DEAD-box RNA helicases in gram-positive RNA decay

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CHAPTER SEVENTEEN

DEAD-Box RNA Helicases in Gram-Positive RNA Decay

Peter Redder *and* Patrick Linder

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Abstract

DEAD-box RNA helicases are important players in eukaryotic and bacterial RNA metabolism. A helicase from *Staphylococcus aureus* was recently shown to affect RNA decay, most likely via its interaction with the proposed Gram-positive degradosome. Some, but not all, RNAs are stabilized when the helicase CshA is mutated, and among the affected RNAs is the *agrBDCA* mRNA, which is responsible for quorum sensing in *S. aureus*. We describe how the stabilization of *agr* mRNA (and others) can be measured and how to conduct assays to measure the effects of quorum-sensing defects, such as biofilm formation and hemolysin production.

1. Introduction

Proteins from the DEAD-box RNA helicase family represent the largest family of helicase proteins throughout eukaryotic and prokaryotic systems (Linder and Jankowsky, 2011; Tanner *et al.*, 2003). DEAD-box

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proteins can be defined by the presence of 12 conserved motifs (Jankowsky, 2011). Although individual motifs may vary as, for example, the DECD motif instead of DEAD in Sub2, the proteins are easily identified by the overall presence of these motifs. Several of these motifs are required for binding and hydrolysis of the ATP, whereas others are involved in RNA binding or intraprotein interactions.

Originally, the DEAD-box protein family was proposed to be a collection of RNA helicases (Linder *et al.*, 1989). This was based on the ability of DEAD-box proteins to alter RNA secondary structures and their presence in dynamic processes such as translation initiation and ribosome biogenesis. Consistent with this idea, a multitude of DEAD-box proteins are required in processes that use guide RNAs to perform RNA modification, RNA processing, or RNA editing. Moreover, genetic evidences suggest that DEAD-box proteins are indeed required for dissociation of guide RNA containing complexes *in vivo* (Chen *et al.*, 2001; Kistler and Guthrie, 2001). Nevertheless, a bona fide dsRNA dissociation activity *in vivo* is extremely difficult to demonstrate.

Whereas eukaryotic genomes encode a multitude of DEAD-box proteins (25 in yeast, 36 in humans), bacteria encode only a few of them (5 in *Escherichia coli*, 4 in *Bacillus subtilis*, 2 in *Staphylococcus aureus*, 6 in *Pseudomonas aeruginosa*, 9 in *Pseudoalteromonas haloplanktis*, 10 in *Vibrio fischeri*, 11 in *Colwellia psychrerythraea*; Iost and Dreyfus, 2006; Lopez-Ramirez *et al.*, 2011). Moreover, many DEAD-box proteins are essential in eukaryotes, but mutations in bacterial DEAD-box protein genes at most give a cold-sensitive phenotype. Interestingly, even a quintuple mutant in *E. coli* is viable, suggesting that these proteins do not perform essential functions under normal laboratory conditions (Jagessar and Jain, 2010).

So far, in bacteria only DEAD-box proteins from *E. coli* were studied in detail: CsdA (originally called DeaD) and SrmB were identified in genetic screens using mutations in ribosomal proteins and their role in ribosome biogenesis has since been confirmed. Extensive and beautiful work has pinpointed a CsdA function to the biogenesis of the 50S subunit (Iost and Dreyfus, 2006). The DbpA protein is famous for its requirement for a specific RNA substrate for efficient ATP hydrolysis (Fuller-Pace *et al.*, 1993). Indeed, this DEAD-box protein is exclusively and heavily stimulated in its activity by the hairpin h92 from the 23S rRNA (Diges and Uhlenbeck, 2001; Nicol and Fuller-Pace, 1995). Recently, it was shown that a dominant-negative mutation in motif VI of *dbpA* results in a ribosome biogenesis defect (Elles and Uhlenbeck, 2008). While the above proteins are involved in ribosome biogenesis, the RhlB protein is part of the degradosome, a multicomponent complex involved in the targeted degradation of a large subset of mRNAs (Carpousis, 2007; Py *et al.*, 1996). The RNA helicase in this complex is associated on the scaffold protein RNase E and is generally assumed to assist the

degradosome on structured RNAs. Nevertheless, the precise role of the helicase in RNA degradation is not known. Interestingly, CsdA can also associate with the degradosome under cold-shock conditions, but this interaction does not use the same region of RNase E involved in the interaction with RhlB (Prud'homme-Genereux *et al.*, 2004). Finally, RhlE is the least-characterized RNA helicase in *E. coli*. Genetic data indicate that RhlE might be involved in ribosome biogenesis together with CsdA and SrmB (Jain, 2008). Other data show that RhlE is also able to associate with the degradosome (Khemici *et al.*, 2004). Clearly further work will be required to elucidate the function(s) of RhlE.

RNA helicases are important players in RNA metabolism and participate in the dynamic rearrangement of RNP (ribonucleoprotein) complexes. It is therefore not surprising that several bacterial DEAD-box proteins popped up in a variety of screens. In cyanobacteria, the RNA helicases CrhC and CrhR are induced by cold and redox stress, respectively (Owttrim, 2006). In *Bacillus cereus*, RNA helicases from the DEAD-box family were identified by mutagenesis to be required for survival at low temperatures, pH, and oxidative stress (Pandiani *et al.*, 2010). Similarly, Tn916 mutagenesis identified an RNA helicase from the Gram-positive *Clostridium perfringens* to be involved in the adaptive response to oxidative stress (Briolat and Reysset, 2002), and random transposon mutagenesis identified a RNA helicase involved in the regulation of phenolic acid metabolism in *Lactobacillus plantarum* (Gury *et al.*, 2004) and a RNA helicase (CshA) in *S. aureus* involved in biofilm formation (Tu Quoc *et al.*, 2007). So far, however, their molecular function remains mostly elusive.

Very recently, bacterial two-hybrid analysis in *B. subtilis* and *S. aureus* identified the CshA RNA helicase as a potential member of a degradosome in Gram-positive bacteria (Lehnik-Habrink *et al.*, 2010; Roux *et al.*, 2011). These results are nicely consistent with data from our laboratory that show that a mutation in *cshA* results in a stabilization of the *agr* mRNA (Oun *et al.*, in preparation). This transcript is part of a quorum-sensing system that is induced at high-cell density to repress the expression of surface proteins and to induce synthesis of secreted virulence factors. Here, we give examples of how to measure RNA decay and phenotypic readouts caused by differences in the mRNA of the *agr* quorum-sensing system.

2. Measuring mRNA Decay

2.1. RNA preparation

In order to measure an effect on RNA decay in bacterial systems, new RNA synthesis is blocked by rifampicin treatment (400 μg/ml), whereupon the level of the RNA in question is measured at different time points (e.g., 0,

2.5, 5, 15, and 30 min; Oun *et al.*, in preparation). Obviously, the strain used needs to be checked for sensitivity to rifampicin, since Rif^R mutations occur readily, and various *S. aureus* strains may differ in the rifampicin concentration needed.

Many RNAs are expressed differently depending on growth phase (Dunman *et al.*, 2001), and while the initial amount of a given RNA might not influence its decay rate significantly, the potential differences in intracellular enzyme composition between a stationary and an exponentially growing cell certainly might. Therefore, it is important to ensure that the wild-type and the helicase mutant are at similar growth phases when rifampicin is added. In praxis, we usually dilute overnight cultures 200 times in fresh medium and then follow the growth until it reaches approximately $OD_{600} = 0.4$, at which point we add the rifampicin. The culture will still be in exponential phase, but we have enough cells to get a sufficient amount of RNA.

For sample preparation, an aliquot (e.g., 1 ml) is taken from the bacterial culture at the indicated time points, immediately centrifuged briefly to remove excess liquid (30 s at 10,000 g), and then half a volume of 1:1 ethanol:acetone is added to stop further degradation of the RNA. The samples can then be stored at $-80\,^{\circ}C$ until they are ready for RNA extraction (Fig. 17.1A). We find that thorough and immediate mixing with rifampicin is very important for obtaining consistent results, especially if short time intervals are chosen. It should also be noted that rifampicin only blocks initiation of new RNA synthesis, thus allowing ongoing RNA synthesis to finish, which can lead to a small increase in RNA levels immediately after the rifampicin treatment.

RNA extraction (Fig. 17.1B), which can be done using a commercially available kit, such as RNeasy Mini Kit (Qiagen, Cat N°74104), should also include a DNase treatment, to remove genomic DNA that will otherwise interfere with subsequent measurements. The RNA should then be checked for quality (lack of degradation) by agarose gel electrophoresis or using a bioanalyzer.

2.2. qRT-PCR determination of RNA decay

Quantitative reverse transcription PCR (qRT-PCR) can be used to determine the amount of the RNA of interest, by using a specific primer-set (Fig. 17.1C). However, care must be taken when interpreting the results, and a second set of primers must be used as an internal reference (choice of reference(s) for RNA decay is discussed below). Total RNA from the various time points and from mutant and wild-type cultures is used as template for the qRT-PCR reaction, and the individual relative quantities for the time points can then be plotted. For comparison of the wild-type and mutant data, we find it convenient to normalize the steady-state levels (e.g., time = 0) to 100% (Fig. 17.2). An exponential regression curve can be

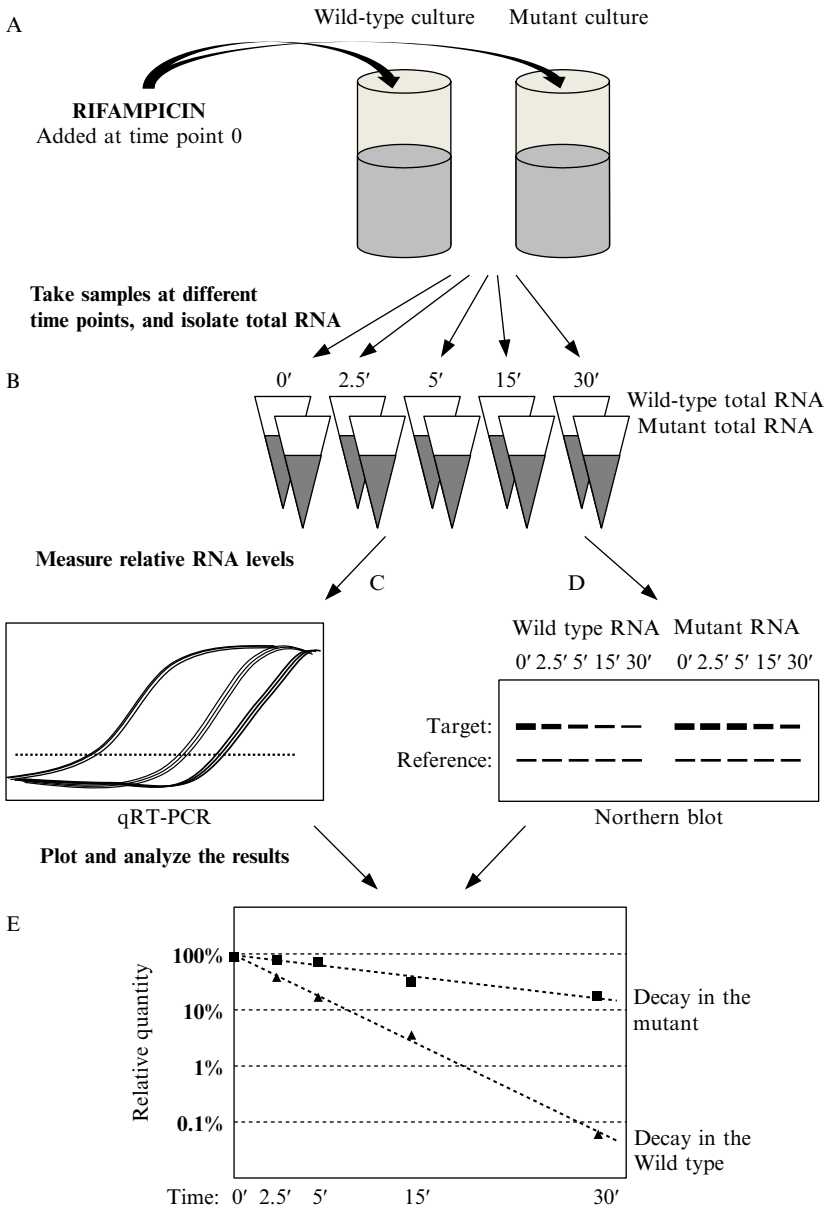

Figure 17.1 Flowchart for RNA-decay assay. (A) Rifampicin is added to exponentially growing cultures of the mutant and the wild type. (B) Samples are taken at specific time points, for example, 0, 2.5, 5, 15, and 30 min after addition of the rifampicin, and RNA is isolated from each sample. (C) qRT-PCR can be used to determine the quantity of the target RNA, using a nonaffected RNA as internal reference. (D) Alternatively, northern blotting can be used to quantify the target RNA. (E) The RNA quantities, relative to the reference RNA, can then be plotted in a semilogarithmic

calculated from this plot, or alternatively, the mutant and the wild type can be compared for each individual time point (Figs. 17.1E and 17 2).

In spite of the strength and accuracy of qRT-PCR method, or perhaps due to it, there are several caveats to observe (Bustin *et al.*, 2009). Not only does the chosen reference RNA have to be "unaffected" by the RNA-decay mechanism being studied (more on choice of reference below), but also the primers used to amplify the reference RNA must have a similar efficiency to the primers used for the RNA of interest. Further, the abundance of reference RNA must be such that the two RNAs can be observed within the same range of detection in the qRT-PCR amplification plot (see below).

2.3. Using northern blotting to determine RNA decay

The use of qRT-PCR allows for more accurate measurements and is also generally less labor intensive than northern blotting. It is therefore the method of choice for the majority of experimental setups. However, northern blotting additionally provides information about the length and particularly on the integrity of the RNA, whereas qRT-PCR does not. Therefore, northern blotting may be the superior technique for situations where it is suspected that one RNA segment is decaying more rapidly than another. An RNA-decay measurement using northern blotting is carried out in essentially the same way as qRT-PCR, using both a probe for the RNA of interest and a reference probe (Fig. 17.1D). Experimental details for northern blotting can be found in Sambrook and Russell (2001).

2.4. Analyses of RNA-decay data

Once RNA levels have been measured for several time points after rifampicin treatment, either by northern blot or by qRT-PCR, it is possible to calculate relative half-lives of the RNAs. Relatively, because each RNA measurement is measured in relation to the reference RNA, and it is therefore not possible to calculate an absolute half-life, but only possible to calculate how much faster (or slower) the RNA of interest decays in comparison to the decay of the reference RNA (Fig. 17.3).

When the relative quantity values are plotted in a semilogarithmic fashion, the decay curves should appear linear (Fig. 17.1E), and it should be possible to generate a linear regression (using Microsoft Excel,

plot, after normalization by defining the time point 0 as having 100% RNA. Black squares indicate the data for the mutant, and black triangles the data for the wild type. Dotted lines indicate exponential regression curves fitted to the data points. A relative RNA half-life can be determined based on the regression curves.

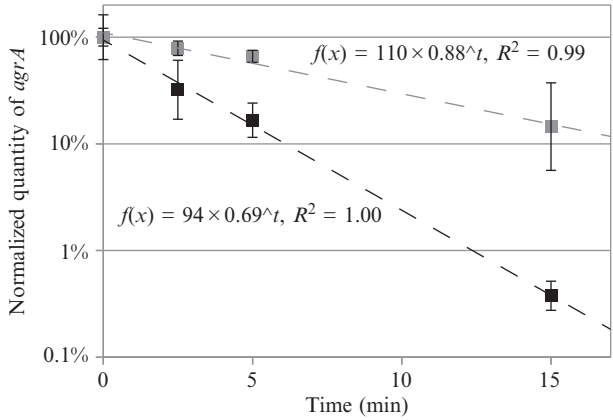

Figure 17. 2 Analyzing RNA-decay data. The graph represents an example of an output from OpenOffice.org Calc, where the data from a single qRT-PCR experiment were analyzed (taken from Oun *et al.*, in preparation). Each measurement of *agrA* and HU (the reference gene) was carried out in triplicate on the same qRT-PCR plate, and the error-bars indicate the 95% confidence level. Black squares: SA564 wild type. Gray squares: *cshA* mutant. Dotted lines show the exponential regression curves with the formulas and R^2 value indicated. Mutant and wild type do not have the same steady-state levels of *agrA* mRNA, and the data was therefore normalized to the measurements at time point 0.

OpenOffice.org Calc, or similar) with an R^2 value close to 1. For example, using OpenOffice.org Calc the formula for the curve will be in the format

$$Y = A \times B^t, \tag{17.1}$$

where A is the offset at $t = 0$, t is the time, and B is a constant that defines the slope of the curve. To calculate the relative half-life, one uses the formula

$$1/2 = B^{t_{1/2}} \tag{17.2}$$

resulting in

$$\ln(1/2)/\ln(B) = t_{1/2}. \tag{17.3}$$

2.4.1. Choosing a reference RNA for RNA-decay assays

A DEAD-box helicase is one of the core components of Gram-positive degradosome, and although very little is as yet known about the function of the degradosome in these organisms, it must be presumed that this protein complex affects the decay rate of many RNAs in the cell. Therefore, it is a

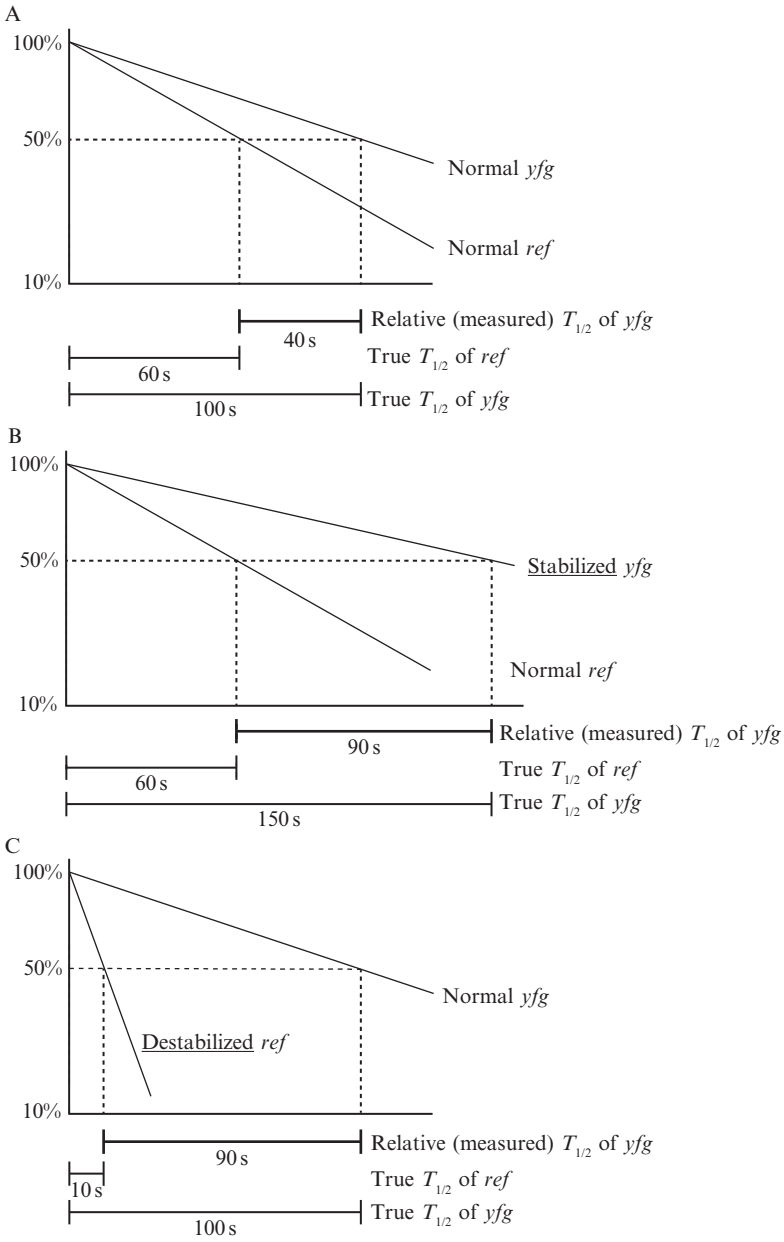

Figure 17.3 The choice of a reference RNA and examples of dangers in interpreting decay data. *yfg*, the gene of interest; *ref*, the chosen reference RNA. Thin lines indicate the decay curves obtained for *yfg* RNA and *ref* RNA. Measured RNA levels of both *yfg* and *ref* are decreasing over time, as expected. However, Rifampicin is an antibiotic and is killing the bacteria during our time course, and part of the observed decrease might be due to fewer cells harvested. Moreover, even with when care is taken to have the same

non-trivial task to choose an "unaffected" RNA to use as a reference RNA for either northern blotting or qRT-PCR (Eleaume and Jabbouri, 2004; Vaudaux *et al.*, 2002). In most publications, rRNA is used as an internal standard, but in this case, it has to be considered that this reference is very abundant and will not appear in a similar abundance window and that ribosome biogenesis and the highly structured nature of rRNA may be influenced by the RNA helicase. Nevertheless, if several mRNAs are shown to be affected to the same degree and to similar extents in wild type and mutant (i.e., the decay rate of one stays the same in relation to the others), then one of these RNAs can be used for normalizing affected RNAs. As an example, in *S. aureus*, a mutation in the DEAD-box helicase *cshA* gene appears to stabilize the *agrBDCA* mRNA, when compared to the *HU* mRNA (compare Fig. 17.3A and B). However, if one only compares the two mRNAs, then the effect might equally be that HU mRNA is destabilized and *agrBDCA* mRNA stays unaffected (Fig. 17.3C). HU mRNA decay was therefore compared to a number of other RNAs to show that it is indeed unaffected by the *cshA* mutation (or at least is affected to the same degree as the other RNAs). Further, in a transcriptome-wide RNA-decay assay, the *HU* mRNA belongs to the (mostly) unaffected group of RNAs, whereas *agrBDCA* mRNA was stabilized in comparison to the global mRNA decay (Oun *et al.*, in preparation).

3. Phenotypic Readouts

In *S. aureus*, disruption of the *cshA* gene causes the cells to exhibit several phenotypic changes, especially regarding expression of virulence factors. This was first noticed by Tu Quoc *et al.* (2007) who observed a reduced capacity for producing biofilm in a *cshA* mutant of the *S. aureus* S30 strain, which is normally a strong biofilm producer. Moreover, *cshA* mutants exhibit stabilization of their *agrBDCA* mRNA, which is responsible for quorum sensing in *S. aureus* (Oun *et al.*, in preparation). The *agr*-system induces a regulatory RNA, RNAIII, which is responsible for

amount of total RNA in each well, it is almost impossible to avoid small fluctuations. Therefore, it is important to use an internal reference RNA (*ref* RNA) to standardize the amount of *yfg* RNA. One consequence of this is that it is not possible with this method to determine an absolute half-life ($T_{1/2}$) for *yfg*, but only a $T_{1/2}$ relative to *ref* RNA. The figure displays three different scenarios: (A) For a wild-type situation, the true $T_{1/2}$ of *yfg* RNA is 100 s and that of ref RNA is 60 s, resulting in a relative $T_{1/2}$ of 40 s for *yfg* RNA. (B) In a mutant, where *yfg* RNA is stabilized, the true $T_{1/2}$ of *yfg* RNA has increased to 120 s while the true $T_{1/2}$ of *ref* RNA stays at 60 s, leading to a relative $T_{1/2}$ for *yfg* of 60 s. (C) In another mutant, *yfg* RNA stability stay the same as in wild type ($T_{1/2} = 100$ s), whereas *ref* RNA is destabilized and now only has a $T_{1/2}$ of 40 s. The relative $T_{1/2}$ of *yfg* RNA in this mutant is therefore 60 s, indistinguishable from the mutant in example B, unless additional experiments are carried out.

up- and downregulation of a number of virulence factors, such as Rot, protein A, and hemolysin alpha (Boisset *et al.*, 2007; Kong *et al.*, 2006). Further, RNAIII includes a small open reading frame, which encodes the hemolysin delta protein. As a consequence, hemolysin alpha and delta are useful markers for disruptions of the *cshA* helicase gene (Fig. 17.4, *agr* pathway).

Hemolysins are small proteins that lyse erythrocytes, and it is possible to distinguish between the alpha and delta hemolysin by the difference in efficiency against rabbit and horse erythrocytes. Hemolysin alpha is highly active against rabbit blood, whereas horse erythrocytes are highly vulnerable to hemolysin delta (*note*: hemolysin beta can be assayed using sheep blood; Garvis *et al.*, 2002).

Mutations in genes to be analyzed in clinical *S. aureus* strains can be obtained by a variety of methods. In the laboratory, we have used the targetron system (Yao *et al.*, 2006) and for making clean deletions, a highly powerful selection/antiselection system that takes advantage of the sensitivity of bacteria to 5-FOA (Redder and Linder, 2012).

3.1. Biofilm assay

To determine the level of biofilm production for a mutant of *S. aureus*, the classical method is via crystal violet staining of biofilm after a specific period of nonagitated growth. The method is described in Mack *et al.* (2001), but an adaptation used in our laboratory can be briefly summarized as follows: Mutant and wild-type cultures are diluted to OD = 0.1 and 1 ml is incubated for 6 h in a polystyrene tube without agitation at 37 °C. It should be noted that biofilm formation depends on the number of cells and moreover may be influenced by the quorum-sensing system, and it is therefore important to take into account potential differences in growth rate and adapt initial dilutions accordingly. For staining, 100 μl of a solution of 1% w/v crystal violet and 2% ethanol are then added and allowed to stain for 15 min, whereupon the liquid is removed and the tube is washed with water. The crystal violet that is fixed by the biofilm is then dissolved in 400 μl ethanol and transferred to a cuvette with 600 μl water. The amount of biofilm can be measured by the absorption at 570 nm (we find that 600-nm work for most purposes as well). Alternatively, biofilm can be grown on glass slides, which can then be studied under the microscope, after staining with crystal violet.

The best possible but also most laborious way of studying biofilm is probably when it is monitored by 3D imaging in a flow cell. If the bacteria are fluorescent, then this method even allows the forming of the biofilm matrix to be followed live. Detailed protocols for growing biofilm in flow cells and examination by 3D imaging can be found in Christensen *et al.* (1999).

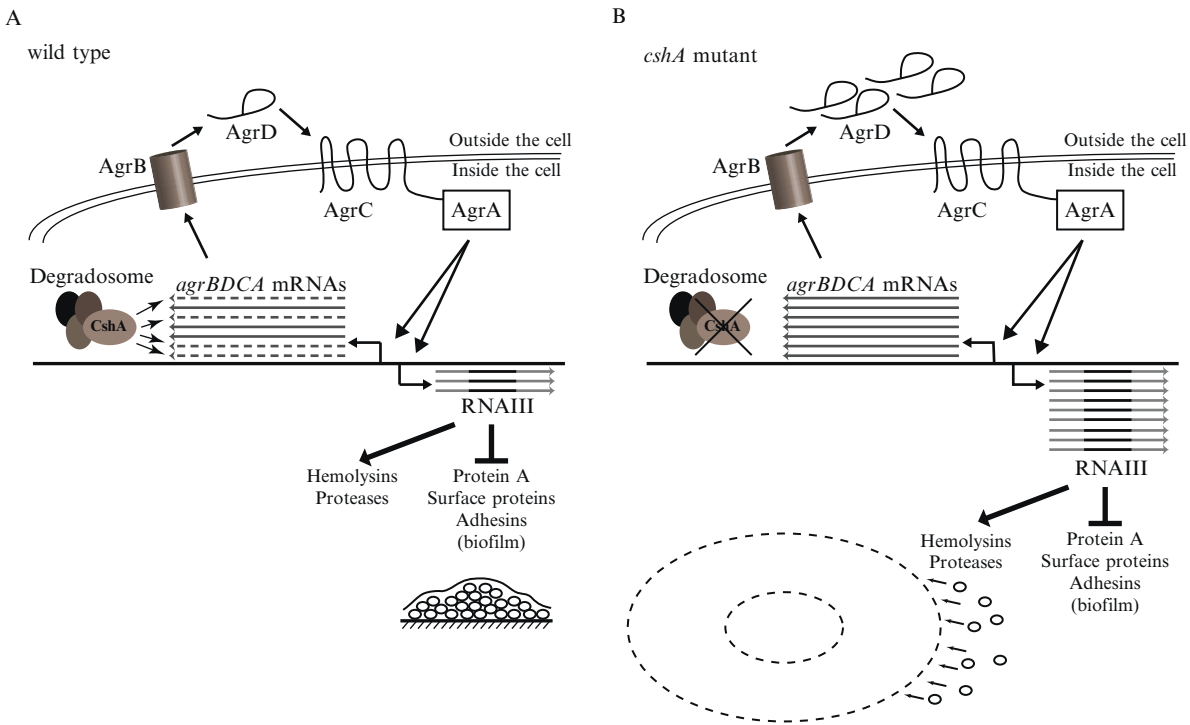

Figure 17.4 The role of the degradosome in quorum sensing. (A) In the wild type, with normal levels of CshA, *agrBDCA* mRNAs are produced (gray arrows) but the degradosome is able to degrade a significant portion of them (dotted gray arrows). The remaining *agrBDCA* mRNAs are translated and the quorum-sensing system is able to respond correctly to cell density to regulate the amount of RNAIII (light gray arrows). The hemolysin delta open reading frame, encoded by RNAIII, is shown in black. (B) In the *cshA* mutant, the degradosome is unable to degrade the *agrBDCA* mRNAs correctly, leading to a much higher level of Agr proteins and results in an overactive quorum sensing that leads to elevated RNAIII levels. The RNAIII in turn overly stimulates production of hemolysins and extracellular proteases and inhibits the production of biofilm components.

3.2. Hemolysis assay

The easiest way of testing hemolysis activity is via blood-agar plates, which will give a semiquantitative estimate of activity (Garvis *et al.*, 2002). Rich medium (e.g., Mueller-Hinton, Becton Dickinson, NJ, USA), autoclaved with 10 g/l agar, is cooled to 43 °C and mixed with 7% defibrinated blood, preheated to 43 °C (TCS Biosciences Ltd., Buckingham, UK), whereupon the mix is immediately poured into petri dishes (antibiotics can be added if desired). The plates should be dried well at 37 °C before use.

For the assay, 10 µl of wild type and mutant overnight cultures are then spotted on the same plate, and after the spot has dried, the plate is incubated at 37 °C for about 20 h and then transferred to 5 °C for 2–3 days to allow hemolysis on the plate to occur. The efficiency of the hemolysis can then be monitored by the size of the zones of transparency around the spots (Fig. 17.5).

When interpreting data from the plate assay, it is important to take into account that one strain might grow significantly slower than another, which means that there might be significantly fewer cells in one spot than in another, until stationary phase is reached. This means that one strain might have much more time to produce hemolysins than another, especially taking into

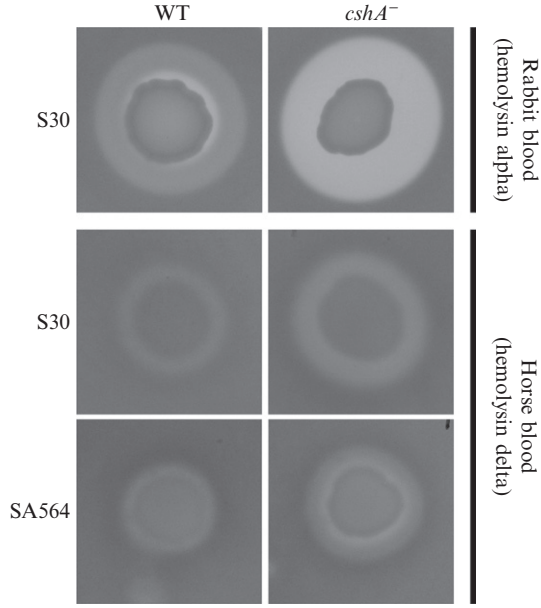

Figure 17.5 Hemolysis. Examples of clearance zones caused by hemolysins on rabbit blood plates and horse blood plates. The clearance zones are consistently larger in the *cshA* mutants, on both rabbit and horse blood. However, there are strain-specific differences in the appearance of the clearance zones, exemplified here by two clinical strains S30 and SA564.

consideration that hemolysins in *S. aureus* are produced in a growth-phase- and quorum-sensing-dependent manner (Dunman *et al.*, 2001; Kong *et al.*, 2006).

An alternative to the blood-agar assay is to measure hemolytic activity in a liquid solution, with the advantage that it is possible to quantify the activity by spectrophotometry. The assay is described in detail in Bernheimer (1988). To prepare the hemolysin for the liquid assay, 2 ml of bacterial culture is centrifuged for 5 min at 8000 rpm, whereupon the supernatant is sterilized with a 0.22-μm filter. The removal of the hemolysin-producing bacteria, by centrifugation and filtration, ensures that only hemolysins that were present at the time of harvesting will be measured in the assay, thus eliminating the experimental error that arises from differences in doubling times, as is the case in the spots of the blood-agar-plate assay. However, the difference in growth rate should still be taken into account when deciding at what point the cells are removed, since fewer cells and/or cells in exponential phase will produce less hemolysins.

4. Concluding Remarks

The Gram-positive degradosome has only recently been discovered by bacterial two-hybrid analyses. Its function and the exact role of the individual components, including the DEAD-box helicase CshA, are still very much on the speculative stage. Here, we have presented various methods and their advantages and caveats necessary for measuring the effects of a *cshA* mutation, via the stabilization of the *agrBDCA* mRNA, although many other RNAs are likely to be affected as well. The field of mRNA turnover is very dynamic and many variations or even alternative methods from those described here can be found in the literature.

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