Detection of mRNA by Reverse Transcription-PCR as an Indicator of Viability in *Escherichia coli* Cells

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The relationship between the detection of mRNA and cellular viability in *Escherichia coli* was investigated in cells killed by heat or ethanol. Reverse transcription-PCR (RT-PCR) methods were developed for detecting mRNA from *rpoH*, *groEL*, and *tufA* genes. mRNA from all three genes was detected immediately after the cells had been killed by heat or ethanol but gradually disappeared with time when dead cells were held at room temperature. In heat-killed cells, some mRNA targets became undetectable after 2 to 16 h, whereas after ethanol treatment, mRNA was still detected after 16 h. In contrast, 16S rRNA was detected by RT-PCR in all samples containing dead cells and did not disappear during a subsequent incubation of 16 h at room temperature. Of the different types of nucleic acid, mRNA is the most promising candidate for an indicator of viability in bacteria, but its persistence in dead cells depends on the inactivating treatment and subsequent holding conditions.

Conventional methods for detecting bacterial pathogens typically involve culturing the organisms in selective media and identifying isolates according to their morphological, biochemical, and/or immunological characteristics. These methods are sensitive and can allow small numbers of organisms ($<10^3$ cells/ml) to be detected in complex environments such as foods and certain clinical samples. However, the methods also require days from initiation to readout, and identification schemes based on phenotypic properties are notoriously difficult to interpret.

Gene probe methods of detection and identification are inherently more specific and can also be extremely sensitive when coupled, for example, with PCR (26). One disadvantage of DNA-based methods is that they do not distinguish between living and dead organisms (22), which limits their use for monitoring purposes. Josephson et al. (18) performed PCR amplification of DNA from cells killed by boiling or exposure to UV light, while Masters et al. (25) showed that there was no relationship between viability and PCR detection of DNA targets in *Listeria monocytogenes* or *Escherichia coli* that had been exposed to heat, acid, hydrogen peroxide, drying, or starvation. In the latter study, even autoclaved cells provided a positive PCR signal.

These disadvantages of DNA-based methods may be partly overcome by including a preenrichment step that allows organisms to multiply before gene probe tests are applied. This procedure increases sensitivity and helps to distinguish between samples that contain living cells and those that do not; however, it also increases the time taken for analysis. There is also a possibility that samples which initially contained large numbers of dead cells would still give positive results with sensitive gene probe methods even though the number of target organisms had not increased during preenrichment.

Nucleic acid-based methods that could be applied directly to samples to give an indication of the viability of any microbes present would be of enormous significance for food, industrial, environmental, and medical applications. mRNA is turned over rapidly in living bacterial cells, with most mRNA species having a half-life of only a few minutes (2, 7). Detection of mRNA might therefore be a good indicator of living cells or those only recently dead at the time of sampling. Detection of mRNA by Northern blot hybridization has been used as an indicator of microbial metabolic activity in aquatic and soil samples (17, 28, 35), and detection of mRNA by reverse transcription-PCR (RT-PCR) was used to monitor gene expression in activated sludge (33). While these studies undoubtedly reflected the activities of viable cells in natural environments, their main purpose was not to distinguish between living and dead cells. Few studies have specifically investigated the relationship between detection of microbial mRNA and viability. Bej et al. (5, 6) used RT-PCR to examine *Legionella pneumophila* and *Vibrio cholerae* exposed to heat or starvation, respectively, and detected specific mRNA only in samples that contained viable cells detected by culturing. Similarly, Patel et al. (27) successfully assessed the viability of heat-killed *Mycobacterium leprae*, detecting a heat shock protein mRNA in living cells. Recently, Klein and Juneja (20) described a method for the specific detection of viable *L. monocytogenes* cells based on RT-PCR. Despite their potential advantages, mRNA-based approaches have proved difficult to exploit because of the complexity of the methods, the practical problems of extracting detectable levels of intact mRNA from small numbers of bacteria, and a lack of basic information about the significance of detecting mRNA in stressed cells. The aim of this work was to develop methods for detecting specific mRNA from *E. coli* and to examine the relationship between viability and the presence of mRNA. Since any relationship between mRNA and viability may depend on the method used to inactivate cells or the type of mRNA sought, we exposed the cells to two different stress treatments (heat and ethanol) and assayed mRNA from three different genes (*rpoH*, *groEL*, and *tufA*). These genes were chosen as representing a gene encoding an abundant cellular housekeeping protein (*tufA*) (8, 36) and genes associated with a stress response regulon (*rpoH* and *groEL*) (3, 12, 14).

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TABLE 1. Oligonucleotide primer sequences used for RT-PCR analysis

Accession no.[a]	Target gene	Primer	Sequence (5′-3′)	Size (positions) of product[b]
J01695	16S rRNA	ICM16SF	CAGCGGGGAGGAAGGGAGTAAAGT	405 (732–1137)
		ICM16SR	ACCACCGCCCGTCACACCATG	
X07850	*groEL*	IMGROEF	CCGTGGCTACCTGTCTCCTTACTT	653 (1058–1711)
		IMGROER	CCAGCAACCACGCCTTCTTCTACC	
M20668	*rpoH*	IMRPOHF	CCACAGGCGGATTTGATTC	450 (351–801)
		IMRPOHR	GGTTTGCCGCCTGCTCTTC	
J01690	*tufA*	IMTUFAF	ACTTCCCGGGCGACGACACTC	578 (624–1202)
		IMTUFAR	CGCCCGGCATTACCATCTCTAC	

[a] EMBL/GenBank accession numbers.
[b] Sizes are in base pairs; positions are nucleotides.

MATERIALS AND METHODS

Bacterial strain and growth conditions. *E. coli* (type strain NCFB 1984) was grown in Luria-Bertani (LB) broth to exponential phase (optical density at 680 nm, 0.2; approximately 2×10^8 cells/ml).

Viable counts. To obtain quantitative estimates of viable numbers in suspensions subjected to heat or ethanol treatment and in experiments to determine the limits of detection by RT-PCR, samples were serially diluted in maximum-recovery diluent (Oxoid, Basingstoke, United Kingdom) and plated onto LB agar. Colonies were counted after incubation for 2 days at 37°C.

To monitor for the presence of viable cells in ethanol- or heat-killed suspensions used for RT-PCR, 0.1-ml samples were removed at the same time that samples were removed for RNA extraction and diluted 1:100 in LB broth. The broth was incubated aerobically at 37°C for 2 days and then examined for visible turbidity. Any survival, recovery, or multiplication of cells exposed to an inactivation treatment and then held at room temperature would thus have been detected. A broth was used as recovery medium to provide the maximum opportunity for resuscitation of injured cells (23).

Inactivation treatments. Volumes (1 ml) of exponential-phase culture (ca. 10^7 cells) were treated in five different ways to kill the bacteria. They were killed by boiling at 100°C for 5 min, heating at 80°C for 10 min or at 60°C for 20 min, or exposure to 50 or 67% ethanol for 7 min. The bacteria treated with ethanol were pelleted by being centrifuged in a bench centrifuge at $10,000 \times g$ for 5 min, washed twice, and resuspended in 100 µl of LB broth. All the treated bacteria were left in their broth at room temperature for 0, 15, or 30 min or 1, 2, 3, or 16 h after treatment before RNA isolation.

RNA isolation. The chaotropic solution used for isolating total *E. coli* RNA consisted of 2% (vol/vol) DivoLab cationic detergent (Diversey Ltd., Northampton, United Kingdom), 50 mM sodium acetate, and 1% (vol/vol) β-mercaptoethanol (pH 4.5) and was prepared immediately prior to use. Chaotropic solution (500 µl) was added to the bacterial suspension (100 µl), and the mixture was transferred to a blue-capped Ribolyser tube (Hybaid Ltd., Teddington, United Kingdom) containing silica beads and 600 µl of phenol-chloroform (5:1, pH 4.7). The tubes were shaken in the Ribolyser at speed setting 6 for 20 s and cooled on ice before being centrifuged at $10,000 \times g$ for 10 min. The aqueous layer was removed and washed twice with chloroform-isoamyl alcohol (24:1) by vortexing for 30 s and centrifuging at $10,000 \times g$ for 10 min between each wash. RNA was precipitated with 2 equal volumes of isopropanol containing 0.01 M polyinositol and left at −20°C for at least 10 min before the nucleic acids were pelleted at $10,000 \times g$ for 30 min. The nucleic acid pellet was washed twice with 75% (vol/vol) ice-cold ethanol, air dried, resuspended in 40 µl of sterile double-distilled diethylpyrocarbonate-treated water (Sigma, Poole, United Kingdom), and stored at −20°C. An aliquot (5 µl) of RNA was examined by gel electrophoresis on a 1.2% (wt/vol) agarose gel running in 1× TAE (0.04 M Tris acetate, 0.001 M EDTA) buffer and then stained with ethidium bromide (25 µg/ml) and examined with a UV transilluminator at 254 nm.

Elimination of contaminating DNA. To remove any contaminating DNA, 10 µl (*ca*. 1 µg) of RNA was incubated at 37°C for 30 min with 30 U of RNase-free DNase (either Boehringer Mannheim, Lewes, United Kingdom, or Life Technologies Ltd., Paisley, United Kingdom) and 1 U of RNasin RNase inhibitor (Promega, Southampton, United Kingdom) in a 50-µl volume. Residual DNase was either heat inactivated at 80°C for 5 min (Life Technologies) or removed by extraction with phenol-chloroform (5:1; pH 4.7) (Boehringer Mannheim), and RNA isolation continued according to the protocol. A PCR was performed to check for any contaminating DNA by using *rTth* DNA polymerase with 1× EZ buffer (Perkin-Elmer, Warrington, United Kingdom) and 16S rDNA primers. DNase-treated RNA (ca. 100 ng) was amplified using with 10 µM forward primer, and 10 µM reverse primer, 5 U of *rTth* DNA polymerase, 1× EZ buffer (50 mM bicine, 115 mM potassium acetate, 8% [wt/vol] glycerol [pH 8.2]), 300 µM each deoxynucleoside triphosphate, and 2.5 mM manganese acetate in a reaction volume of 50 µl. The reaction mixtures were overlaid with mineral oil before being subjected to amplification for 35 cycles of 95°C for 30 s, 60°C for 45 s, and 72°C for 1 min with a final cycle of 72°C for 10 min. The PCR products

(15 µl) were run on a 1.2% (wt/vol) agarose gel, stained with ethidium bromide (25 µg/ml), and photographed with 35-mm film.

Southern blotting. To confirm the identity of PCR products, gels were Southern blotted onto Hybond N⁺ (Amersham International, Amersham, United Kingdom) nylon (32). PCR products from 16S rDNA, *tufA*, or *rpoH* genes were randomly fluorescein labelled as specified by the manufacturer (Tropix Inc., Bedford, Mass.) and hybridized to the blot at 65°C for 16 h in hybridization buffer (1 mM EDTA, 7% sodium dodecyl sulfate [SDS], 0.25M disodium phosphate [pH 7.2]). The blots were washed twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at 25°C, twice for 15 min with 1× SSC–0.1% SDS at 65°C, and twice for 5 min with 0.1× SSC–0.1% SDS at 25°C. The hybridized probe was detected with the Southern-Star (Tropix) nucleic acid detection system as specified by the manufacturer and exposed to BioMax X-ray film (Sigma) before being developed.

One-step RT-PCR with *rTth* DNA polymerase. DNase-treated RNA samples containing any contaminating DNA (as identified by PCR) were not analyzed further. The presence of three target mRNAs, *groEL*, *tufA*, and *rpoH*, was analyzed by a one-step RT-PCR method in which *rTth* DNA polymerase (Perkin-Elmer) is used to synthesize cDNA from RNA and also to amplify the product in the subsequent PCR. DNase-treated RNA (ca. 100 ng) was amplified with 5 U of *rTth* DNA polymerase, 1× EZ buffer 10 µM each forward and reverse primers, 300 µM each deoxynucleoside triphosphate, and 2.5 mM manganese acetate made up to 50 µl with sterile double-distilled water. The RT-PCR samples were overlaid with 50 µl of mineral oil and amplified in a two-step thermal program: cDNA was synthesized at 60°C for 30 min and then amplified in a two-step PCR for 40 cycles (denaturing at 95°C for 45 s and annealing at 50°C for 45 s) with a final extension at 60°C for 10 min. The RT-PCR samples (15 µl) were electrophoresed on a 1% (wt/vol) agarose gel in 1× TAE buffer. As a positive control for each sample, RT-PCR was performed on the same extract with primers specific for 16S rRNA. This target was chosen as a control because it is very stable and is present in much greater quantities than is mRNA.

Two-step RT-PCR with *rTth* DNA polymerase. The one-step RT-PCR system with EZ buffer was designed for use as a screening or detection tool and allows a certain degree of enzymatic transcriptional and synthetic infidelity. A change in reaction buffering conditions in a two-step RT-PCR with *rTth* DNA polymerase ensures high enzymatic fidelity; therefore, to compare the limits of mRNA detection, both one- and two-step RT-PCR methods were used. In the first step, DNase-treated RNA (ca. 100 ng) was subjected to RT with 2.5 U of *rTth* DNA polymerase–1× RT buffer (10 mM Tris-HCl, 90 mM KCl [pH 8.3]), 300 µM each dNTP–10 mM MnCl₂–10 µM reverse primer in a total volume of 20 µl (made up to volume with sterile double-distilled water) at 60°C for 30 min. To this mixture, 80 µl of PCR components was added and a PCR was carried out with 40 cycles of 95°C for 45 s, 50°C for 1 min, and 72°C for 1 min, finishing with an extension at 60°C for 10 min. The PCR buffer contained 2 mM MgCl₂, 10 mM forward primer, 1× chelating buffer (10 mM Tris-HCl, 0.1 M KCl, 0.05% [wt/vol] Tween 20, 0.75 mM EGTA [pH 8.3]), and sterile double-distilled water. RT-PCR products (15 µl) were electrophoresed on a 1% (wt/vol) agarose gel as described above.

Two-step RT-PCR with Superscript II and *Taq* DNA polymerase. DNase-treated RNA was subjected to RT into cDNA with Moloney murine leukemia virus reverse transcriptase (Superscript RNase H⁻ reverse transcriptase [Life Technologies Ltd.]), as specified by the manufacturer, in the presence of 1 U of RNasin. cDNA (3 µl) was then PCR amplified with *Taq* DNA polymerase (Applied Biosystems) under the thermal conditions and by the procedure detailed for the one-step RT-PCR system.

Oligonucleotide primer sequence design. Oligonucleotide primers (Table 1) were designed by using the PRIMERSELECT program from a genetic analysis computer package (DNASTAR Inc., Madison, Wis.), from DNA sequences submitted to the EMBL/GenBank databases. The primers amplify between 40 and 50% of the transcribed gene.
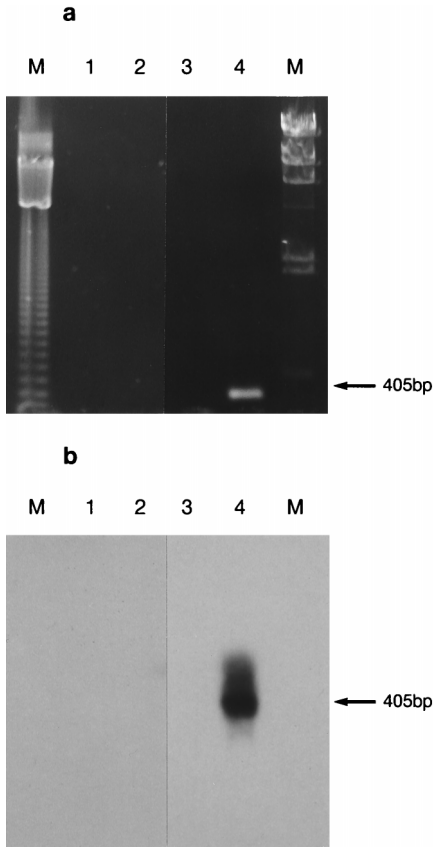
a

← 405bp

b

← 405bp

FIG. 1. (a) PCR test to demonstrate the absence of residual contaminating 16S rDNA in DNase-treated mRNA samples. *E. coli* cells (10^7 cells/ml) were heat inactivated at 100°C for 5 min and then left at room temperature for 16 h (lane 2) after the killing treatment. Lane 1 contains an unheated control sample, lane 3 is a negative control containing sterile water in place of nucleic acid, and lane 4 is a positive control containing *E. coli* DNA. Lanes M contain molecular size standards. (b) Southern blot analysis of PCR products from amplification of residual contaminating 16S rDNA in DNase-treated mRNA samples. The gel in panel a was blotted and probed with a fluorescein-labelled 16S rDNA fragment.

RESULTS

Development of the RT-PCR protocol. Several methods of extracting RNA, involving hot- and cold-phenol extractions and the use of different chaotropic agents and lytic enzymes, were tested (1, 11, 13, 21, 30). The most reproducible results were obtained when cells were rapidly disrupted with silicon beads in a chaotropic buffer and phenol-chloroform mix with the Hybaid Ribolyser system.

The choice of detergent used as a chaotrophic agent was specific to the type of bacteria being investigated (24). From a comparison of different concentrations of a range of detergents (e.g., Tween 80, SDS, Teepol), 2% Divo Lab detergent was found to be optimal for *E. coli* cells. Moreover, the time and speed at which the Ribolyser tubes were shaken were critical, since low speeds and short shaking times favoured the isolation of DNA from *E. coli* while high speeds and long times favored the isolation of RNA. The quality of total cellular RNA extracted was monitored by gel electrophoresis, but, surprisingly, the efficiency of extraction of total RNA was not a reliable guide to the success of subsequent RT-PCR of mRNA.

We observed no significant difference between the two different reverse transcriptase enzymes and three different protocols used for detecting mRNA from initial cell concentrations of 10^7 *E. coli* cells/ml. However, the method involving

rTth DNA polymerase with EZ buffer was the most suitable for our purposes because it was the quickest method and required the fewest manipulations. In the one-tube method, all the reverse-transcribed RNA (cDNA) is available for amplification, whereas in the two-tube method, only a subsample is amplified. By using this RT-PCR regimen, it was possible to detect *rpoH* mRNA from suspensions containing 10^3 cells/ml, and Southern blot analysis confirmed the amplification fidelity of this enzyme (data not shown).

The method is not valid without an assurance of the absence of DNA. This is done quite simply by performing a control PCR on the DNase-treated RNA sample, which should give negative results. This was done for all our RNA samples. 16S rDNA was the target chosen for detecting contaminating DNA, and samples were analyzed by using *rTth* DNA polymerase with EZ buffer (Fig. 1a), since this was the enzyme system used to analyze mRNA in killed cells. Blotting and probing PCR agarose gels with a 16S rDNA fragment (Fig. 1b) confirmed that no contaminating DNA was present in DNase-treated RNA samples, eliminating the chance of a false-positive result in the subsequent RT-PCR analysis.

Detection of mRNA in heat-killed cells. Detection of *tufA* mRNA in heat-killed cells is shown in Fig. 2. mRNA from *groEL*, *rpoH*, and *tufA* genes was detected by RT-PCR in all the cell suspensions immediately after heating at 60, 80, or 100°C (Table 2). However, during subsequent incubation at room temperature, these mRNA species became undetectable and were absent at 16 h. No viable cells were detected in samples immediately after treatment or during subsequent incubation at room temperature. The time at which mRNA became undetectable varied slightly and depended on the target. Generally, target mRNA from all three genes was detectable for up to 2 h but disappeared after 16 h (Table 2). However, *groEL* mRNA was undetectable at 2 h after heating at 60°C for 20 min. By contrast, 16S rRNA was detected immediately after heating and also at 16 h in all the samples.

Detection of mRNA in ethanol-treated cells. As with heat treatment, mRNA from the *groEL*, *rpoH*, and *tufA* genes was detected immediately after the cells had been killed (Table 3). However, these mRNA species were more stable in ethanol-treated cells than in heat-killed cells, since *rpoH* and *tufA* mRNAs were still detectable 16 h after treatment with 50% (vol/vol) ethanol and *tufA* was also detected 16 h after treatment with 67% (vol/vol) ethanol (Fig. 3). mRNA from *groEL* became undetectable before that from *rpoH* or *tufA*, disap-
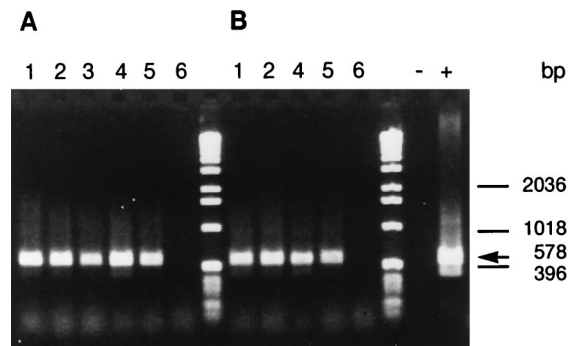

FIG. 2. RT-PCR detection of *tufA* mRNA from *E. coli* cells (10^7 cells/ml) killed by heat treatment at 100°C for 5 min (A) or 80°C for 10 min (B) and left at room temperature for 0 min (lanes 2), 30 min (lanes 3), 60 min (lanes 4), 120 min (lanes 5), or 16 h (lanes 6). Lanes 1 contain control unheated *E. coli* samples, and the RT-PCR-positive (lane +) and -negative (lane −) controls contain *E. coli* DNA and sterile water, respectively.

TABLE 2. mRNA species detected by RT-PCR after incubation of heat-killed *E. coli* at room temperature

Treatment	Target	Detection after[a]:					
		Un-treated	0 min	30 min	1 h	2 h	16 h
100°C for 5 min	16S rRNA	Y	Y	NP	Y	Y	Y
	groEL	Y	Y	Y	Y	Y	N
	rpoH	Y	Y	NP	Y	Y	N
	tufA	Y	Y	Y	Y	Y	N
80°C for 10 min	16S rRNA	Y	Y	Y	Y	Y	Y
	groEL	Y	Y	Y	Y	Y	N
	rpoH	Y	Y	Y	Y	Y	N
	tufA	Y	Y	Y	Y	Y	N
60°C for 20 min	16S rRNA	Y	Y	Y	Y	Y	Y
	groEL	Y	Y	N	Y	N	N
	tufA	Y	Y	Y	Y	Y	N

[a] Y, positive RT-PCR amplification; N, negative RT-PCR amplification; NP, not performed.

pearing by 16 and 2 h in cells killed with 50 and 67% (vol/vol) ethanol, respectively. As with the heat-killed cells, 16S rRNA was present in all ethanol-treated samples.

DISCUSSION

The terms "life" and "living" have proved difficult to define because there is no unique property common to all things normally regarded as living. Hence, definitions tend to consist of a collection of attributes, none of which is shown by all organisms and some of which also apply to inanimate objects (29). Microbiologists tend to adopt a pragmatic approach and usually define viable organisms as those that can multiply to form colonies on agar plates or visible turbidity in broth. This definition may need qualification to accommodate the so-called viable but nonculturable organisms (31) and those that can multiply in liquid media but only to low maximum cell densities (9). We propose that a reasonable working definition of a viable bacterial cell is one that has the potential to multiply under suitable conditions.

Since replication involves the coordinated activity of homeostatic, biosynthetic, and energy-conserving systems, there is no a priori reason to suppose that any single indicator of metabolic activity will correlate with viability, so defined, under all circumstances. Nevertheless, metabolic indicators such as membrane potential, the ability to generate reducing power, or

TABLE 3. mRNA species detected by RT-PCR after incubation of ethanol-killed *E. coli* at room temperature

Treatment	Target	Detection after[a]:						
		Un-treated	0 min	10 min	30 min	1 h	2 h	16 h
50% for 7 min	16S rRNA	Y	Y	Y	Y	Y	Y	Y
	groEL	Y	Y	Y	Y	Y	Y	N
	rpoH	Y	Y	Y	Y	Y	Y	Y
	tufA	Y	Y	Y	Y	Y	Y	Y
67% for 7 min	16S rRNA	Y	Y	Y	Y	Y	Y	Y
	groEL	Y	Y	Y	Y	Y	N	N
	rpoH	Y	Y	Y	Y	Y	Y	N
	tufA	Y	Y	Y	Y	Y	Y	Y

[a] Y, positive RT-PCR amplification; N, negative RT-PCR amplification.
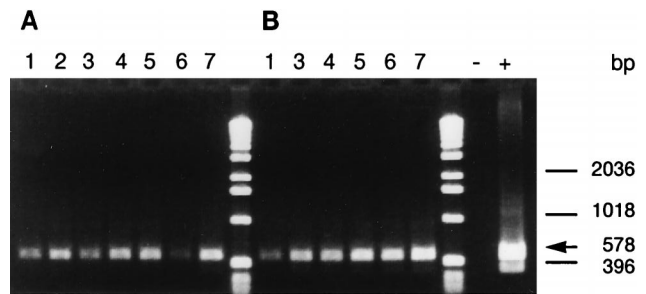

FIG. 3. RT-PCR detection of *tufA* mRNA from *E. coli* cells (10^7 cells/ml) killed with 50% (A) or 67% (B) ethanol for 7 min and left at room temperature for 0 min (lanes 2), 10 min (lanes 3), 30 min (lanes 4), 60 min (lanes 5), 120 min (lanes 6), or 16 h (lanes 7). Lanes 1 contain control untreated *E. coli* sample, and the RT-PCR positive (lane +) and -negative (lane −) controls contain *E. coli* DNA and sterile water, respectively.

the ability to undertake DNA synthesis have proved useful indicators of the viability of cells in the natural environment (4, 19). Nucleic acids as viability indicators would have the added advantage of specificity.

In principle, the presence of one or another type of nucleic acid (DNA, rRNA, mRNA or tRNA) in bacterial cells might be a useful indicator of viability if (i) it is present only in viable cells, (ii) the kinetics of its disappearance is related to loss of viability, or (iii) it disappears from cells soon after death.

In this work, we have developed RT-PCR methods for detecting specific mRNA from *E. coli* and have shown that mRNA was initially present in cells killed by heat or ethanol but subsequently disappeared at a rate depending on the inactivating treatment. Because mRNA was detected in dead *E. coli* cells by RT-PCR, it was not an absolute indicator of viability according to criterion (i) given above. Our findings are different from those of Bej et al. (6), who detected no mRNA in cells of *Vibrio cholerae* that were killed by heat and starvation. Similarly, Patel et al. (27) did not detect a heat shock protein mRNA from *Mycobacterium leprae* killed by heat treatment, probably because the sample was left at room temperature for 5 h before analysis, allowing for any surviving mRNA to become degraded. An RT-PCR method for the specific detection of viable *L. monocytogenes* was recently developed (20) based on the detection of mRNA from *iap*, *hly*, or *prfA* genes. The greatest sensitivity was achieved with *iap* mRNA as the target, which allowed the detection of 10 to 15 viable cells/ml after a 1-h enrichment in broth. Cells killed by autoclaving were not detected, but the method was not tested with cells killed by other treatments.

Because of the presence of the 2′-hydroxyl group of ribose, the phosphodiester bonds of RNA are more susceptible to hydrolysis than those of DNA, particularly in the presence of divalent cations. RNA is therefore more labile than DNA and more susceptible to degradation caused directly by deleterious treatments, such as heating or acidification. However, the extent of that degradation will vary according to the type and severity of treatment, and mRNA would be expected to survive in some circumstances. Indeed, because cells have to be killed before their nucleic acid can be extracted, there can be no absolute correlation between the presence of mRNA and viability.

The three different mRNA species detected in this study were selected to represent two genes involved in stress responses (*groEL* and *rpoH*) and a gene encoding an abundant cellular housekeeping protein (*tufA*). Of these mRNAs, *tufA* and *rpoH* mRNAs were detected for a longer time after treat-

ment with both heat and ethanol than was *groEL*. This may be correlated with the stability of the different mRNA sequences targeted by the oligonucleotide primers designed here or to the relative abundance of each mRNA type in a given cell. Either way, it appears that the type of mRNA selected for detection of viable bacteria will be important.

Detectable mRNA disappeared more quickly from heat-killed cells than from ethanol-killed cells (based on the time at which RT-PCR gave a negative result). Since the incubation conditions after heat or ethanol treatment were identical, the difference in detection times after treatment must be related to the different effects of heat and ethanol on the capacity for RNA breakdown in dead cells. In living cells, most mRNA turns over rapidly, reflecting a balance between the synthesis of mRNA and its degradation by RNases (2, 7). In dead cells, mRNA synthesis (if any) is likely to be slow and nuclease activity will continue to degrade any mRNA present. The factors controlling mRNA longevity in dead cells are not understood, but presumably mRNA would disappear most rapidly from cells killed by treatments that do not inactivate the degradative RNase enzymes. Conversely, mRNA may remain intact for longer periods in cells killed by treatments that also inactivate RNases or render the RNA resistant to attack. The relatively rapid disappearance of mRNA from dead cells shown here means that it is likely to be a much better indicator of viability than DNA according to criterion (iii) above. The factors that influence the longevity of mRNA in dead cells require clarification before its usefulness as a general indicator of viability can be judged. In particular, the effects of different inactivating treatments and holding conditions must be investigated, along with any differences in abundance or rates of disappearance of different target mRNAs.

rRNA has been suggested as an indicator of viability in *Mycobacterium smegmatis* (37). In cells exposed to rifampin and ofloxacin, the presence of 16S rRNA, as detected by the nucleic acid sequence-based amplification (NASBA) method, corresponded well to numbers of viable cells and a lack of signal coincided with loss of viability. A correlation between viability and cellular rRNA content was also observed in *E. coli* cells undergoing starvation (10) but did not occur in starved *Azotobacter agilis* cells (34). In heat-killed bacterial cells, the breakdown of ribosomes and rRNA is highly dependent on the medium in which cells are heated and is not necessarily related to loss of viability (16). In our study, 16S rRNA from heat- or ethanol-killed *E. coli* cells did not disappear after 16 h of incubation in broth at room temperature. It was not, therefore, a useful indicator of viability for the time frame over which our experiments were conducted (i.e., 16 h).

There is very little information about the persistence of tRNA in injured or dead cells. Davis et al. (10) found that tRNA survived much longer than rRNA in cells of *E. coli* that had died of starvation, and it is therefore unlikely to be a good indicator of viability.

This work has demonstrated that of the four species of nucleic acid, mRNA is the most promising candidate as an indicator of viability in bacteria. Klein and Juneja (20) showed a good correlation between the presence of mRNA and the viability of *L. monocytogenes* when comparing growing cells with those killed by autoclaving. However, in cells killed by milder treatments, the correlation is not absolute and in some cases the mRNA can persist for a while. The detection of mRNA therefore indicates either that a cell is alive or has died "fairly recently." The length of time that mRNA persists and hence the time envelope described by the phrase "fairly recently" will depend on both the method by which cells were killed and the postmortem holding conditions. We have estab-

lished that mRNA can persist for at least 16 h, but further work is required to characterize the decay rates of mRNA in dead cells under a range of conditions before the limitations of the method are fully defined. However, it should be possible to identify conditions under which mRNA disappears very rapidly and those where it does not. In this way, RT-PCR methods may be developed for particular applications that will provide a good indication of the presence of viable cells in food, clinical, or environmental samples. Methods now exist for detecting mRNA in single bacterial cells (15), and further improvements in technology are likely to increase the application of these methods in microbial monitoring and ecology.

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