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Quantification of Bacterial tmRNA using *in vitro* Transcribed RNA Standards and Two-Step qRT-PCR

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Abstract: tmRNA transcript copy number was determined in total RNA extracted from *Streptococcus pneumoniae* and *Staphylococcus aureus* exponential and stationary phases of growth using 5' exonuclease probes and two-step qRT-PCR. For each species, standard curves were prepared using Real-Time RT-PCR of homologous RNAs generated by *in vitro* transcription (cRNA standards) of cloned *ssrA* PCR product. Total RNA was prepared from 3 strains of each species and tmRNA copy number data was determined by real-time RT-PCR of the total RNA and interpolation to the cRNA standard curves. The mean tmRNA transcript level μg^{-1} of total RNA from *S. pneumoniae* was 9.63×10^9 (Range 2.32×10^9-2.15×10^{10}). For *S. aureus* the mean tmRNA copy number was 1.42×10^{10} transcripts μg^{-1} (Range 8.4×10^9-2.4×10^{10}). When normalised to total cellular RNA content, it was determined that there are 923 tmRNA transcripts cfu^{-1} in *S. pneumoniae* (Range 508-1384) and 1063 transcripts cfu^{-1} in *S. aureus* (Range 262-3192). This study presents the first absolute quantification data for tmRNA levels in bacterial species other than *Escherichia coli.* These results confirm the previously reported observation that tmRNA is present at high copy numbers in bacteria.

Key words: tmRNA, high copy number RNA, qRT-PCR, *in vitro* transcription, quantification of bacterial

INTRODUCTION

Bacterial transfer-messenger RNA (tmRNA, also known as 10Sa RNA) is the RNA transcript of the *ssrA* gene, first identified in *Escherichia coli* and subsequently found in all bacterial phyla (Keiler *et al.,* 2000; Williams, 2002). The molecule displays structural and functional properties of both tRNA and mRNA. The secondary structure and function of tmRNA has been well defined for *E. coli* and has been shown to be similar in other bacterial phyla (Muto *et al*., 1996, 1998). The 5' and 3' ends of the molecule fold in a self-complementary manner into a typical tRNA "cloverleaf" structure while the internal part of tmRNA contains an mRNA open reading frame encoding a proteolysis tag peptide (Hou and Schimmel, 1988; Komine *et al*., 1994). The most highly conserved structural features of tmRNA involve the alanyl-tRNA-like 5' and 3' ends, some properties of the degradation tag sequence and the overall secondary structure. Evaluation of diverse microbial populations suggests that apart from these core regions much of the tmRNA structure can vary with little effect on the basic function (Nameki *et al*., 1999; Kelley *et al*., 2001).

The function of tmRNA in bacteria is to add a C-terminal peptide tag to incomplete nascent polypeptides translated from a broken mRNA lacking a stop codon (Tu *et al*., 1995). In this manner tmRNA rescues stalled ribosomes and facilitates the degradation of incomplete polypeptides by proteolysis specifically targeting the tagged molecules (Muto *et al*., 1996). *In vivo,* tmRNA is a component of a ribonucleoprotein complex composed of tmRNA and Small protein B (SmpB) (Saguy *et al*., 2005). All known biological activities of tmRNA require SmpB and it is this complex that functions as the rescue element to free ribosomes stalled on defective mRNAs (Haebel *et al*., 2004). The *ssrA*-SmpB system seems to be a universal feature of all eubacteria. Some exotic rearrangements of the *ssrA* gene appear in the α-proteobacteria where a two piece tmRNA is assembled post-transcriptionally, while the presence of this RNA in a bacterium like *Mycoplasma genitalium* which has fewer than 500 genes provides further support for an important biological role (Karzai *et al*., 2000). The s*srA*-SmpB system is not required for growth in *E. coli* but is essential for growth of *Neisseria gonorrhoeae, M. genitalium* and *M. pneumoniae.* It is dispensable for normal growth of *Salmonella typhimurium* but is required for survival of

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this bacterium within macrophages (Karzai *et al*., 2000). Genes for tmRNA have also been identified in some plastid, mitochondrial and bacteriophage genomes (Zwieb *et al*., 2003; Wower *et al*., 2005).

Bacterial tmRNA has been the extensively studied at a structural (Felden *et al*., 1997; Hanawa-Suetsugu *et al*., 2001; Gutmann *et al*., 2003) and functional (Himeno *et al*., 1997; Okada *et al*., 2004; Shpanchenko *et al*., 2005) level. Studies of the effect of tmRNA expression levels on bacterial viability have employed relative quantification techniques such as northern blotting and densitometry (Muto *et al*., 2000) while the only experimentally determined estimate of tmRNA copy number came from a 1978 study which incorporated radiolabeled nucleotides into exponential phase *E. coli*. This study estimated that exponentially growing *E. coli* had between 500-1000 copies of tmRNA per cell (Lee *et al*., 1978). Real-time PCR has been shown to be suitable for the quantification of gene copy number (Stults *et al*., 2001; Bustin, 2002; Bustin and Mueller, 2005). For estimation of RNA copy number in samples, an *in vitro* transcribed cRNA standard homologous to the transcript being quantified is recommended for the generation of the standard curve (Bustin, 2002; Fronhoffs *et al*., 2002). We have applied these techniques to determine the tmRNA transcript copy number of *S. pneumoniae* and *S. aureus*. This study provides the first quantitative data for tmRNA copy number in bacteria other than *E. coli* and also demonstrates the general application of *in vitro* transcribed RNA standards and real-time PCR for the quantification of RNA transcripts in bacteria.

MATERIALS AND METHODS

Preparation of cRNA for use in standard curves: Amplification primers for the *ssrA* genes of *S. pneumoniae* and *S. aureus* are listed in Table 1. Cycling conditions were 95°C for 5 min followed by 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 45 s and a final extension step of 72°C for 5 min. PCR products were purified using QIAquick® PCR purification columns (Qiagen) and cloned into TOPO II cloning vector (Invitrogen). Following transformation into TOP 10 cells and selection on Luria Bertani (LB) plates containing 50 μg mL^{-1} ampicillin and 40 μl of 40 mg mL^{-1} X-gal (Sigma-Aldrich), white colonies containing inserts were selected and plasmids were prepared using QIAprep® plasmid preparation kit (Qiagen).

Purified plasmids were sequenced (Sequiserve, Germany) to determine the orientation of the insert relative to the T7 RNA promoter site on the plasmid. Plasmids containing an insert with the forward primer adjacent to the promoter site were used to generate a sense RNA suitable for RT-PCR (Technical bulletin 156, www.ambion.com).

cRNA templates for the preparation of standard curves were generated by *in vitro* transcription of plasmid vector containing cloned *ssrA* PCR product inserts from each species using previously described methods (Glynn, 2006).

Bacterial strains and growth conditions: *S. pneumoniae* and *S. aureus* strains used in this study are listed in Table 2. Both species were plated onto Mueller Hinton agar supplemented with sheep blood (LIP Ltd., Galway, Ireland) and grown overnight at 37°C in a gas jar. Following overnight incubation, single colonies of *S. pneumoniae* were isolated and inoculated into 20 mL of Brain Heart Infusion (BHI) broth (Oxoid) and incubated overnight at 37°C with shaking. *S. aureus* colonies were inoculated into 20 mL LB broth and grown under the same conditions. Two millilitres of overnight culture were transferred into 100 mL of appropriate broth and incubated at 37°C with shaking. Growth curves were prepared for each species by taking optical density readings (600 nm) at 1 h intervals. A plot of optical density versus time was used to identify mid-exponential and stationary phase of growth for each strain.

DNA extraction: Genomic DNA was prepared from 10 ml overnight culture using the QIAamp® DNA mini-kit (Qiagen) according to manufacturer's instructions.

Preparation of total RNA from *S. pneumoniae* and *S. aureus*: The Ambion RiboPure™ Yeast RNA purification kit was used for the preparation of total RNA from *S. pneumoniae* and *S. aureus* exponential and stationary phase cultures. RNA preparations were analysed using Agilent 2100 Bioanalyser and RNA 6000 LabChip® system (Agilent Technologies).

Determination of RNA content per cell: The total amount of RNA recoverable from 1 mL of culture was calculated using the Ambion kit and Agilent analysis. Total cfu mL^{-1} was quantified by plating triplicate serial dilutions of culture from both growth phases for each strain onto appropriate agar plates. Following overnight incubation, colonies were counted and the total cfu mL^{-1} of the original culture was calculated. The recoverable RNA content per cfu was calculated by division of the quantity of RNA recovered from 1 mL of broth by the number of cfu mL^{-1} for the relevant strain and growth phase.

Two-step real time RT-PCR quantification of tmRNA transcripts: Two-step qRT-PCR incorporating *in vitro*

Table 1: Oligonucleotide primers and probes used during this study

PCR primers	Function	Sequence 5'-3'
Spnu_quantF	*S. pneumoniae* tmRNA amplification 5' primer	AAC GCT CAG TTA AAT
Spnu_quantR	*S. pneumoniae* tmRNA amplification 3' primer. First strand cDNA synthesis.	TCT AGA AAC TGC GAG
S.aur F_2	*S. aureus* tmRNA amplification 5' primer	GAA GTT CAT GGA TTC GAC A
S.aur R_2	*S. aureus* tmRNA amplification 3' primer. First strand cDNA synthesis.	ATA CAC ATC CTT TCT ACG TGT
DNA Probes		
QtmRNA_SP	5' FAM, 3' TAMRA labelled quantification probe for *S. pneumoniae* tmRNA.	TAT TAG CGA GAT ACG ATT AAG CCT TGT
SaTM2	5' FAM, 3' TAMRA labelled quantification probe for *S. aureus* tmRNA.	TGT TTA TCA CTT TTC ATG ATG CGA

Table 2: Bacterial strains used during this study. DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen, (German Collection of Microorganisms and Cell Cultures), Braunschweig, Germany. NCTC: National Collection of Type Cultures, Health Protection Agency, London

Species (Strain)	Source	Reference	Comment
S. pneumoniae (6303)	NCTC	NCTC 6303	Type strain
S. pneumoniae (11865)	DSMZ	Serogroup 9V	Clinical source
S. pneumoniae (20566)	DSMZ	NCTC 7465	Type strain
S. aureus (20231)	DSMZ	NCTC 8532	Type strain
S. aureus (6732)	DSMZ	ATCC 25178	Veterinary mastitis strain
S. aureus (MRSA 252)	Dept of Microbiology, NUI, Galway	Holden *et al.* (2004)	Clinical isolate

transcribed cRNA standards was used to quantify tmRNA copy numbers in RNA extracted from exponential and stationary phase cultures of *S. pneumoniae* and *S. aureus* (Glynn, 2006). Standard curves were generated from this data by the LightCycler® software as described. At least 3 repeats of each qRT-PCR were performed. A standard curve prepared from the appropriate cRNA template was included in each LightCycler® run.

Data analysis: Integrated LightCycler® software (Version 3.5.1, Feb, 2001) was used to measure the slope of a plot of the ct values for a dilution series of the cRNA standards (1×10^2-1×10^8 copies) and the cDNA samples generated from total RNA of *S. pneumoniae* and *S. aureus* cultures during exponential and stationary phases. PCR amplification efficiency was calculated by the formula $E = 10^{-1/S}$, where E is the amplification efficiency and S is the slope of the plotted ct values. Quantification data was only considered for reactions where the E value for the standards closely matched the E value for the total RNA samples (Values were all within 0.2). Standard curves were run in parallel with samples from both mid-log and stationary phase total RNA from all strains both *S. pneumoniae* and *S. aureus*.

Copy number of tmRNA was calculated by interpolation from the appropriate standard curves and expressed as tmRNA molecules per μg total RNA. Quantification values were also normalised against the total recoverable RNA content per cfu of *S. pneumoniae* and *S. aureus* during the appropriate growth phase and expressed as tmRNA transcripts per cfu.

RESULTS AND DISCUSSION

Figure 1 shows a representative set of amplification curves from *in vitro* transcribed cRNA standards and a dilution series of total RNA from *S. pneumoniae* exponential phase culture. Results of at least 3 independent quantitative real-time RT-PCR experiments for each species, strain and growth phase are summarised in Table 3. These results demonstrate that tmRNA is present at high transcript number μg^{-1} of total RNA prepared from different strains and at different growth phases for *S. pneumoniae* and *S. aureus*. *S. pneumoniae* exponential phase cultures contained a mean of 9.22×10^9 transcripts μg^{-1} total RNA (Range 7.05×10^9-1.2×10^{10}) while stationary phase cultures contained a mean of 1.0×10^{10} transcripts μg^{-1} (Range 6.3×10^9-2.15×10^{10}). *S. aureus* exponential phase RNA had a mean of 1.93×10^{10} tmRNA transcripts μg^{-1} (Range 1.6×10^{10}-2.4×10^{10}) while total RNA prepared from stationary phase cultures had a mean of 9.17×10^9 tmRNA transcripts μg^{-1} (Range 8.4×10^9-1×10^{10}).

Figure 1 shows amplification curves of standards and unknown total RNA samples and plot of ct values. A dilution series of cRNA standards for *S. pneumoniae* tmRNA containing from 1×10^8-1×10^4 copies per reaction was reverse transcribed and 2μL of each cDNA amplified on the LightCycler® (Fig. 1a). A total RNA dilution series consisting of from 10 pg-1 ng *S. pneumoniae* 20566 mid-exponential phase RNA was also reverse transcribed and the cDNA amplified in the same LightCycler® run (Fig. 1b). Standard curves were prepared by plotting the ct values of the standards (Fig. 1c) and the total RNA sample (Fig. 1d). Amplification efficiencies were calculated from the slope of this plot using the formula $E = 10^{-1/S}$.

Measurement of total RNA cfu^{-1} from exponential and stationary phase cultures of *S. pneumoniae* and *S. aureus*: In order to normalise tmRNA results to transcript number cfu^{-1} the amount of recoverable total RNA cfu^{-1}

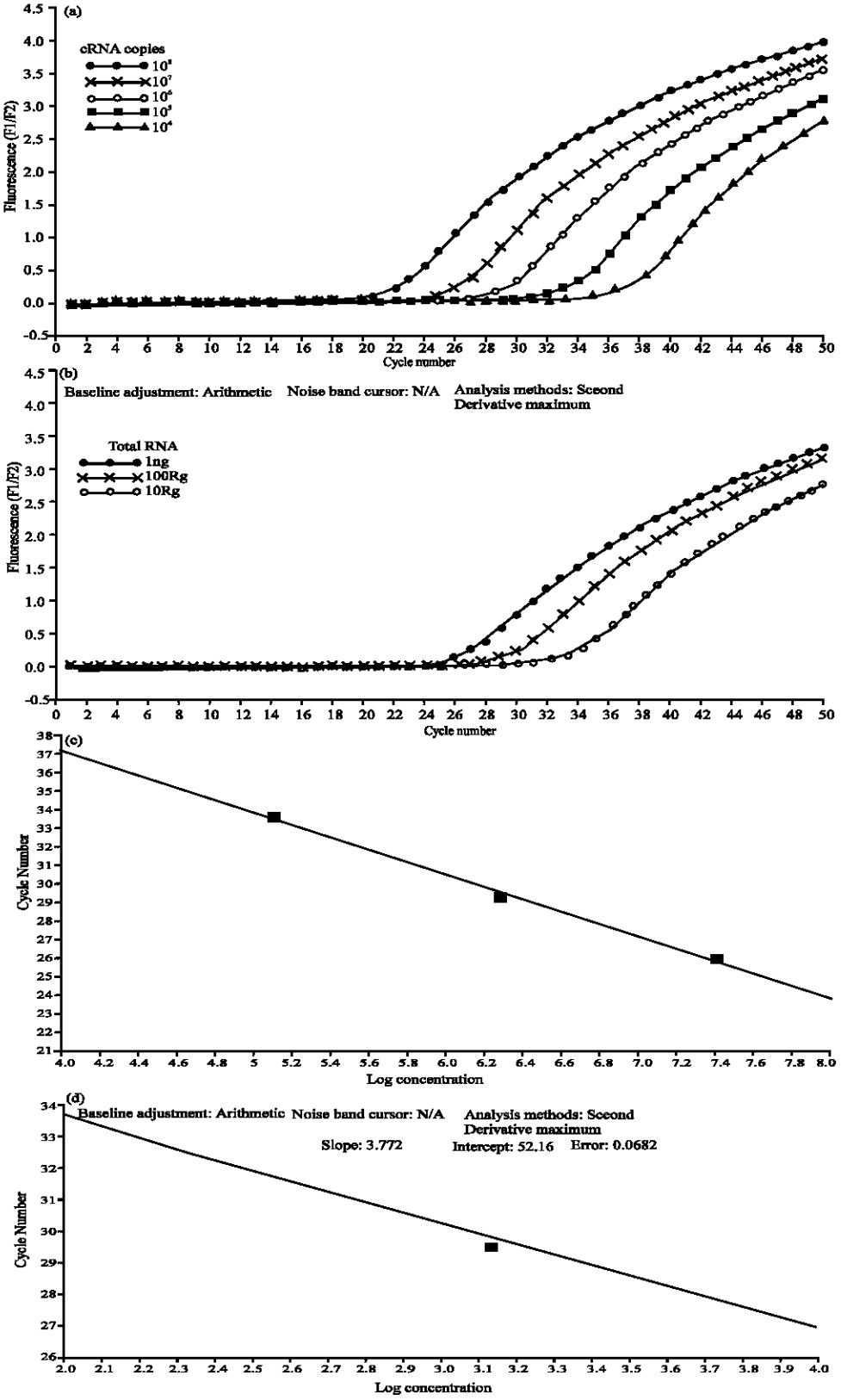

Fig. 1: Amplification curves of standards and unknown total RNA samples and plot of ct value

Table 3: tmRNA transcript levels μg^{-1} and cfu^{-1}

Species (Strain)	Growth phase	Mean tmRNA copy number μg^{-1}	Standard Deviation (SD)	Mean RNA content (fg cfu^{-1})	SD	Mean tmRNA content cfu^{-1}	SD
S. pneumoniae							
(NCTC6303)	Exponential	7.05×10^9	2.23×10^9	113	44	797	289
	stationary	2.32×10^9	6.75×10^8	219	144	508	148
S. pneumoniae							
(DSM11865)	Exponential	8.82×10^9	9.16×10^8	110	10.4	971	101
	stationary	2.15×10^{10}	1.03×10^9	64.5	3.5	1384	62
S. pneumoniae							
(DSM20566)	E×ponential	1.18×10^{10}	3.81×10^9	70.5	14.5	832	269
	stationary	6.3×10^9	1.01×10^9	159	53.5	1046	76.2
S. aureus							
(MRSA252)	Exponential	1.6×10^{10}	1.53×10^9	45.4	7.6	3192	75
	stationary	8.4×10^9	2.07×10^9	28.8	4.9	1034	255
S. aureus							
(DSM 6732)	Exponential	2.4×10^{10}	4.94×10^9	38.6	7.6	1039	215
	stationary	8.5×10^9	7.74×10^9	2.9	0.6	262	24
S. aureus							
(DSM 20231)	Exponential	1.8×10^{10}	1.56×10^9	24.6	1.5	553	48
	stationary	1.06×10^{10}	1.21×10^9	5.8	0.4	297	34

was established for each of the *S. pneumoniae* and *S. aureus* strains at both exponential and stationary phases of growth. In this laboratory, the RiboPure™-Yeast Kit (Ambion) consistently gave the highest yields of total RNA from *S. pneumoniae* and *S. aureus* compared to other methods. Addition of a Lysostaphin pre-treatment step did not improve total RNA yield from *S. aureus* using this kit (Data not shown). *S. pneumoniae* was *S. aureus* total RNA yield cfu^{-1} are summarised in Table 3. For two *S. pneumoniae* strains (6303 and 20566) a higher level of RNA was recovered from stationary phase cultures compared to mid-exponential phase cultures. From the third *S. pneumoniae* strain (11865) the total RNA recovered was higher at mid-exponential phase compared to stationary phase. The total RNA recovered from *S. aureus* strains was consistently lower than from *S. pneumoniae* cultures. For stationary phase *S. aureus* cultures there was a large reduction in RNA yield for two of the strains studied (6732 and 20231). The RNA yields obtained may be results of a reduction in the RNA content of the cell during stationary phase of growth. However, the total RNA yields obtained may be reflecting the relative efficiencies of the RNA extraction method used for different cell types. There is a possibility that the RNA extraction protocol applied may not have achieved efficient lysis of the *S. aureus* stationary phase cell wall and this may have contributed to the lower yield of total RNA cfu^{-1} in these strains.

Normalisation of tmRNA copy number to total RNA cfu^{-1}: Recoverable total RNA yield cfu^{-1} was used to normalise the tmRNA transcript numbers (Table 3). The *S. pneumoniae* strains had a mean of 923 tmRNA transcripts cfu^{-1} (Range 508-1384). As can be seen, the transcript levels did not always vary in proportion to recoverable total RNA yield cfu^{-1}. However, the overall tmRNA copy number in *S. pneumoniae* was relatively stable between exponential and stationary phase samples and may suggest that this molecule is transcribed during periods of maximum growth and remains stable within the cell into stationary phase growth.

Mean *S. aureus* exponential phase tmRNA transcript numbers cfu^{-1} was 1593 (Range 553-3192). Stationary phase *S. aureus* had a mean of 531 tmRNA transcripts cfu^{-1} (Range 262-1034). It may be of note that the *S. aureus* strain determined to have the highest stationary phase tmRNA transcript number cfu^{-1} (MRSA252) also gave the highest total RNA cfu^{-1} yield.

The previously published figure for tmRNA (10Sa) copy number in bacteria involved radio-labelling of total RNA followed by cell lysis and polyacrylamide gel electrophoresis to separate RNAs by size (Lee *et al*., 1978). The novel 10S RNA fraction was recognised as comprising a mixture of 2 RNA molecules (Designated 10Sa and 10Sb) and while it was not possible to separate them, it was estimated that the total 10S RNA content was 1000 copies and that each of the components was present at approximately 500 copies $genome^{-1}$. The 10Sb fraction was later characterised as a precursor molecule for the RNA moiety of the processing enzyme RNase P (Gurevitz *et al.*, 1983).

In this study, we have employed a more selective methodology for the quantification of tmRNA transcripts. Quantitative real-time RT-PCR has become the benchmark technology for the measurement and comparison of RNA levels (Bustin *et al.*, 2005). qRT-PCR assays are becoming established in clinical settings for the detection of viral load and therapy monitoring (Bustin and Mueller, 2005). However, the use of qRT-PCR for accurate transcript measurement requires control of key variables including

template quality, variability in the RT step itself as well as variation that may be introduced in data analysis and interpretation (Bustin and Nolan, 2004). We have taken steps to limit these variations by monitoring of template quality using the Agilent Bioanalyser, parallel amplification of cRNA standards and total RNA templates and measurement of tmRNA from multiple strains of each species.

CONCLUSION

High copy number RNAs, such as tmRNA, have the potential to be exploited as species-specific recognition elements when combined with emerging non-amplified nucleic acid detection technologies (Baeumner *et al*., 2004; Xie *et al*., 2004). In this study, we have conclusively demonstrated that tmRNA is present in both *S. pneumoniae* and *S. aureus* at a high copy number. Bacterial tmRNA is known to contain regions of sequence variably which may be exploited for the species-specific detection and identification of bacteria A Fluorescent *In Situ* Hybridisation (FISH) assay targeting *Lactococcus lactis* tmRNA has already demonstrated the potential application of this molecule in diagnostics applications (Schonhuber *et al.,* 2001).

Quantification of specific RNA copy number in a clinical, environmental or food sample may also provide a means to rapidly monitor the number of viable bacteria present in the sample. The application of a RNA quantification methodology strategy to monitor bacterial load during an anti-bacterial treatment may also provide useful information that cannot currently be obtained from DNA based diagnostics or conventional microbiological approaches (Picard and Bergeron, 2002).

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