Relationship between nucleic acid ratios and growth in *Listeria monocytogenes*

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***Listeria monocytogenes* is a pathogen whose distribution in a range of foodstuffs requires the development of methods for sensitive and rapid detection. Molecular biological methods usually rely on specific detection of *L. monocytogenes* rDNA directly amplified by the application of PCR to DNA extracts. Information on the metabolic status of *L. monocytogenes* populations would be valuable and can, in theory, be provided by quantitative detection of rRNA itself. Both fluorometry and oligonucleotide probe assays were applied to *L. monocytogenes* cultures to quantify RNA and DNA and produced more meaningful data than previous estimates for bacteria based on eukaryotic nucleic acid standards. In batch culture, the RNA–DNA ratio was found to be greatest at the end of exponential growth, after which RNA became degraded in accordance with the rapid decrease in viability. When the pH of the medium was controlled at neutrality, culture viability was dramatically extended and although RNA was degraded, intact DNA was maintained for the duration of the experiment. Ribosome numbers per cell were estimated to decrease from about 25 000 observed during mid-exponential growth to about 600 during stationary phase, under pH-controlled conditions. Like *Escherichia coli*, therefore, *L. monocytogenes* loses viability and rRNA rapidly once exponential growth has ceased in batch culture. However, much improved survival of a culturable *L. monocytogenes* population when pH is controlled has clear implications for the persistence of this species in buffered environments such as dairy products.**

Keywords: rRNA, fluorometry, RNA–DNA ratio

INTRODUCTION

Listeria monocytogenes is an important foodborne pathogen responsible for systemic infections (listeriosis) that predominantly occur in neonates, geriatric and immunocompromised patients (Farber & Peterkin, 1991). The organism is widely distributed, particularly in unpasteurized dairy foods and cooked meat products (Farber & Peterkin, 1991; Ferron & Michard, 1993; Gilbert *et al.*, 1989; Pine *et al.*, 1989; Verheul *et al.*, 1995; Young & Fogeding, 1993). Consequently, detection, enumeration and viability assessment of *L. monocytogenes* is of importance to both the food industry and clinicians alike (Gilbert, 1989; Hof & Rocourt, 1992; Nørrung & Skovgaard, 1993).

Conventional methods for detection of *L. monocytogenes* use enrichment and selective isolation followed by biochemical tests (Farber & Perkin, 1991; McLauchlin & Pini, 1989). Such procedures are valuable, but speed and sensitivity can be improved by the use of PCR-based methods (Bessesen *et al.*, 1990; Cooray *et al.*, 1994; Scheu *et al.*, 1999). However, detection of DNA does not discriminate between viable and non-viable cells and DNA can be associated with detrital material (Dell'Anno *et al.*, 1998). In theory, RNA detection is indicative of metabolically active organisms, but often requires an enrichment step prior to the molecular biological analysis (Blais *et al.*, 1997; Klein & Juneja, 1997). This precludes simple, rapid quantification and suffers from poor sensitivity when attempted directly on food samples (Powell *et al.*, 1994; Wang *et al.*, 1992; Cano *et al.*, 1995; Makino *et al.*, 1995).

It has been established that bacterial growth rate and

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cellular RNA concentration are positively correlated in a number of bacterial species (Kjellgaard & Kurland 1963; Rosset *et al.*, 1966; Gausing, 1977; Kerkhof & Ward, 1993; Amann *et al.*, 1990a, b; Muttray & Mohn, 1998). Increased levels of rRNA associated with increased growth rates (Bremer & Dennis, 1987) would effectively increase *L. monocytogenes* detection sensitivity if rRNA targeted probes were used. However, the correlation is not absolute for the growth of all bacterial species and the relationship is poor at low growth rates (Kramer & Singleton, 1992; Kerkhof & Ward, 1993; Kerkhof & Kemp, 1999). Under such conditions the cellular rRNA concentration approached the detection limits of the techniques used.

Definition of the relationship between *L. monocytogenes* growth and cellular RNA content is required prior to the development of meaningful RNA-based detection methods. In this paper, we use fluorometry and quantitative nucleic acid probing to determine RNA-DNA ratios for *L. monocytogenes* in relation to the batch culture growth cycle, and culture viability in the presence and absence of pH control.

METHODS

Nucleic acid extraction. All solutions were prepared following standard procedures for the prevention of RNase activity (Blumberg, 1987). Nucleic acids were extracted from cells by an enzymic digestion method. Briefly, biomass was harvested by centrifugation for 10 min at 740 g, cell pellets were resuspended in 70% (v/v) ethanol and stored at 4 °C until required. Cell pellets were resuspended in 200 µl lysis buffer (50 mM Tris/HCl, pH 8.0, 1 mM CaCl₂, 1%, w/v, SDS) to which Proteinase K (Roche Diagnostics) was added to a final concentration of 500 µg ml⁻¹. Samples were incubated in Eppendorf tubes at 55 °C for 60 min and then to each was added 1 ml phenol/chloroform/isoamyl alcohol (25:24:1; Sigma Aldrich). Tubes were mixed by inversion and centrifuged at 9440 g for 5 min at room temperature. The aqueous phase was retained and to each was added 1 ml absolute ethanol. Nucleic acids were precipitated at -20 °C for at least 1 h before centrifugation at 9440 g for 30 min at 4 °C. Pellets were resuspended in 200 µl TE buffer (10 mM Tris/HCl, pH 8.0, 1 mM EDTA) and stored at -70 °C before examination and subsequent quantification by either fluorometry or oligonucleotide hybridization.

Agarose gels (0.8–1.2%, w/v) were prepared in 1 × TAE buffer (2 ml 50 × TAE; 0.5 M Tris/HCl, pH 7.6; 0.05 M EDTA; 57.1 ml glacial acetic acid) and 2 µl ethidium bromide (10 mg ml⁻¹) was added to 100 ml molten agarose prior to pouring the gel. Gel equipment was suitably treated to inactivate RNases and used exclusively for RNA work. Nucleic acid samples (1–15 µl) were mixed with 4 µl loading buffer (50%, v/v, glycerol, 50%, v/v, TE buffer, 0.05%, w/v, bromophenol blue) prior to loading onto the gel. Nucleic acids were separated by electrophoresis at 90 V cm⁻¹ for 1 h and visualized on a UV transilluminator.

Fluorometric quantification of nucleic acid extracts. Standard curves for fluorometric determination of DNA or RNA concentration were prepared using serial dilutions of chromosomal DNA from *L. monocytogenes* ATCC 19111 (undiluted concentration 5.68 µg ml⁻¹ determined by A₂₆₀) and *E. coli* 16S and 23S rRNA mixture (Roche Diagnostics 206938, 4 µg ml⁻¹)

in molecular grade water. Fluorescence of nucleic acid samples was recorded on a Perkin Elmer 3000 Spectrofluorometer. Samples (10 µl) were added to fluorometry cuvettes and to each was added 3 µl staining solution (10 mg ethidium bromide ml⁻¹ in 5 mM Tris/HCl, pH 8.0, 10 mM EDTA) prior to addition of PBS to a final volume of 3 ml. Samples were mixed and equilibrated at 37 °C for 15 min and fluorescence measured (excitation λ₂₆₀, emission λ₅₉₀; LePecq & Paoletti, 1966) using slit widths of 10 and 20 nm for excitation and emission, respectively. No signal expansion was required throughout and all solutions were checked prior to use for quenching or enhancement of fluorescent signal. Unstained nucleic acids and PBS mixed with stain solution were used as negative controls. The data were subjected to linear regression analysis by a least squares method. Correlation coefficients were calculated by a Pearson's product moment coefficient (Sokal & Rohlf, 1995).

L. monocytogenes nucleic acid extracts were digested with either DNase or RNase, as appropriate, before fluorescent quantification. To digest RNA, DNase-free RNase (Roche Diagnostics 1119915) was added (3–15 U µl⁻¹). Nucleic acids were also incubated in the presence of 3 µl RNase-free DNase (Roche Diagnostics 776785) after addition of DNase buffer (20 mM Tris/HCl and 10 mM MgCl₂, pH 7.4) to each sample. All digestions were performed for 4 h at 37 °C. The efficacy of DNA or RNA selective digestion had been assessed previously (data not shown). Fluorescence was determined as described above.

Quantitative oligonucleotide probe hybridization. Nucleic acids were applied to positively charged nylon membranes (Positive; Appligene) with a Minifold II vacuum manifold (Schleicher and Schuell) which had a sample footprint size of 6 mm². For RNA immobilization, the manifold was soaked overnight in 0.5% (v/v) diethylpyrocarbonate (DEPC)-treated water to inhibit RNase activity. Samples of RNA (maximum volume 50 µl) were mixed with 3 vols of a solution that contained 70% (v/v) deionized formamide, 24% (v/v) of a 37% (v/v) filtered formaldehyde solution and 6% (v/v) 20 × SSC (3 M NaCl, 0.3 M sodium citrate, dissolved in 800 ml DEPC-treated water and adjusted to pH 7.0 before adjusting the volume up to 1 l). Samples were heated for 15 min at 68 °C, chilled on ice and 2 vols of ice cold 20 × SSC added before storage on ice until required. RNA was applied to the manifold according to manufacturer's instructions and slots were rinsed twice with 200 µl 20 × SSC under vacuum.

DNA samples (maximum volume 50 µl) were heated to 95 °C, chilled on ice and 1 vol. ice cold 20 × SSC added before storage on ice until required. Subsequent application of DNA to the membrane followed the procedure described above for RNA. Both DNA and RNA were fixed onto the nylon membrane by air-drying for 1 h followed by cross-linkage at 80 °C for 1 h. Membranes were wrapped in aluminium foil and stored at 4 °C prior to further treatment.

A probe, MV9RP2, was designed to be specific for *L. monocytogenes* rRNA (5'-ATAGTTTATGGGATTAGC-TC-3', position 1301–1281). This probe was designed by comparison of rDNA sequences deposited in the EMBL database (accession nos X56148–X56154; Collins *et al.*, 1991). The CHECKPROBE package (Ribosomal Database Project, <http://www.cme.msu.edu/RDP>) was used to screen candidate sequences for diagnostic value and possible artefact formation. Experimental evaluation of this probe against rDNA obtained from a large collection of *Listeria* reference strains and clinical isolates showed cross-reactivity with a type strain of *Listeria innocua* ATCC 33090 (data not shown).

Probes were end-labelled with [γ - 32 P]dATP (ICN Supplies) and efficiency of labelling was determined using the procedure described by Hiorns *et al.* (1995). It was intended to use probe MV9RP2 to specifically detect *L. monocytogenes* DNA also, but preliminary experiments showed this to have poor sensitivity (data not shown). Consequently, oligonucleotide probe pA, designed to be specific for all Eubacteria (5'-AGAGTTTGATCCTGGCTCAG-3'; position 8-28; Edwards *et al.*, 1989), was used to detect *L. monocytogenes* rDNA. To monitor the relationship between *L. monocytogenes* rRNA and rDNA, subsequent experiments were performed using this organism in pure culture.

Membranes were prehybridized for at least 1 h in a buffer that comprised 5 $\times$ SSPE (0.78 M NaCl, 0.155 M Na₂HPO₄·H₂O, 74 mM EDTA, pH 7.4, made up to 800 ml and adjusted to pH 7.4 prior to dilution to 1 l), 20% (v/v) deionized formamide, 0.02% SDS, 0.1% (w/v) N-lauryl sarcosine and blocking reagent [2%, w/v, of a solution that comprised 0.1 M maleic acid and 0.15 M NaCl adjusted to pH 8.0 prior to addition of blocking reagent (10%, w/v, Roche Diagnostics 1096176)]. Prehybridization solution was removed and membranes rinsed briefly in hybridization solution prior to addition of hybridization solution that contained 5 $\times$ SSPE, 20% (v/v) deionized formamide, 0.02% SDS, 0.1% N-lauryl sarcosine and 10 pM radiolabelled oligonucleotide probe. Membranes were hybridized overnight at 40 °C prior to washing twice for 15 min at room temperature in the above solution. Filters were wrapped in cling film and X-ray film exposed by autoradiography for 2 and 7 d at -70 °C. The autoradiograph signal was quantified with ImageQuant version 3.2 software running on a Molecular Dynamics Personal Densitometer. Signal (pixel) intensity above background was transferred to Microsoft Excel version 3.0 software where data manipulation was performed.

A preliminary experiment determined the response of oligonucleotide probe signal in relation to nucleic acid concentration. Briefly, titration series of either *L. monocytogenes* RNA or DNA were applied to nylon membranes and hybridized overnight in the presence of 10 pM radioactively labelled oligonucleotide probe pA (DNA) or MV9RP2 (RNA) as outlined above. Autoradiographs were quantified and data analysed as described above.

Growth of *L. monocytogenes* in shake flask and pH-controlled batch culture. Conical flasks (5 l volume) containing 2 l Tryptone Soya broth (30 g l⁻¹) supplemented with 0.3% (w/v) yeast extract and 0.5% (w/v) D-glucose (TSYGB) were inoculated with 2.5×10^{10} c.f.u. of an overnight culture of *L. monocytogenes* ATCC 19111. Cultures were incubated at 37 °C with shaking at 100 r.p.m. and samples (10 ml) removed aseptically at intervals. Growth was determined by measurement of OD₆₆₀ and viable counts determined on NAB plates (30 g Nutrient broth l⁻¹, pH 7.2; 12 g Agar No.2 Lab M l⁻¹) supplemented with 0.5% (v/v) defibrinated horse blood (Sigma Aldrich). In addition, pH was recorded by insertion of a pH probe into an aliquot of growth medium. Biomass was harvested by centrifugation of 1 ml aliquots at 740 g for 5 min. Nucleic acid extraction was performed prior to fluorescent and oligonucleotide assessment of RNA or DNA concentrations as described in detail above.

Fermenter vessels (LH Inceltech 501 series) that contained 1.5 l TSYGB medium buffered with KH₂PO₄ (8.5 g l⁻¹) were prepared and maintained at 37 °C, pH 6.8, for 24 h prior to inoculation with 10^{10} c.f.u. of an overnight culture of *L. monocytogenes* ATCC 19111. Constant rates of agitation, air flow and pH were maintained throughout. Samples were

removed to determine OD₆₆₀, viable counts and nucleic acid concentrations at intervals up to 456 h, as described above.

RESULTS AND DISCUSSION

Fluorometric quantification of nucleic acids

Titration series of DNA from *L. monocytogenes* and a mixture of 16S and 23S rRNA from *E. coli* were prepared and stained with ethidium bromide and fluorescence recorded after equilibration. A linear relationship between nucleic acid concentration and fluorescence was obtained (Fig. 1). For DNA and RNA, linear regression analysis gave equations of form $y = mx + C$: $y = 119.64x + 14.81$ ($r^2 = 0.99$) and $y = 31.05x + 7.22$ ($r^2 = 0.98$), respectively. Hence, the ratio of the DNA/rRNA slopes was 3.85. Consequently, for determination of RNA-DNA ratios, all values of RNA fluorescence were multiplied by 3.85. A linear relationship between nucleic acid concentration and ethidium-bromide-mediated fluorescence has been observed (LePecq & Paoletti, 1966), but calf thymus DNA and rat liver RNA were used and the slope ratio (2.82) was significantly different to that reported here. Kerkhof & Ward (1993) calculated nucleic acid ratios for bacteria based on this conversion factor which may not be appropriate for bacterial nucleic acids. The nucleic acid ratios reported here are based on analysis of DNA from *L. monocytogenes* and rRNA from *E. coli*, which gave a significantly larger and presumably more reliable conversion factor for bacteria. Measurement of ethidium-bromide-mediated nucleic acid fluorescence will record amounts of all double-stranded nucleic acid. Consequently, fluorescent determination of RNA concentration will have included 5, 16 and 23S rRNA and tRNA, and may overestimate RNA-DNA ratio. However, this effect will probably be less pronounced at high growth rates as it has been reported previously that in rapidly growing *E. coli* cultures, 85% of RNA is rRNA (Bremer & Dennis, 1987).

Fig. 1. Relationship between bacterial nucleic acid concentration and fluorescence. ●, *L. monocytogenes* DNA; ○, *E. coli* rRNA. Fluorescence was determined by treatment with ethidium bromide as described in Methods. Data are means of triplicates $\pm$ SD. Linear regression was determined as described in Methods.

Fig. 2. Relationship between *L. monocytogenes* nucleic acid concentration and relative hybridization signal intensity. (a) rRNA hybridized to *L. monocytogenes* species-specific oligonucleotide probe MV9RP2. (b) DNA hybridized to eubacterial specific oligonucleotide probe pA ($y = 22.376x + 92.644$, $r^2 = 0.9135$).

Quantitative oligonucleotide probing

In preliminary experiments, titration series of either *L. monocytogenes* RNA or DNA were immobilized onto nylon membranes and hybridized with radioactively labelled oligonucleotide probes. Subsequent examination of autoradiographs showed predictable relationships between nucleic acid concentration and signal intensity (pixel intensity above background).

Titration series of RNA showed a sigmoidal relationship between the logarithm of the nucleic acid concentration and pixel intensity (Fig. 2a). At the lowest amounts of nucleic acids applied (< 740 ng rRNA), the hybridization signal was below the detection threshold. This rRNA detection limit was equivalent to about 100 c.f.u. of a late-exponential-phase culture. Saturation of the nylon membrane occurred at >5 µg per slot. A linear relationship was observed between *L. monocytogenes* DNA concentration and pixel intensity for the range studied (Fig. 2b). Pixel intensities obtained for DNA were significantly lower than those obtained from RNA analysis (Fig. 2a) and the detection limit was about 1 µg DNA. This is significantly higher than that determined by Kerkhof & Ward (1993) and may be due to the many variables involved when preparing filters for oligonucleotide probing. However, the difference in amount of target sequence as a proportion of total

nucleic acid applied is significant. This is estimated to be 0.4% of total RNA and 0.004% of total DNA (assuming lengths of 5S + 16S + 23S rRNA to be 4566 bp and an *L. monocytogenes* genome size of 3.15×10^6 bp) (Michel & Cossart, 1992). In subsequent experiments, titration series of nucleic acids of known concentration and experimental samples were immobilized onto nylon membranes simultaneously. Calibration curves for DNA and RNA were prepared for each experiment and used to determine the nucleic acid concentration from experimental samples. These values were used to determine the RNA–DNA ratio.

Growth of *L. monocytogenes* in batch culture

Growth of *L. monocytogenes* ATCC 19111 in the complex unbuffered medium showed predictable exponential, stationary and death phases as determined by A_{660} (Fig. 3a). Viable counts determined on NAB plates, however, rapidly decreased to zero after 24 h incubation from a peak of 10^{10} c.f.u. ml⁻¹ after 10 h incubation (data not shown). No further growth was observed over the remainder of the experiment (168 h). Culture pH decreased from 6.3 to 4.3 during the exponential growth phase of 13 h duration, probably due to production of lactic and acetic acids that reduced the pH to a level inhibitory to growth (Pine *et al.*, 1989), a phenomenon that has been observed previously in *L. monocytogenes* (George & Lund, 1992; ter Steeg & Pieterman, 1991). Use of a *Listeria* selective recovery medium, for example modified Vogel–Johnson agar (Smith & Buchanan, 1990), may have enabled the detection of viable cells beyond 24 h.

Electrophoresis of nucleic acid extracts from this experiment gave clear bands of DNA and rRNA up until the 24 h sample in which nucleic acids had clearly undergone degradation (Fig. 3b). Both fluorometric and oligonucleotide probing analysis of nucleic acid extracts removed at intervals showed similar trends (Fig. 3c). RNA–DNA ratios were greatest at the end of the exponential growth phase (19 and 12.5 h, as determined by oligonucleotide probing and fluorometry, respectively) and decreased rapidly thereafter. The RNA–DNA ratio decreased to about 2 after 72 h (Fig. 3c) and remained at that value thereafter (data not shown). Nucleic acid ratios may be expressed as ribosome numbers using the formula: ribosome number = (RNA–DNA ratio $\times$ genome length)/the sum of the lengths of 5S + 16S + 23S rRNA (Kerkhof & Ward, 1993). After 12 h growth, ribosome numbers were calculated to be about 8600 per cell for an assumed genome length of 3.15×10^6 bp and rRNA length of 4566 bp. At the end of the sampling period (168 h), the ribosome number per cell was calculated to be about 1400. However, these data should be regarded as tentative estimates. During exponential phase in batch culture the expected growth rate (μ) of the cell population will tend towards $\mu_{\max}$ and cell samples will be relatively homogeneous (Neidhardt *et al.*, 1990). However, samples taken outside exponential phase will

Fig. 3. Growth of *L. monocytogenes* in complex unbuffered medium. (a) ●, OD₆₆₀; ○, pH. Data are means of triplicates and standard deviations were always within 10% of the mean. (b) Agarose gel electrophoresis of nucleic acid extracts of cells harvested at intervals: 4, 8, 10, 12, 13, 24, 48, 72, 120 and 144 h incubation. M, Molecular mass marker VI (Roche Diagnostics). (c) Nucleic acid ratios determined by fluorometry (●) and oligonucleotide hybridization (○).

contain a heterogeneous population of cells growing at different rates. Therefore, the RNA–DNA ratios reported here are a mean of the population status at a given time. Also, it is noteworthy that nucleic acid ratios do not discriminate between lag phase and starved cells. At these stages of the growth cycle, nucleic acid concentrations (in particular RNA) were at the limits of detection for both methods of analysis applied (Figs 1 and 2).

Growth of *L. monocytogenes* in pH-controlled batch culture

To determine the effects of prolonged culture of *L. monocytogenes* on survival and nucleic acid content, a pH-controlled batch culture was inoculated and growth was monitored. Growth of the culture showed a predictable exponential phase of about 10 h duration (Fig. 4a) followed by a prolonged death phase. Viable counts were 10^{10} c.f.u. ml⁻¹ after 10 h and 10^9 c.f.u. ml⁻¹ at the end of the experiment (456 h). The promotion of *L. monocytogenes* survival by maintaining the pH close to neutrality would appear to be correlated with the role of buffered dairy products as a vector for this human pathogen. During exponential growth, intact RNA and DNA were recovered (Fig. 4b) in accordance with the data from batch culture in the absence of pH control (Fig. 3b). The rapid degradation of DNA in prolonged

shake flask culture (Fig. 3b) was not apparent under pH control where intact DNA was maintained for the 96 h duration of the experiment (Fig. 4b), while 16S rRNA and 23S rRNA were lost. Estimation of RNA–DNA ratios by fluorometry and oligonucleotide probing gave similar patterns (Fig. 5). Nucleic acid ratios increased rapidly to a maximum value observed towards the end of the exponential growth phase and decreased rapidly thereafter. Nucleic acid ratios obtained from samples taken between 96 and 456 h were generally < 3, although viable counts were $> 10^9$ c.f.u. ml⁻¹. Nucleic acid ratios estimated from the starvation phase by fluorometry were consistently greater than those determined by oligonucleotide probing. This could be due to partial nucleic acid degradation and loss of oligonucleotide probe targets in samples taken from the starvation phase.

A number of studies have investigated the effects of prolonged culture or starvation on nucleic acid content and survival in various bacterial species. Maximum RNA–DNA ratio has been observed at, or close to the end of, exponential growth in *E. coli* (Davis *et al.*, 1986) and resin acid-degrading bacteria (Muttray & Mohn, 1998), although marine *Proteobacteria* showed strain-specific responses (Kerkhof & Kemp, 1999). When *E. coli* was starved and inoculated into fresh medium, a rapid increase in RNA–DNA ratio at the onset of

Fig. 4. Growth of *L. monocytogenes* in pH-controlled batch culture. (a) ●, OD₆₆₀; ○, c.f.u. ml⁻¹. Data are means of triplicates and standard deviations were always within 10% of the mean. (b) Agarose gel electrophoresis of nucleic acid extracts of cells harvested at intervals: 2, 4, 6, 8, 10, 12, 24, 48, 96 h incubation. M, Molecular mass marker VI (Roche Diagnostics).

Fig. 5. Nucleic acid ratios in relation to growth of *L. monocytogenes* in pH-controlled batch culture. ●, Ratio determined by fluorometry; ○, ratio determined by quantitative oligonucleotide hybridization. Data are means of triplicate determinations; error bars are standard deviations.

exponential growth was not observed (Cangelosi & Brabant, 1997). During exponential growth, bacteria will tend towards $\mu_{\max}$ and, in theory, contain maximum cellular concentrations of both rRNA and ribosomes. We have calculated from the data in Fig. 5 that the number of ribosomes per cell at the end of exponential growth was 24000 and 26000, as determined by oligonucleotide probing and fluorometry, respectively. The data show that whilst a relatively high viable count can be maintained after exponential growth has ceased (20–456 h), the RNA–DNA ratios decrease rapidly (Fig. 5). Indeed, measurement by oligonucleotide probing and

fluorometry estimated ribosome numbers of between 200 and 900 per cell, respectively, for this period. This is lower than that determined in a starved *Vibrio alginolyticus* culture (10^8 c.f.u. ml⁻¹) that contained an estimated 8000 ribosomes per cell (Flardh *et al.*, 1992), but in the same magnitude as that estimated in a marine strain of *Pseudomonas stutzeri* (Kerkhof & Ward, 1993).

The rate and degree of ribosomal loss at the onset of bacterial starvation appears to vary, depending upon the species studied and the nature of the starvation imposed. For example, *E. coli* cells have been reported to lose functional ribosomes rapidly after the onset of starvation (Davis *et al.*, 1986). However in *Vibrio alginolyticus*, RNA concentration decreased by up to 99% of exponential levels, but gradually over a 15 d starvation period (Kramer & Singleton, 1992). Starvation responses can be remarkably specific, as Kramer & Singleton (1992) also reported that *Vibrio furnissii* lost most of its rRNA during only 3 d of starvation. Maintenance of an active ribosomal pool would appear to be essential for cell survival and recovery. Starvation of *E. coli* cells resulted in rapid loss and degradation of functional ribosomes that led to rapid loss of viability (Kaplan & Apirion, 1975a, b; Davis *et al.*, 1986), but a pool in excess of translational requirements was maintained in marine *Vibrio* spp. (Flardh *et al.*, 1992). Ribosomes may represent a valuable source of metabolites to starved cells, but a critical number must be retained to maintain cell viability (Davis *et al.*, 1986). Differential retention of ribosomes shown by different bacterial species may also affect recovery rates from a stressed environment (Kramer & Singleton, 1992). It is possible that ribosome

dimers (100S ribosomes) reported to be of increased resistance to protease and nuclease degradation in *E. coli* (Wada *et al.*, 1990) formed during the prolonged stationary phase, although these were not found in starved *Vibrio* spp. cells (Flardh *et al.*, 1992).

In this paper, we have demonstrated that *L. monocytogenes* behaves in a similar manner to *E. coli*, i.e. viability and rRNA are lost rapidly once exponential growth has ceased. Viability can be maintained under buffered or pH-controlled conditions, enabling persistence of about 10% of the maximum exponential population, supported by the recovery of intact DNA, but not RNA.

In conclusion, the data presented here for *L. monocytogenes* show that it is possible to assess RNA–DNA ratios by fluorometry and oligonucleotide probing. In particular, we have shown that *L. monocytogenes* populations exhibit maximal RNA content late in the exponential growth phase and maintain viability over an extended period in pH-controlled batch culture, with greatly reduced cellular ribosome content when exponential growth has ceased. Having defined the *L. monocytogenes* RNA–DNA characteristics in relation to the growth cycle and maintenance of culture viability, modification of molecular detection methods for this pathogen to also provide information on their physiological status can be addressed.

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