157:H7 grown in TSB and was significantly larger than for LB-grown *E. coli.* When the period of freezing at −18°C was extended, both the cryotolerance and starvation effects (%) decreased significantly in TSB-grown *E. coli* after 120 h, but the cryotolerance and starvation effects (%) were only reduced marginally in LB-grown *E. coli* culture.

Specific fluorescence intensity of TSB- and LB-grown control and starved *E. coli* p*uspA*::*gfp*$_{uv}$, p*grpE*::*gfp*$_{uv}$, and p*rpoS*::*gfp*$_{uv}$ was monitored over a 6-h period. A significant increase in the fluorescence of starved cells was observed for TSB-grown *E. coli,* whereas fluorescence of LB-grown *E. coli* did not increase on starvation compared with respective control cells (Fig. 3). The change in p*uspA*::*gfp*$_{uv}$, p*grpE*::*gfp*$_{uv}$, and p*rpoS*::*gfp*$_{uv}$ expression on starvation, as measured by a change in fluorescence, was significantly higher in TSB-grown *E. coli* compared with LB-grown *E. coli* (data not shown). These results indicate that *uspA, grpE,* and *rpoS* could play an important role in starvation-induced cryotolerance of *E. coli* grown in TSB broth, whereas LB-grown *E. coli* did not show starvation-induced cryotolerance because *uspA, grpE,* and *rpoS* genes were not up-regulated on starvation.

The gene expression results were further confirmed by RT-PCR (data not shown). For *uspA,* it showed a maximum 12-fold increase in expression on starvation *(6).* Serial dilutions of total RNA indicated that under TSB growth conditions, *uspA* mRNA was detected through 1:100 dilutions for control cells and through 1:10,000 dilutions for starved *E. coli* cells, whereas following growth in LB, *uspA* mRNA was detected through 1:1,000 dilutions for control cells and through 1:100 dilutions for starved *E. coli* cells. RT-PCR results for the expression of *tufA* (positive RNA control), a housekeeping gene encoding EF-Tu in *E. coli,* were consistent among all treatments across replication.

Expression of *uspA, grpE,* and *rpoS* in *E. coli* O157:H7 grown on half-strength TSB and 1.5×-strength LB. *E. coli* O157:H7 was grown in 0.5× TSB and 1.5× LB

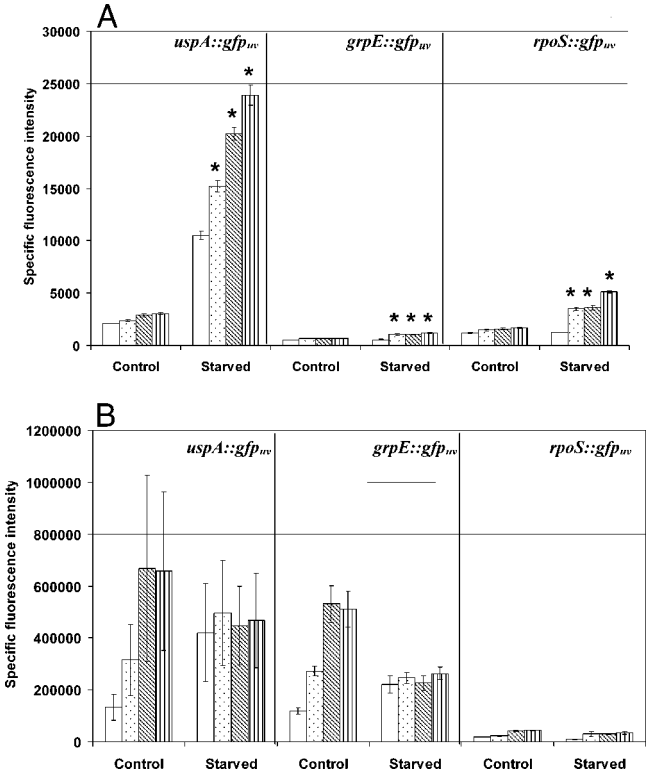

FIGURE 3. *Effect of 0 (□), 2 (▨), 4 (▧), and 6 h (▥) of starvation on expression of* puspA::gfp$_{uv}$, *p*grpE::gfp$_{uv}$, *and p*rpoS::gfp$_{uv}$ *in (A) TSB-grown and (B) LB-grown* E. coli *O157:H7. Bars marked * showed significant increase in green fluorescence (*$P < 0.01$*) from the respective control. Specific fluorescence intensity (SFI) is defined as the fluorescence obtained from* 10^9 *CFU/ml.*

before starvation to determine the effect of total nutrient content of the media on expression of *uspA, grpE,* and *rpoS* (Fig. 4). When *E. coli* was grown in 0.5× TSB, cells did not show starvation-induced expression of *uspA, grpE,* and *rpoS.* On the contrary, 1.5× LB-grown *E. coli* showed that starvation increased green fluorescence from 36,245 to 122,793 SFI, 17,334 to 52,028 SFI, and 11,367 to 69,924 SFI for p*uspA*::*gfp*$_{uv}$, p*grpE*::*gfp*$_{uv}$, and p*rpoS*::*gfp*$_{uv}$, respectively.

DISCUSSION

Previous studies have shown that at the onset of starvation, many bacteria exhibit the coordinated expression of protective mechanisms that allow the cells to survive exposure to other multiple stresses *(1, 8, 11, 13, 23).* Very few reports are available on starvation-induced cryotolerance and freeze-thaw tolerance *(1, 8, 11).* This has particular relevance to the food industry because water, used to clean or rinse food contact surfaces or even incorporated directly into foods, is generally of low nutrient status. Our study showed that starvation-induced cryotolerance of *E. coli* O157:H7 depends on growth media (Figs. 1 and 2). However, it is acknowledged that this work involved only one strain of *E. coli* O157:H7, and the results for other strains could differ. Other work has suggested a link between nutrient availability and cryotolerance. Givskov et al. *(8)* showed 100% reduction of *Pseudomonas putida* cells

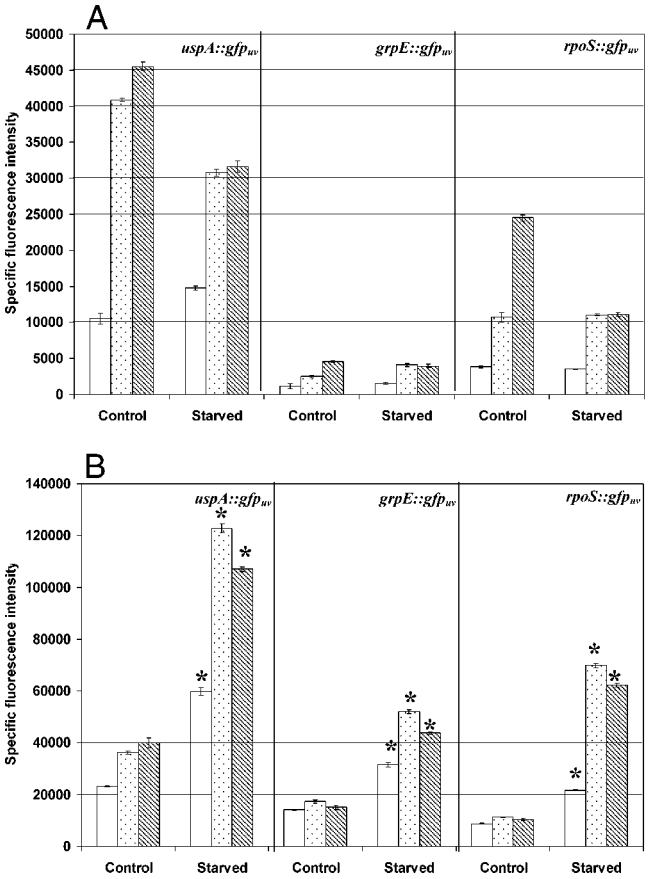

FIGURE 4. *Effect of 0 (□), 2 (⬚), and 4 h (▨) of starvation on expression of* puspA::gfp_{uv}, *p*grpE::gfp_{uv}, *and p*rpoS::gfp_{uv} *in (A) 0.5× TSB-grown and (B) 1.5× LB-grown* E. coli *O157:H7. Bars marked * showed significant increase in green fluorescence (*$P < 0.01$*) from the respective control. Specific fluorescence intensity (SFI) is defined as the fluorescence obtained from* 10^9 *CFU/ml.*

on freezing at −20°C for 24 h, but a culture starved of carbon for 5 days showed a fivefold reduction in viable count. In addition, Gelinas et al. *(7)* showed enhanced cryotolerance of bakers' yeast (*Saccharomyces cerevisiae*) when grown in fed batch culture compared with batch culture. Bacterial morphology, structure, and bacterial attachment are affected by the properties of the medium used for growth *(3, 5, 10).* In the case of TSB-grown *E. coli*, the increase in cryotolerance was significant, indicating that the stress imposed by starvation altered the ability of *E. coli* O157:H7 to survive subsequent freezing. It has been shown that ATP, DNA, and RNA levels did not decline during starvation, indicating that the bacterial cells remain metabolically active *(4, 17, 18).*

Heat shock proteins *(16, 23)* and RpoS protein *(11, 21)* play a central role in the cross-protection system of bacteria. The results of this study demonstrate that the expression of p*uspA*::gfp_{uv}, p*grpE*::gfp_{uv}, and p*rpoS*::gfp_{uv} genes was induced in *E. coli* O157:H7 by starvation when cells were grown in TSB, but the expression of p*uspA*::gfp_{uv}, p*grpE*::gfp_{uv}, and p*rpoS*::gfp_{uv} genes was not induced in *E. coli* O157:H7 by starvation when cells were grown in LB (Fig. 3). Therefore, UspA, GrpE, and RpoS proteins are possibly involved in the induction of starvation-mediated cryotolerance of TSB-grown *E. coli* O157:H7.

The differences observed in starvation-induced cryotolerance of TSB- and LB-grown *E. coli* and corresponding expression of p*uspA*::gfp_{uv}, p*grpE*::gfp_{uv}, and p*rpoS*::gfp_{uv} was thought to be the result of differences in the composition of the growth media. Both media supported similar growth in terms of total viable count, and the pH of growth media after 14 to 16 h of growth was not significantly different (data not shown). However, TSB contains 20 g/liter protein (pancreatic digest of casein and enzymatic digest of soybean meal) along with dextrose as a carbon source, sodium chloride, and dipotassium phosphate, whereas LB broth contains 15 g/liter protein (pancreatic digest of casein and yeast extract) and sodium chloride. To study the effect of total nutrient content of TSB and LB on expression of *uspA, grpE,* and *rpoS* on starvation, *E. coli* O157:H7 was grown in half-strength (0.5×) TSB and 1.5× LB (Fig. 4A and 4B). Changing the growth medium concentration provided a mechanism with which to affect the prior history of the bacterial cultures before the assessment of expression of *uspA, grpE,* and *rpoS.* As expected, there was no significant increase in the expression of p*uspA*::gfp_{uv}, p*grpE*::gfp_{uv}, and p*rpoS*::gfp_{uv} on starvation following growth of *E. coli* O157:H7 in nutrient-depleted (0.5×) TSB. On the other hand, starvation induced the expression of p*uspA*::gfp_{uv}, p*grpE*::gfp_{uv}, and p*rpoS*::gfp_{uv} following growth of *E. coli* in 1.5× LB. These results indicate that *E. coli* O157:H7 grown in full-strength TSB or 1.5× LB did not induce stress tolerance genes (*uspA, grpE,* and *rpoS*), but the genes are subsequently expressed on starvation. In contrast, stress tolerance genes (*uspA, grpE,* and *rpoS*) were induced during growth of *E. coli* O157:H7 in nutritionally poor LB or 0.5× TSB media, but subsequent starvation failed to further increase gene expression. The results of this study support those of previous work with whole-genome analysis of *E. coli (22),* indicating that the expression of stress tolerance genes are repressed in cells grown in rich medium but are induced when the organism is grown in nutritionally poor medium.

The data presented here indicate that the stress imposed by starvation altered the ability of TSB-grown *E. coli* O157:H7 to survive subsequent cryotolerance. Starvation-induced cryotolerance was observed in TSB-grown cells. The finding correlated well with levels of *uspA, grpE,* and *rpoS* gene expression. Starvation-induced cryotolerance was not observed in LB-grown *E. coli* cells, and nutrient limitation did not induce expression of *uspA, grpE,* and *rpoS.* In future, to predict behavior of similar organisms in foods, the influence of growth media to survive freezing at low temperature on starvation and the role of *uspA, grpE,* and *rpoS* need to be studied in different *E. coli* strains before extrapolating results from pure culture on lab media.

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