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The use of molecular beacons to directly measure bacterial mRNA abundances and transcript degradation

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ABSTRACT

The regulation of mRNA turnover is a dynamic means by which bacteria regulate gene expression. Although current methodologies allow characterization of the stability of individual transcripts, procedures designed to measure alterations in transcript abundance/turnover on a high throughput scale are lacking. In the current report, we describe the development of a rapid and simplified molecular beacon-based procedure to directly measure the mRNA abundances and mRNA degradation properties of well-characterized *Staphylococcus aureus* pathogenicity factors. This method does not require any PCR-based amplification, can monitor the abundances of multiple transcripts within a single RNA sample, and was successfully implemented into a high throughput screen of transposon mutant library members to detect isolates with altered mRNA turnover properties. It is expected that the described methodology will provide great utility in characterizing components of bacterial RNA degradation processes and can be used to directly measure the mRNA levels of virtually any bacterial transcript.

1. Introduction

Staphylococcus aureus is a major cause of hospital- and community-associated infections, causing approximately 90,000 invasive infections and 18,000 deaths in the US annually (Klevens et al., 2007). The organism's proficiency at causing infection is largely mediated by its ability to temporally regulate the mRNA titers and consequently protein production, of an expansive repertoire of virulence and antibiotic resistance determinants. The regulatory networks that govern *S. aureus* virulence factor expression are complex. Indeed, the DNA binding protein, SarA, the SarA family of homologues, and at least seven two-component regulatory systems have been shown to affect virulence factor transcript synthesis (Bronner et al., 2004). Moreover, we and others have shown that regulated changes in RNA stability affect the expression of *S. aureus* virulence and antibiotic resistance determinants (Anderson et al., 2006; Huntzinger et al., 2005; Roberts et al., 2006).

Recent studies have revealed that the modulation of mRNA turnover constitutes a common bacterial regulatory mechanism [reviewed in Takayama and Kjelleberg, 2000]. Despite this, advances in methodologies designed to characterize this phenomenon are lacking. Most studies rely on Northern blotting, quantitative reverse transcriptase-mediated polymerase chain reaction (qRT-PCR), or similar technologies to compare the mRNA turnover properties of individual transcripts during control and test conditions. While useful, these procedures are too laborious and/or costly to perform in a high throughput manner, a requisite for screening mutants and other procedures that have aided characterization of bacterial processes. Accordingly, our goal was to develop a simple and efficient system to accurately measure the mRNA abundances and turnover properties of bacterial cells in a high throughput manner.

To do so, we exploited the inherent properties of a class of fluorescent probes, termed molecular beacons. Each beacon is composed of a small nucleic acid hairpin structure which contains a fluorophore and a non-fluorescent quencher moiety covalently attached to their 5′ and 3′ termini (Tyagi and Kramer, 1996). The loop portion of the beacon can be engineered to contain sequence that is complementary to ~25 nucleotides (nt) of a nucleic acid target of interest. In the absence of the target, the beacon assumes a hairpin structure placing the quencher molecule and fluorescent dye in close proximity, resulting in little or no fluorescence. Conversely, in the presence of target, the beacon hybridizes which separates the fluorescent and quencher molecules and results in fluorescence (Fig. 1). The amount of fluorescence serves as a measure of target abundance. Indeed, molecular beacons have had a profound impact in advancing PCR based detection methodologies, allowing real time quantification of targeted amplicons in a homogeneous and rapid format [reviewed in Marras et al., 2006].

The current study describes the development of a simplified molecular beacon-based procedure to simultaneously measure the mRNA abundances of multiple mRNA species within a single bacterial RNA sample. This procedure does not require any *in vitro* PCR-based amplification steps and can be used to accurately measure the

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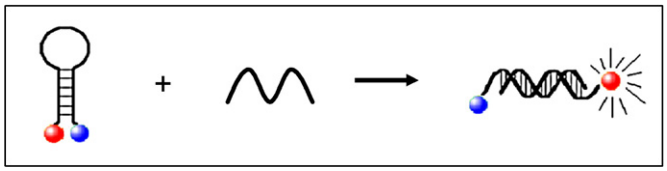

Fig. 1. Overview of molecular beacon technology. Beacons are engineered to conform to a hairpin structure placing a conjugated fluorophore (red circle) in close proximity to a quencher molecule (blue circle), resulting in little fluorescence. In the presence of a target nucleic acid (represented by single wavy line) hybridization can occur. This disrupts hairpin formation, separating the fluorophore and quencher molecules and resulting in fluorescence.

degradation properties of bacterial transcripts. Further, the procedure is amenable to screening large numbers of bacterial samples, as evidenced by our ability to identify members of a *S. aureus* transposon mutant library with altered mRNA turnover properties. We anticipate that the molecular beacon-based approach described here will have great utility in characterizing other bacterial RNA degradation processes and can be used to directly measure the mRNA levels of virtually any bacterial transcript.

2. Materials and methods

2.1. Bacterial strains

S. aureus strain Newman and the *Bursa aurealis* mariner transposon system plasmids, pFA545 and pBursa, were obtained from Dr. D. Missiakas (University of Chicago, Chicago, IL). A *S. aureus* Newman mariner transposon mutant library was constructed essentially as previously described (Bae et al., 2004). Briefly, plasmids pFA545 and pBursa were electroporated into the restriction deficient *S. aureus* strain RN4220 then transferred to strain Newman via ϕ11 phage transduction. Plasmids were then cured by high temperature growth and colonies were screened for transposition events (Erm^R, Tet^S, Cam^S) by replica plating onto appropriate antibiotic medium. Southern blotting confirmed that one random transposition event had occurred per mutant. In total, 9600 mutants were stored in 96-well microtiter plates at −80 °C. *S. aureus* strain UAMS-1 was obtained from Dr. M. Smeltzer (University of Arkansas Medical Center, Little Rock, AR). Strain KLA16 (UAMS-1 Δ*spa*) was generated by exchanging the UAMS-1 *spa* locus with an erythromycin resistance cassette by allelic replacement.

2.2. Bacterial growth conditions

For molecular beacon validation assays, overnight *S. aureus* cultures were diluted 1:100 in fresh Brain Heart Infusion medium (BHI; BD, Spark, MD) and incubated at 37 °C at 225 rpm until they reached early-exponential phase growth ($OD_{600\ nm}$=0.25). Cultures were then either allowed to continue incubation for an additional 30 min (mock treated) or treated with mupirocin (60 μg ml^{-1}; AppliChem, Cheshire, CT) for 30 min to induce the stringent response, as previously described (Anderson et al., 2006). Transcript synthesis was then arrested by the addition of rifampicin (200 μg ml^{-1}; Sigma-Aldrich, St. Louis, MO). Aliquots were removed at 0, 10, 15, and/or 30 min post-transcriptional arrest and stored for RNA isolation at −80 °C in ethanol:acetone solution (1:1), as previously described (Anderson et al., 2006; Roberts et al., 2006). For transposon mutant screening assays, overnight cultures were diluted in BHI medium, grown to early-exponential phase, transcription was arrested, and cell suspensions were stored, as described above.

2.3. Reverse transcriptase-mediated PCR (RT-PCR) and quantitative reverse transcriptase-mediated PCR (qRT-PCR)

Frozen cell suspensions were thawed on ice and total bacterial RNA was purified using RNeasy kits with on-the-column DNase treatment (Qiagen Inc., Valencia, CA), as previously described (Anderson et al., 2006; Roberts et al., 2006). RNA concentrations were determined by spectrophotometry ($OD_{260\ nm}$ 1.0=40 μg ml^{-1}) then used for RT-PCR and/or molecular beacon assays. Titan One Tube RT-PCR kits were used for RT-PCR reactions, following the manufacturer's recommendations (Roche Applied Science; Indianapolis, IN). Reactions included 5 ng of bacterial RNA template and 50 ng each of *spa*-specific primers (5′ CAGATAACAAATTAGCTGATAAAAACAT and 5′ CTAAGGCTAATGATAATCCACCAAATAC) or *norA*-specific primers (5′ AGTGATTTAGGGTTACTTGTTGCTG and 5′ CAACTGCAAACATAAATTCTGACAC). RT-PCR products were assessed by gel electrophoresis in a 1% agarose gel for 3 h at 75 V and visualized by ethidium bromide staining. The aforementioned *norA*-specific primers and 25 ng of indicated RNA sample were used for quantitative real time-PCR. Bacterial RNA was reverse transcribed, amplified, and measured using a LightCycler RNA Master SYBR Green I kit (Roche Applied Science, Indianapolis, IN), following the manufacturer's recommendations.

2.4. Molecular beacon design

Molecular beacons were obtained from Biosearch Technologies, Inc. (Novato, CA). For beacon design, the mRNA structural features of *S. aureus spa* and *norA* transcripts were determined using Mfold prediction software with temperature constraints set at either 50 °C or 60 °C [version 3.0; www.//mfold.burnet.edu.au/ (Zuker, 2003)]. The top five predicted structural features for each transcript were compared and common single-stranded RNA (ssRNA) regions of ≥20 nt were identified. *norA*- and *spa*-mRNA specific molecular beacons were engineered to be complementary to predicted regions of ssRNA (Table 1). With the exception of Spa2, the 5′ terminus of each molecular beacon contained a fluorescein (FAM; fluorophore) molecule covalently linked to the hexanucleotide sequence 5′ CCCAGG; a central region (loop) containing *norA* or *spa* ssRNA complementary sequence (20–29 nt); and a 3′ terminus ending with the

Table 1
Molecular beacons used in this study

Designation	Molecular beacon sequence[a]	Target sequence[b]	Fluorophore[c]
NorA1	5′ CCCAGGACTACCAGTTAATCCCAAATCCTGGG	*norA* mRNA (95–115)	FAM (520 nm)
NorA2	5′ CCCAGGAAATGGCATACGATGTGAAACTTCTGCCCCTGGG	*norA* mRNA (450–479)	FAM (520 nm)
NorA3	5′ CCCAGGTTGGAAAAGTGCACCAAAGATACCGCCCCTGGG	*norA* mRNA (738–765)	FAM (520 nm)
Spa1	5′ CCCAGGCCGTCTTCTTTGCCAGGTTTTTTGTTGTCCCTGGG	*spa* mRNA (1078–1106)	FAM (520 nm)
Spa2	5′ CCCAGGCCGTCTTCTTTGCCAGGTTTTTTGTTGTCCCTGGG	*spa* mRNA (1078–1106)	Pulsar® 650 (650 nm)

[a] A fluorophore and a black hole quencher molecule were covalently linked to the 5′ and 3′ termini of each sequence. Underlined regions are predicted to form a 6 base pair stem portion of a hairpin structure placing the fluorophore and quencher moieties in close proximity. The central portion of each molecular beacon is complementary to the indicated region of *norA* or *spa* mRNA (annealing temperatures 54–56 °C) and is predicted to form the loop portion of a hairpin structure.

[b] The loop portion of each molecular beacon is engineered to anneal to the indicated *S. aureus* mRNA sequence. Numbers in (parentheses) indicate region of target mRNA complementarity.

[c] Shown are the fluorophore attached to each molecular beacon. Number in (parentheses) indicates fluorophore's maximum emission wavelength.