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A major checkpoint for protein expression in *Rhodobacter sphaeroides* during heat stress response occurs at the level of translation

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Summary

Temperature above the physiological optimum is a stress condition frequently faced by bacteria in their natural environments. Here, we were interested in the correlation between levels of RNA and protein under heat stress. Changes in RNA and protein levels were documented in cultures of *Rhodobacter sphaeroides* using RNA sequencing, quantitative mass spectrometry, western blot analysis, *in vivo* [^{35}S] methionine-labelling and plasmid-borne reporter fusions. Changes in the transcriptome were extensive. Strikingly, the proteome remained unchanged except for very few proteins. Examples include a heat shock protein, a DUF1127 protein of unknown function and sigma factor proteins from leaderless transcripts. Insight from this study indicates that *R. sphaeroides* responds to heat stress by producing a broad range of transcripts while simultaneously preventing translation from nearly all of them, and that this selective production of protein depends on the untranslated region of the transcript. We conclude that measurements of transcript abundance are insufficient to understand gene regulation. Rather, translation can be an important checkpoint for protein expression under certain environmental conditions. Furthermore, during heat shock, regulation at the level of transcription might represent preparation for survival in an unpredictable environment while regulation at translation ensures production of only a few proteins.

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Introduction

In their natural environments, bacterial populations are often exposed to drastic changes. Conditions that do not allow optimal growth are considered stress conditions. Bacteria mount responses that allow toleration of these stresses. Exposure to temperatures above the physiological optimum, also referred to as heat stress or heat shock, induces a response in gene expression. The heat shock response has been studied in a range of model organisms, including Drosophila and mammalian cells (Panniers, 1994) and most intensively in the model organism *Escherichia coli*. In *E. coli*, temperature upshift results in increased levels of multiple proteins (called heat shock proteins), which include molecular chaperones and proteases that counteract the accumulation of unfolded proteins (Rodriguez *et al*., 2008; Schumann, 2016). A major mechanism accounting for this response are the RpoH (σ^H or σ^{32}) sigma factors, which activate transcription of large numbers of genes (Grossman *et al*., 1984).

Most of the previous studies on the heat shock response have focused on mechanisms operating at transcription initiation (Roncarati and Scarlato, 2017). Recently, however, when comparing the impact of the heat shock on the transcriptome versus the translatome in *E. coli*, Zhang *et al*. found differences between total transcript abundance and ribosome-associated transcript abundance. This indicated that interactions between the mRNA and the ribosome are altered upon heat stress, and that this alteration affects translation. Moreover, at least some of these differences were attributed to an unknown mechanism, which caused the ribosomes to pause at the starting regions of open reading frames (ORFs) (Zhang *et al*., 2017). Another recent study involving polysomal RNA analysis during heat shock on the model actinomycete bacterium, *Streptomyces coelicolor*, found 'little correlation between the transcriptome and translatome' and proposed an 'unprecedented level of translational control of gene expression' during stress (Bucca *et al*., 2018).

Multiple studies have focused on the global (transcriptional) heat shock response of alphaproteobacteria

(Münchbach *et al*., 1999; Rosen *et al*., 2002; Audia *et al*., 2008; Gürgan *et al*., 2015; Varano *et al*., 2016). Similarly to the gammaproteobacterium *E. coli*, the role of sigma 32-like factors controlling transcription initiation in the heat shock response of alphaproteobacteria has been established (Mantis and Winans, 1992). Many alphaproteobacteria harbour two or more RpoH sigma factors, such as *Bradyrhizobium japonicum*, *Sinorhizobium meliloti* (Oke *et al*., 2001; Ono *et al*., 2001) and *Rhizobium etli* (Martinez-Salazar *et al*., 2009), all of which have three RpoH sigma factors, and *Azospirillum brasiliensis* which has five (Kumar *et al*., 2012). In *Rhodobacter sphaeroides*, two RpoH sigma factors (RpoH1 and RpoH2) are involved in the heat shock response (Karls *et al*., 1998; Green and Donohue, 2006).

The alphaproteobacterium *R. sphaeroides*, the model organism for this study (recently reclassified as *Cereibacter sphaeroides* based on thorough taxonomic analyses; Hördt *et al*., 2020), is mostly found in fresh or brackish water environments with little changes in nutrients and salinity but with frequent changes in temperature, oxygen concentration and light intensities. As a facultative phototroph, *R. sphaeroides* can form pigment protein complexes to perform anoxygenic photosynthesis when the oxygen concentration is low and aerobic respiration is restricted. The simultaneous presence of bacteriochlorophyll, oxygen and light, however, leads to the generation of the harmful singlet oxygen causing photooxidative stress. To minimize damage from singlet oxygen, *R. sphaeroides* uses cellular surveillance and regulatory mechanisms with respect to light and oxygen availability to strictly control the formation of photosynthetic complexes (Gregor and Klug, 2002; Zeilstra-Ryalls and Kaplan, 2004), and to rapidly respond to singlet oxygen to prevent the damage to cellular components (Anthony *et al*., 2005; Ziegelhoffer and Donohue, 2009; Berghoff *et al*., 2011; Nam *et al*., 2013; Berghoff and Klug, 2016; Licht *et al*., 2020).

Proteome and transcriptome studies have provided a global view on various stress responses in *R. sphaeroides* (Anthony *et al*., 2005; Glaeser and Klug, 2005; Berghoff *et al*., 2009; Berghoff *et al*., 2013). A recent study revealed a marked overlap at the transcriptome level between the singlet oxygen stress response and the transcriptional response associated with recovery in fresh medium (outgrowth) following stationary phase (Remes *et al*., 2017). Genes that respond to multiple stresses may have a general role in adaptation to stress. In some cases, a gene can belong to more than one regulon. For example, RpoH1 and RpoH2 each have their own regulons, but these partially overlap (Nuss *et al*., 2009; Nuss *et al*., 2010; Dufour *et al*., 2012; Adnan *et al*., 2015; Billenkamp *et al*., 2015; Remes *et al*., 2017). Additionally, the studies cited above show that both RpoH1 and RpoH2 are also important for other responses, such as the transcription response during outgrowth following stationary phase (Remes *et al*., 2017). The roles of RpoH1 and RpoH2 are not necessarily redundant, however, since *rpoH1* transcription is more induced by heat while *rpoH2* transcription is more induced by singlet oxygen and has a major role in the defence against singlet oxygen (Nuss *et al*., 2010).

While RpoH sigma factors are known to be important for the transcriptional response to heat stress, the proteomic response to heat stress has not been investigated in *R. sphaeroides*. Low correlation between transcripts and protein levels has been reported for *R. sphaeroides* during batch culture growth at physiologically optimal temperature of 32°C (Bathke *et al*., 2019). To learn more about the heat stress response in *R. sphaeroides*, we performed transcriptome analysis by RNA sequencing and a quantitative proteome analysis via mass spectrometry (MS) after a temperature upshift. The transcriptomic response was extensive. To our surprise, however, the proteomic response was limited to only a few proteins. Moreover, the extent of the response of some of these proteins was surprisingly strong. To gain more confidence in the data and to obtain a more detailed view of translation control during heat shock, we created translational fusions between target genes and the gene for a fluorescence reporter, mVenus, and used these to follow expression in liquid cultures undergoing a heat stress response. Additionally, hybrid fusions between promoters and the 5' untranslated regions (UTRs) captured the impact of the UTRs on translation during heat stress. This study reveals that the translation response to heat stress is rather distinct from the transcription response and emphasizes the importance of translation as a checkpoint for controlling the proteome during heat stress.

Results

Transcriptomic response to heat stress is extensive

RNA sequencing (RNAseq) data used in this study were obtained as part of an earlier study (Förstner *et al*., 2018) and are accessible at NCBI's Gene Expression Omnibus (GSE104278). Analysis of these data was used to evaluate the expression of the *R. sphaeroides* coding genes at 20 min following a shift of exponentially growing cultures from 32°C to 42°C. Changes in transcript abundance are shown in Supporting Information Table S1 and are derived from triplicate analyses, where the RNA for each triplicate was harvested from three independently grown cultures. Three sequencing runs from three biological replicates revealed strong reproducibility of the data sets (Supporting Information Fig. S1). The use of a $\log_2$ fold change ($\log_2$fc) of ≤ -1 or ≥ 1 as an arbitrary cutoff

revealed that 43% of all genes responded to heat shock. A total of 603 transcripts responded with increased levels and 760 transcripts with decreased levels. Increasing the cutoff to a $\log_2$fc ≤ −2 or ≥ 2 resulted in 459 increased and 566 decreased transcripts, some 32% of all genes. Only these genes were considered further in this study and are indicated in red in the volcano plot as shown in Fig. 1.

Figure 2 shows the distribution of the annotated genes within the different functional categories or clusters of orthologous groups (COGs). In most categories, there are more genes with lower transcript levels under heat shock than genes with higher levels. For example, in category N (motility), 24 genes out of a total of 74 had lower transcript levels, no genes had increased transcript levels, while the transcript levels of the majority of genes in this category (50 genes, not shown in Fig. 2) were unchanged. For a few categories, the number of genes with increased transcripts outnumber the genes with decreased transcripts. This was the case for COG-K (transcription: 35 increased transcripts, 15 decreased transcripts), for COG-L (replication, recombination and repair: 19 increased transcripts, 12 decreased transcripts), and for COG-O (post-translational modification, protein-turn-over chaperons: 33 increased transcripts, 12 decreased transcripts). In COG-K, several genes with increased transcript levels code for alternative sigma factors including RpoH1, RpoH2, RpoE and the anti-sigma factor ChrR. These proteins are known to have an important role in the heat shock response and other stress responses in *R. sphaeroides* (Karls *et al*., 1998; Green and Donohue, 2006; Nuss *et al*., 2009; Nuss *et al*., 2010; Billenkamp *et al*., 2015). Other genes of COG-K with increased transcript levels include two component regulators and transcription factors of different protein families such as the uncharacterized sigma factors RSP_0415 and RSP_3095 (and its anti-sigma factor RSP_3094). RSP_3095 was recently found to be important for its own expression activation in cultures entering the stationary phase upon carbon limitation and to mildly slow growth during the exponential phase upon over expression (McIntosh *et al*., 2019). Hence, it is likely that RSP_3095 supports the adaptation to a variety of stress conditions via the adjustment of transcription. The regulon of RSP_3095 is unknown.

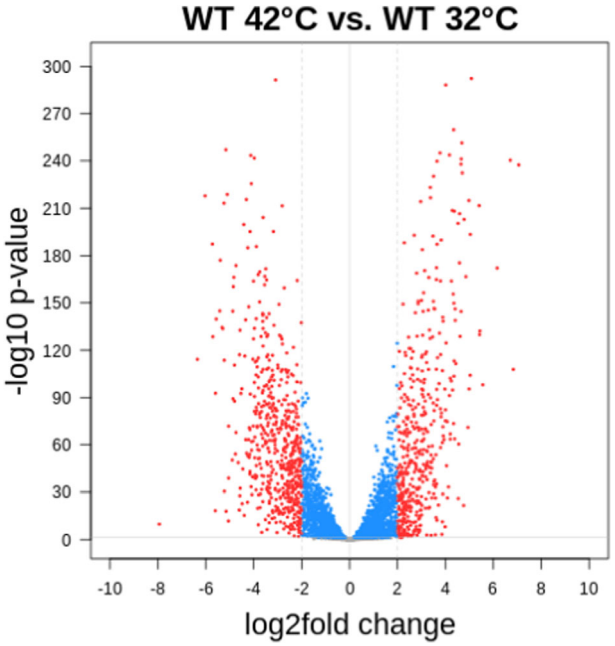

Fig. 1. Heat shock induces changes in the levels of many transcripts. Volcano scatterplot showing response of the transciptome (approximately 32% of all genes) to heat shock. Represented as red points, 459 genes showed increased levels of transcript and 566 genes showed decreased levels of transcript, using a cutoff of a $\log_2$ fold change of ≤ −2 or ≥ 2. Genes with changes in transcript levels below this cutoff are represented as blue points. Only transcripts with *P*-values lower than 0.05 were considered, while transcripts with *P*-values higher than 0.05 are indicated in grey. The -ype (WT) strain for this study was *Rhodobacter sphaeroides* 2.4.1.

The increase in transcripts of genes of COG-L fits to the requirement to repair DNA that is damaged at increased temperatures. Genes of COG-O that are induced upon heat shock encode proteins with a chaperone function, with a role in protein repair/detoxification (such as MsrA and MsrB that repair proteins with oxidized methionine), and proteins of the thioredoxin and glutaredoxin system that are required to reduce oxidized proteins. Among the genes with strongest decrease in expression are those for bacteriochlorophyll synthesis, *nuo* genes (for NADH-quinone oxidoreductase), and genes with a role in cell division, chemotaxis, or cobalamin synthesis.

We compared the heat shock data with the data from previously published studies on adaptation of *R. sphaeroides* to singlet oxygen stress and adaptation associated with the various growth phases (lag, exponential and stationary phase). Figure 3A shows the scatter plots of transcript level changes detected during heat shock compared with changes detected during singlet oxygen stress, transition from exponential growth phase to stationary phase, and following transfer from stationary phase to fresh growth medium (outgrowth). Compared with the other conditions, the changes under heat shock were more extensive, as is evident from the high degree of scattering along the x-axes in contrast to the relatively low degree of scattering along the y-axes (Fig. 3A, top row). Changes detected in the previous studies were fewer, as is reflected by a lower degree of scattering along both axes (Fig. 3A, bottom row).

Do the various stress conditions induce changes in the same set of transcripts or is there a specific set of genes

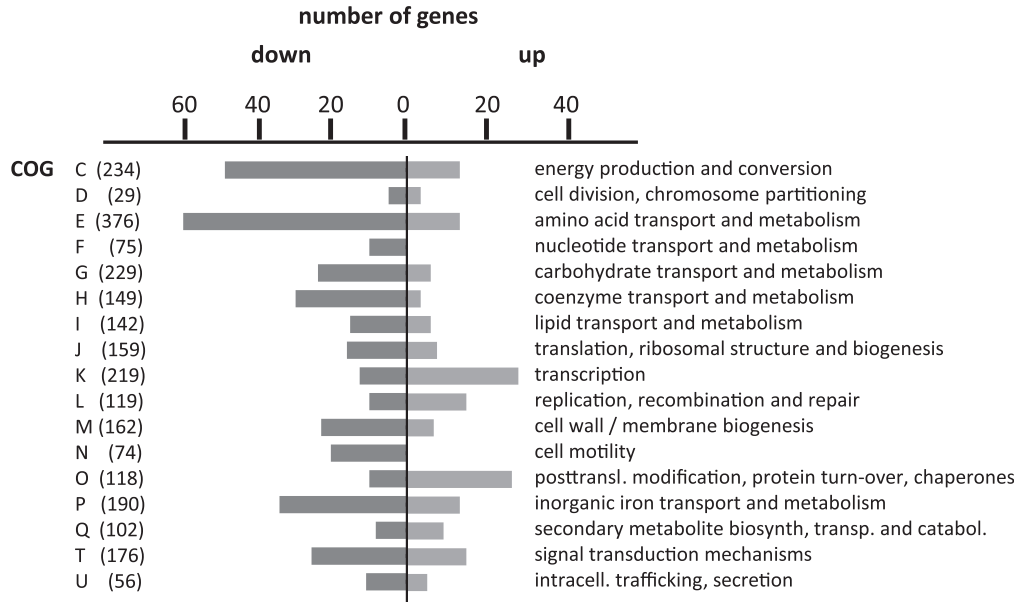

Fig. 2. Distribution of the annotated genes in the clustered orthologous groups (COGs) whose transcripts respond to heat shock using a cutoff of a $\log_2$ fold change of ≤ -2 or ≥ 2. Numbers within brackets indicate the total number of genes within a COG.

for each stress response? A Pearson's correlation coefficient (PCC) matrix between the various conditions is provided in Fig. 3B. Correlation between the heat shock changes and the changes detected by comparing the transcriptome at the exponential and stationary phases was very low (PCC = <0.05). Correlation was higher between heat shock and singlet oxygen stress (PCC = 0.24–0.32), and even higher between heat shock and outgrowth (PCC = 0.47–0.48). This result suggests that a portion of the transcriptional responses to heat stress is not necessarily specific to heat stress. Rather, up to almost half of the transcripts involved in heat shock are also involved in outgrowth.

Proteomic response to heat stress is limited

Quantitative MS analysis was performed in triplicates, each from three independent biological experiments. Samples from cultures grown at 32°C were compared with samples from cultures, which were initially grown at 32°C and then shifted to 42°C and further incubated for up to 90 min. Levels of 1712 detected proteins were compared at 0 min and 60 min of incubation at 42°C, and levels of 1827 detected proteins were compared at 0 and 90 min of incubation at 42°C. The number of proteins with changed levels was drastically lower than that of the transcripts, as seen in the volcano plots (Fig. 4A). Considering proteins with a $\log_2$fc ≥ 1, only nine proteins were increased after 60 min at 42°C and 18 proteins after 90 min. Similarly, for proteins with a $\log_2$fc ≤ -1 levels, only 10 proteins decreased after 60 min at 42°C and 18 proteins after 90 min. Most of these did not exceed a cutoff of $\log_2$fc $\geq \pm 2$. This is a remarkable result, given that the transcription response was far more extensive. Indeed, the number of transcripts using a cutoff of $\log_2$fc $\geq \pm 2$ was 459 increased and 566 decreased after only 20 min. A comparison between transcriptome (x-axis) and proteome (y-axis) changes is shown in the scatter plot in Fig. 4B and clearly shows the much higher number of changes in the transcriptome response.

Of the 11 proteins with increased levels (cutoff of $\log_2$fc ≥ 1) upon heat stress after 60 min, 5 are hypothetical proteins. The others include two heat shock proteins (RSP_1016 and RSP_1572) of the Hsp20 family, a peptide methionine sulfoxide reductase (RSP_2617), a putative aminotransferase (RSP_0442), a putative calcium-binding EF-hand domain protein (RSP_0416), and a possible dioxygenase/glyoxylase family protein (RSP_3053). The protein with the highest increase upon heat shock was a small heat shock protein, RSP_1016. Remarkably, the levels of RSP_1016 increased by a massive 128-fold ($\log_2$fc ≈ 7) after 60 min at 42°C. Such an increase in protein levels surpassed the changes observed for any transcript in the RNAseq data, including its own transcript which showed a more modest 32-fold increase ($\log_2$fc ≈ 5) after 20 min at 42°C (see Fig. 4B, upper right quadrant). This result indicates that the dynamic of the proteome response is not necessarily lower than the transcription response, nor is it limited by the detection method.

The protein with the second highest increase was RSP_0557, a hypothetical protein containing a DUF1127

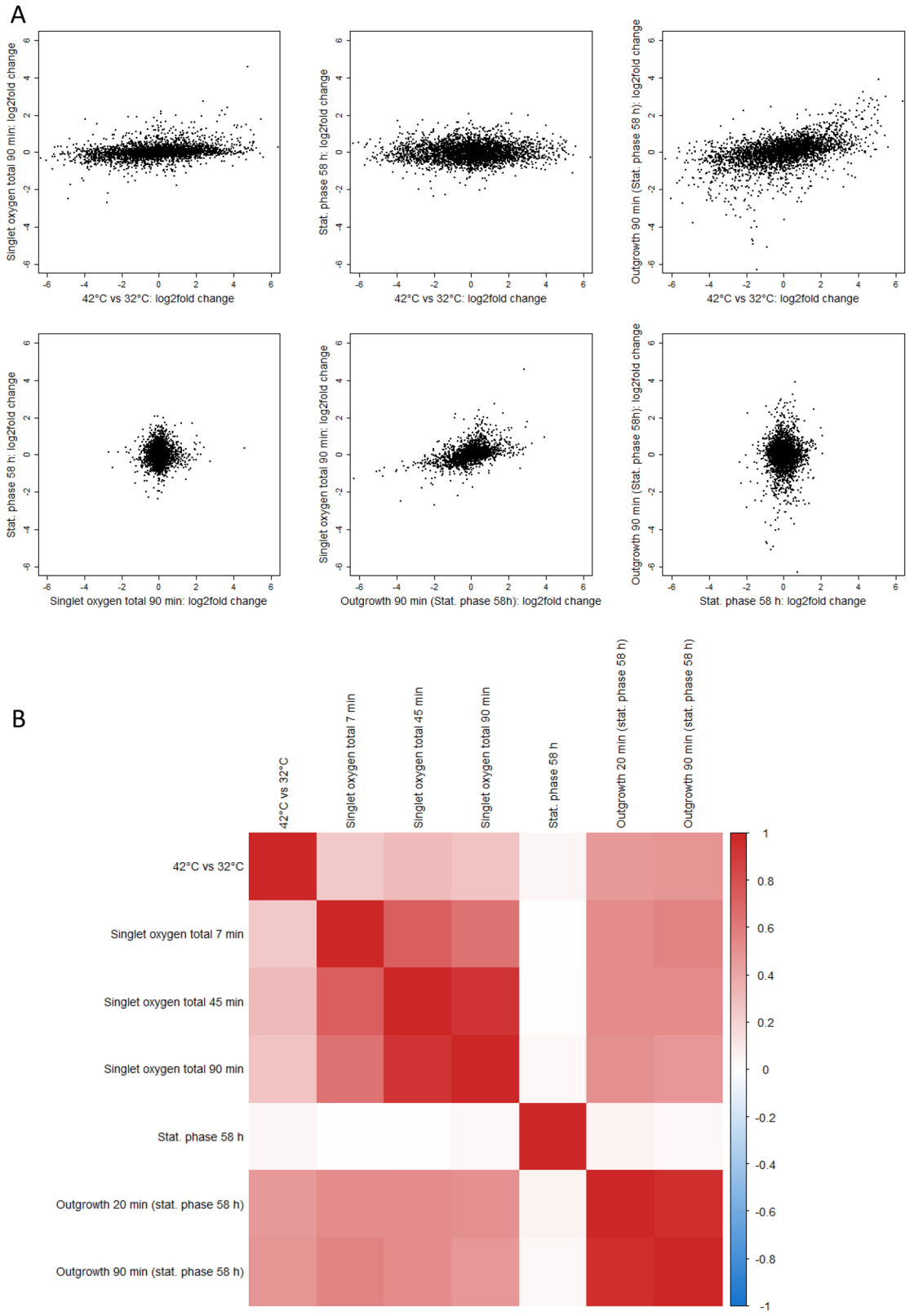

Fig. 3. Legend on next page.

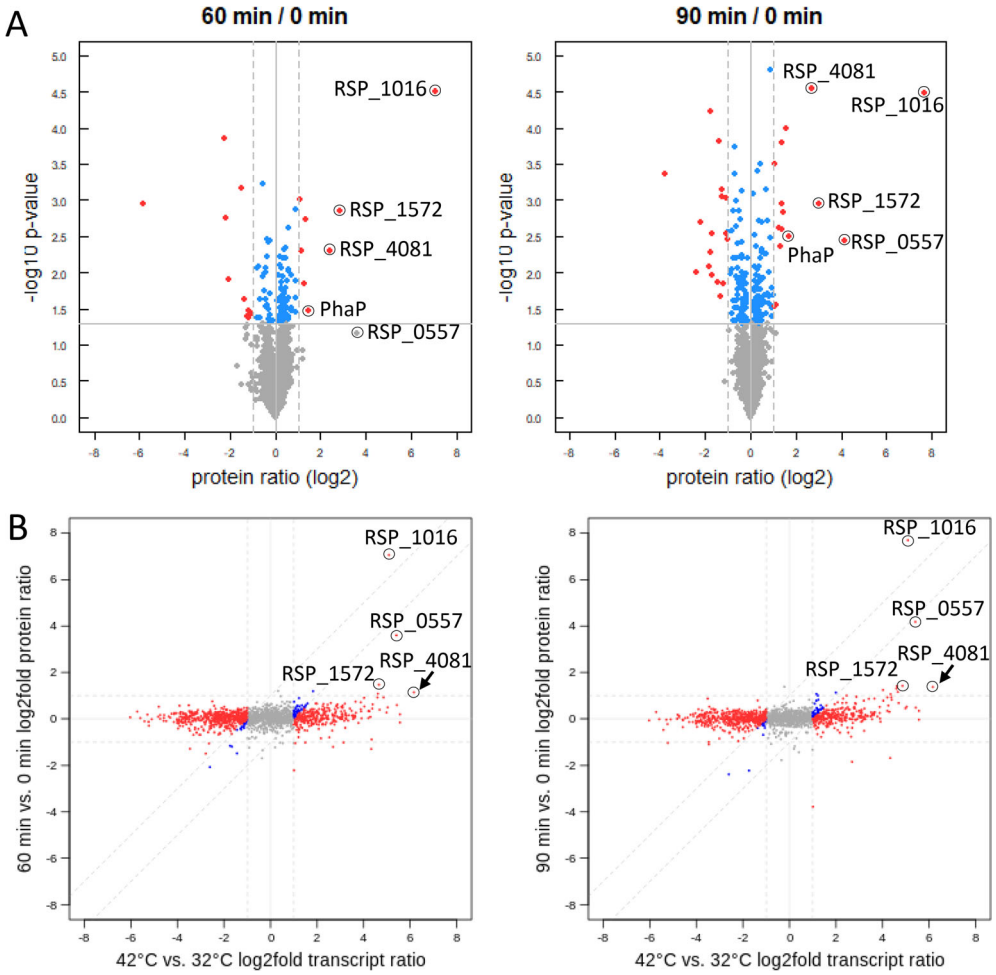

Fig. 4. Heat shock induces changes in the levels of only a few proteins. Volcano scatterplot of proteins detected via MS following heat shock induces changes.
A. Proteins with a ≤ −1 or ≥ 1 $\log_2$ fold change (x-axis) after 60 min (left panel) and 90 min (right panel) of heat stress are represented as red dots. Blue dots represent detected proteins with > −1 or < 1 $\log_2$ fold changes. Grey dots represent proteins with a p value >0.05 (y-axis). Note that the y-axis is comparable to that in Fig. 1, except that the scale here is smaller (0–5 in Fig. 4B, 0–30 in Fig. 1).
B. Transcript level (y-axis) for each gene compared to its protein level (x-axis) after 60 min (left panel) and 90 min (right panel) of heat stress. Genes with linear correlations between protein and transcript levels are indicated as blue dots (for transcripts with showing ≤ −1 or ≥ 1 $\log_2$ fold changes). Red dots represent genes with ≤ −1 or ≥ 1 $\log_2$ fold changes at the transcript level, but lack a corresponding linear change at the protein level. Grey dots represent the bulk of genes whose levels of transcripts or proteins do not change (using the ≤ −1 or ≥ 1 $\log_2$ fold change cutoff) during heat shock. Dots within circles represent genes whose proteins showed the strongest increases in abundance.

domain. The RSP_0557 gene was previously found to be strongly induced during oxidative stress by Pos19, a sRNA that also carries an ORF (Müller *et al*., 2016). Interestingly, an increase in RSP_0557 mRNA was also detected during the adaption to stationary phase upon carbon limitation in well-aerated cultures (McIntosh *et al*., 2019). MS data from this study revealed a 12-fold ($\log_2$fc ≈ 3.6) increase in levels of the RSP_0557 protein after 60 min at 42°C.

A scatterplot, which compares the levels of each transcript (x-axis) with the levels of its corresponding protein (y-axis) shows the small heat shock protein RSP_1016

Fig. 3. Heat shock induces changes in levels of a broad range of transcripts.
A. Comparison of transcriptomes during heat shock, singlet oxygen stress, stationary phase and outgrowth. Each dot represents a transcript. Top row, values on the x-axis represent the changes in transcript levels upon heat shock. Values on the y-axis represent changes in transcript levels in the following conditions: singlet oxygen stress, stationary phase and outgrowth from stationary phase. Bottom row, changes in transcript levels were also compared between various other conditions.
B, PCC matrix of a comparison between the changes in the transcriptome over a range of conditions.

localized in the upper right quadrant (Fig. 4B), indicating high correlation, i.e., that both the transcript and protein levels of RSP_1016 accumulate very strongly upon heat shock. A similar but less extreme location was also observed for RSP_0557. These are represented as red points in the scatterplot in Fig. 4B. Altogether, comparison of the RNAseq data with the MS data revealed a striking result. Contrary to our expectations, the vast majority of transcripts with significantly increased levels during heat shock did not result in changes in protein levels.

Western blot analysis of selected proteins confirms a heat shock response

Because the changes in protein levels upon heat stress were radically different from the changes in transcript levels, we used western blot analysis to check the abundance of selected proteins. As preparation for the western blot analysis, growth of *R. sphaeroides* was compared at 32°C and 42°C (Fig. 5). Furthermore, two other model bacteria in which the heat shock response has been studied, *E. coli* and *Agrobacterium tumefaciens*, were included. Growth of all three bacteria was poorer after the shift to 42°C (indicated by arrow head), although *E. coli* showed the best growth. A shift to 45°C completely stopped the growth of *E. coli* (Fig. 5A).

Then, as further preparation for western blot analysis, a DNA sequence encoding three copies of the Flag tag (a total of 23 amino acids) was fused to the 3'end of the coding region of each of the selected genes using the chromosomally integrating plasmid pK18mob2 (Schäfer *et al*., 1994). Proteins selected for this analysis were based on the MS analysis and are included in Table 1. Figure 5B shows the abundance of the Flag-tagged proteins at intervals over 90 min. The small heat shock protein RSP_1016 and RSP_0557 showed the strongest response at 10 min at 42°C. Abundance of RSP_0557 decreased thereafter, indicating that degradation plays a significant role in its abundance. In contrast, RSP_3095, an uncharacterized sigma factor protein (not detected in the MS analysis) showed a modest and gradual increase (Fig. 5B). Also included in the western blot analysis were proteins which showed little or no change in abundance in the MS analysis but whose corresponding transcripts strongly increased levels upon heat shock. These were PhaP and CerI. PhaP belongs to a group of proteins known as phasins which associate with polyhydroxyalkanoate (PHA) granules (Mezzina and Pettinari, 2016). Despite the large increase in transcript levels upon heat shock ($\log_2$fc = 5.6), our MS data indicated that the PhaP protein level increased only slightly ($\log_2$fc = 1.3–1.7, Table 1), and the western blot analysis revealed a similar outcome (Fig. 5B). CerI is a quorum sensing inducer synthase (Puskas *et al*., 1997) that is important for exponential growth, since a knockdown of *cerI* expression resulted in extremely poor growth and strong aggregation of cells (McIntosh *et al*., 2019). Upon heat shock, *cerI* transcript levels increased significantly ($\log_2$fc = 3.7), while the MS data indicated almost no change in protein levels ($\log_2$fc = 0.7–0.9, Table 1). Western blot analysis revealed that levels of CerI decreased, rather than increased, upon heat shock (Fig. 5B). Similarly, while the sRNA Pos19 showed strongly increased levels ($\log_2$fc = 4.3) upon heat stress, its small uncharacterized protein ($Pos19^{ORF}$ or RSP_7581, not detected in the MS analysis) showed only a modest increase after 10 min of the western blot analysis. Two other proteins, which showed no change in abundance in the western blot analysis, were the sigma factor RpoH1 and the chromosome replication initiator DnaA. DnaA has been studied in the context of heat shock activated protein degradation in the alphaproteobacterium *Caulobacter crescentus* (Jonas *et al*., 2013). Interestingly, the *dnaA* transcript decreased ($\log_2$fc = −1.78) while that of RpoH1 increased ($\log_2$fc = 4.0). Despite this response at the RNA level, the MS analysis revealed virtually no change at the protein level ($\log_2$fc = −0.4 and 0.5, respectively). Importantly, this is also reflected in the western blot analysis (Fig. 5B). Altogether, the western blot analysis confirmed that for a set of genes whose transcripts showed strongly increased levels under heat stress, some of their proteins (RSP_1016 and RSP_0557) showed correspondingly increased levels, while other proteins (PhaP, CerI and $Pos19^{ORF}$) did not.

This unexpected result raised the question of whether our RNA-seq data was reliable. Therefore, we performed an additional RNA extraction and used quantitative real-time RT-PCR and northern analysis to check for changes in the levels of a set of RNA transcripts. The results of the RT-PCR analysis are shown in the Supporting Information Fig. S2. Although not as sensitive as the RNA-seq data, the RT-PCR data show a similar pattern of transcript level increases and decreases. For example, while the transcript of *dnaA* showed no increase following the heat shock, that of *cerI* showed a modest increase, while those of *rpoH1*, RSP_3095, *phaP*, RSP_1016 and RSP_0557 all showed strong increases. This result provided verification of the RNA-seq data. Furthermore, the RNA-seq data showed that the transcript levels of RSP_0557 and Pos19 (RSP_7581) increased by $\log_2$fc ≈ 5.3 and 4.3, respectively, after 20 min of heat stress, and we have confirmed these strong increases via northern blot analysis (Supporting Information Figs S3 and S4).

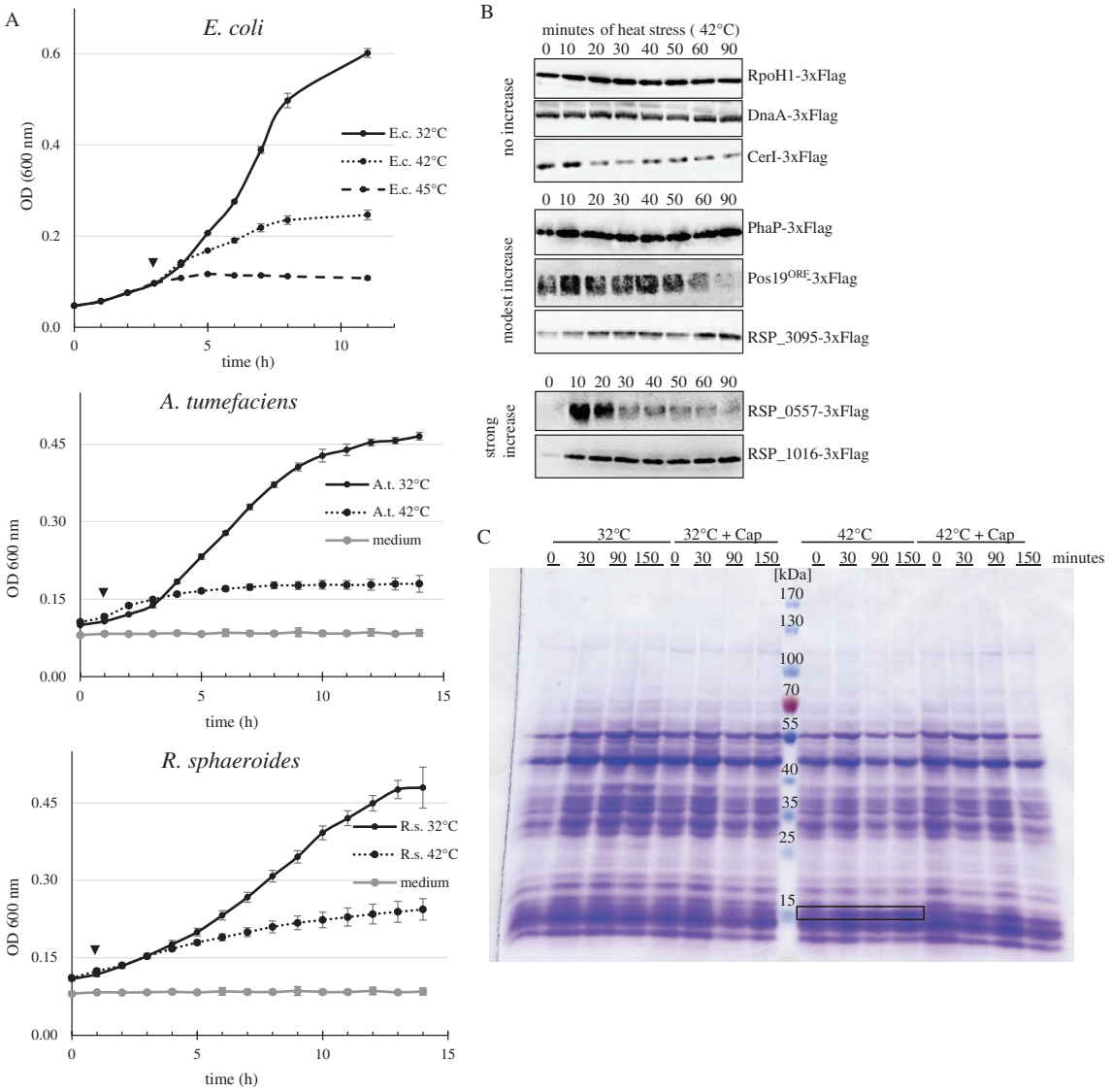

Fig. 5. A. Growth in response to heat stress. *E. coli*, *A. tumefaciens* and *R. sphaeroides* were grown at 32°C and 42° for over 10 h, and growth was monitored by measuring optical density at 600 nm. For growth at 42°C or at 45°C, cultures were initially incubated at 32°C and then shifted to a higher temperature at the time point indicated by a black arrow. Each bacterium was grown in a defined minimal medium (see Materials and Methods). For *A. tumefaciens* and *R. sphaeroides*, the cultures were incubated and monitored using a Tecan reader with automated measurements every hour. For *E. coli*, the measurements were made on cultures grown in 20 ml volumes in 100 ml flasks. Each data point represents the average of 3–8 independently grown replicates. The experiments were performed three times.
B and C. Western blot and SDS-PAGE analysis. Each exponentially growing culture was divided into two cultures, one which was grown at 32°C, the other was incubated at 42°C for up to 150 min. Samples from liquid cultures were normalized according to OD before loading. B, western blot analysis of various 3x Flag-tagged proteins from liquid cultures incubated at 42°C for up to 90 min. The time points (minutes) at which samples were taken for western blot analysis are indicated. Protein standards indicate molecular size (kDa). The expected sizes of the proteins, including the Flag tag, are as follows: CerI: 26.2 kDa, DnaA: 54.4 kDa, Pos19^{ORF}: 8.5 kDa, RSP_1016: 20.7 kDa, RSP_3095: 22.6 kDa, PhaP: 18.6 kDa, RSP_0557: 10.6 kDa. The Flag tag alone was 2.9 kDa. C, SDS-PAGE (8%–18% acrylamide) analysis of proteins from whole cell fractions. Where indicated, chloramphenicol (Cap) was added at 500 μg ml^{-1} to stall translation prior to the shift to 42°C. Black box represents the excised region shown to contain the RSP_1016 protein.

In vivo *[^{35}S]-methionine labelling and SDS-PAGE analysis reveal synthesis of specific proteins*

We next considered whether the discrepancy between transcript levels and the levels of certain proteins might be caused through protein degradation. Western blot analysis (see Fig. 5B above) had indicated that at least some proteins were degraded. To see whether massive degradation of protein was occurring under our heat shock conditions, protein from whole cells was analysed by SDS-PAGE (Fig. 5C, see Materials and Methods for

Table 1. List of genes showing changes in transcripts and protein levels at specified time intervals following a shift to 42°C.

Gene	Description	20 min	60 min	90 min	Sequence of RBS, translation start and coding region included in translational fusion to mVenus
		Transcript (log_2fc)	Protein (log_2fc)	Protein (log_2fc)	
phaP RSP_0381	phasin	5.6	1.3	1.7	C**AGGAGA**CAAGGCA**ATG**accaagaccccgacttcagcaaagtgatgcaggac::mVenus
RSP_0557	DUF1127	5.4	3.6	4.2	A**AAGGAC**AGACGCC**ATG**gca::mVenus
RSP_1016	heat shock protein	5.1	7.1	7.7	CTT**GGAGGA**TGACC**ATG**cgtagctatgatttctcgccg::mVenus
RSP_1572	heat shock protein	4.8	2.8	2.7	**AAGGACA**TGACGGA**ATG**accaaactgacttcgggggccat::mVenus
cerI RSP_0123	AHL synthase	3.7	0.7	0.9	**TTGGGGA**TTGAAGA**ATG**::mVenus
rpoH1 RSP_2410	sigma factor	4.0	0.5	0.8	G**AGGGGGG**TCAACGA**ATG**agcact::mVenus
RSP_3095	sigma factor, leaderless	4.3	ND	ND	AAG**ATG**::mVenus
Pos19	ORF, uncharacterized	4.3	ND	ND	**AAAGGAC**CTGACCG**ATG**agc::mVenus

Transcript and protein levels are represented as log_2-fold change (log_2fc) based on comparison to levels at 32°C. ND = not detected. On the right side of the table, the DNA sequence of the ribosome binding site (RBS; bolded), translation start (bolded) and part of the coding sequence of each gene (lowercase letters) fused to the gene for mVenus is shown. RSP_3095 lacks a conventional RBS; its transcript is leaderless.

details on sample preparation). Additionally, we added chloramphenicol to the cultures immediately prior to the heat shock to disrupt protein synthesis at the mRNA–ribosome complex. However, this revealed no major decreases in protein abundance, even after 150 min at 42°C in the presence of chloramphenicol. This approach, however, did reveal an increase in the level of a protein of approximately 17 kDa (see Fig. 5C, black box). The band was excised and subjected to MS analysis and confirmed as the heat shock protein RSP_1016. Apart from RSP_1016, there were no obvious increases in proteins from whole cells under heat shock. Therefore, we used *in vivo* labelling with [^{35}S]-methionine to better follow *de novo* protein synthesis under heat shock. Based on the MS analysis, we anticipated that very few proteins would be labelled with [^{35}S]-methionine under heat stress. Figure 6A confirmed our expectations, showing only two clearly labelled bands under heat stress. Based on comparison to the expected sizes, the upper band is likely from RSP_1016 (17.8 kDa), while the lower, weaker band is likely from RSP_0557 (10.6 kDa). Thus, this approach supports the conclusion from the MS analysis that *R. sphaeroides* responds to heat stress by generally decreasing translation of all but a few specialized proteins.

Because the *R. sphaeroides* heat stress response at the protein level appeared to be more selective than that of other well-studied bacteria, we also used the [^{35}S]-methionine labelling method for another well-studied alphaproteobacterium, *Agrobacterium tumefaciens*, and the gammaproteobacterium *E. coli* (Fig. 6B). The response of *R. sphaeroides* was indeed different from that of both *E. coli* and *A. tumefaciens*, since *A. tumefaciens* produced at least six labelled bands (see black arrows), while *E. coli* produced at least 13.

mVenus reporter translational fusions reveal heat shock response

Another approach to detect the expression of protein during heat stress is the use of translational fusions between the gene of interest and a reporter gene. The advantage with using a reporter protein is that it can provide an approximation of the production rate of the protein of interest while ignoring its degradation rate. To ensure this, most of the coding region of the gene of interest was excluded from the translational fusion. In the best case scenario, only the ATG translation start of the gene of interest was included. Genes selected for translational fusions with the mVenus fluorescence reporter gene included those from the western blot analysis: RSP_1016, RSP_0557, *cerI*, *phaP*, *rpoH1*, Pos19^{ORF} and RSP_3095. For each mVenus fusion construct, the promoter, 5' UTR and start of each target gene was fused

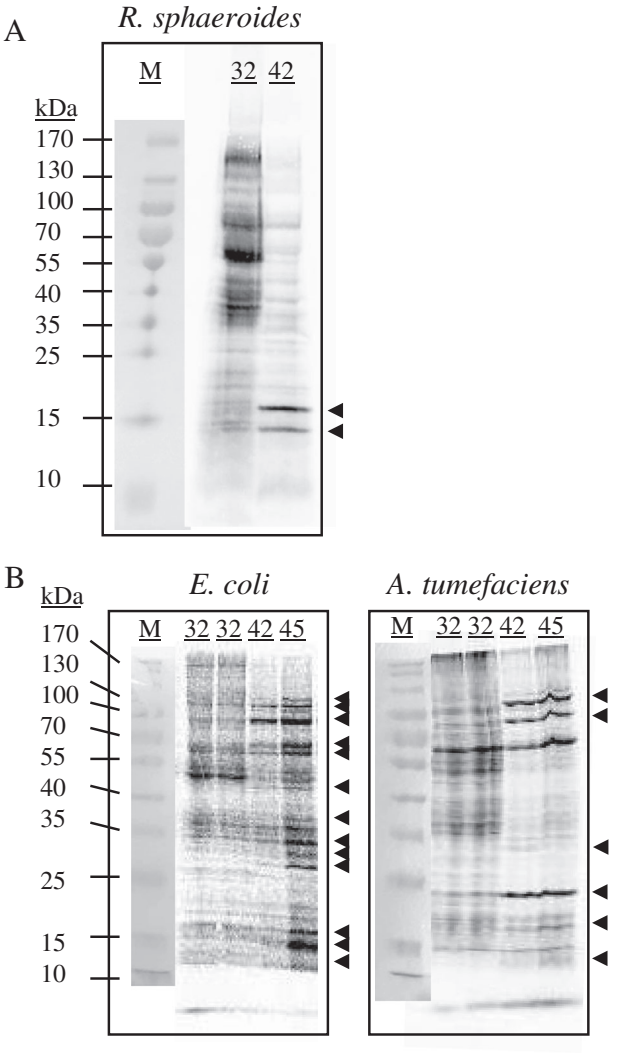

Fig. 6. *In vivo* [^{35}S]-methionine labelling reveals the number of proteins with increased abundance. Increases in abundance of proteins are indicated by the black arrows.
A. For *R. sphaeroides*, these are likely to be RSP_1016 (upper band) and RSP_0557 (lower band).
B. While for *E. coli* and *A. tumefaciens*, the labelled proteins were not identified in this study. The number of arrow heads provide an approximation for the number of proteins with changed levels. For the labelling during heat shock, the [^{35}S]-methionine was added to the minimal media cultures 10 min after the shift to 42°C. Cultures were incubated for an additional 10 min with [^{35}S]-methionine before harvesting. Samples from liquid cultures were normalized according to OD before loading for SDS-PAGE (12% acrylamide) analysis.

to the gene for mVenus. In some cases, part of the protein coding sequence (1–12 codons) was also included in the fusion, depending upon primer design constraints (see Table 1 for the DNA sequence fused to the mVenus gene). In addition to the genes listed above, we included as a comparison the promoter of the 16S rRNA gene fused to the Shine–Dalgarno sequence from *cerI*. Expression activity of the highly active 16S promoter fused to the RBS of *cerI* was previously found to correlate well with growth (McIntosh *et al*., 2019), similarly to the expression of the rRNA genes during growth of *E. coli* (Maeda *et al*., 2015).

All fusion constructs were carried on the broad host range low-copy replicating plasmid pPHU213. *R. sphaeroides* was grown in 96-well plates and incubated in a Tecan reader with an automated 60 min cycle for measuring OD and fluorescence. The 96-well plate was initially incubated for 3 h at 32°C and then either shifted to 42°C or held at 32°C for up to 30 h. As expected, growth at 42°C was slower and failed to reach the OD of the cultures grown at 32°C (Fig. 7, top left). Remarkably, mVenus fusions to RSP_1016 and RSP_0557 showed strong and clear responses to 42°C (Fig. 7, top row), providing excellent correlation with the MS analysis. Maximal mVenus production from the fusions with RSP_1016 and RSP_0557 was observed at approximately 2 h following the shift to 42°C. Other fusions showed weaker responses. The mVenus fusion to Pos19^{ORF} showed a modest response (Fig. 7, bottom right). As expected, *cerI* and 16S fusions (Fig. 7, middle row) showed poor mVenus production at 42°C. The *rpoH1* fusion (Fig. 7, middle left) showed a temporary weak increase in mVenus fluorescence at 42°C. This was also seen in the case of PhaP (PhaP + 39), Fig. 7, bottom left), which fits well with the MS data (a $\log_2$fc of 1.7 increase in protein levels after 90 min of heat stress, see Table 1). For PhaP, we also constructed a second mVenus fusion, which included the whole coding region of *phaP* (PhaP + W) (Fig. 7, bottom) to see whether mVenus production was additionally affected by the coding region of *phaP*. The PhaP + 39 and PhaP + W fluorescence profiles were comparable at 32°C, while the PhaP + W fluorescence was slightly (50%) weaker at 42°C.

Altogether, the fluorescence profiles matched well with the MS analysis, the western blot analysis and the *in vivo* [^{35}S]-methionine labelling analysis. RSP_1016 and RSP_0557 showed clear, strong expression under heat shock while CerI, PhaP and RpoH1 did not.

Heat shock expression responses of RSP_1016 and RSP_0557 depend upon their 5' UTRs

Results described above indicate that mVenus translational fusions provide a suitable approach for studying the heat shock response. However, one limitation with these mVenus fusions is the inability to distinguish between transcription regulation and translation regulation, since the readout of fluorescence is the sum of both transcription and translation activity. Therefore, we fused the UTRs of RSP_1016 and RSP_0557 to the promoter of *cerI*, replacing the native UTR of *cerI*. mVenus expression from these hybrid fusions (PcerI::UTR1016::mVenus

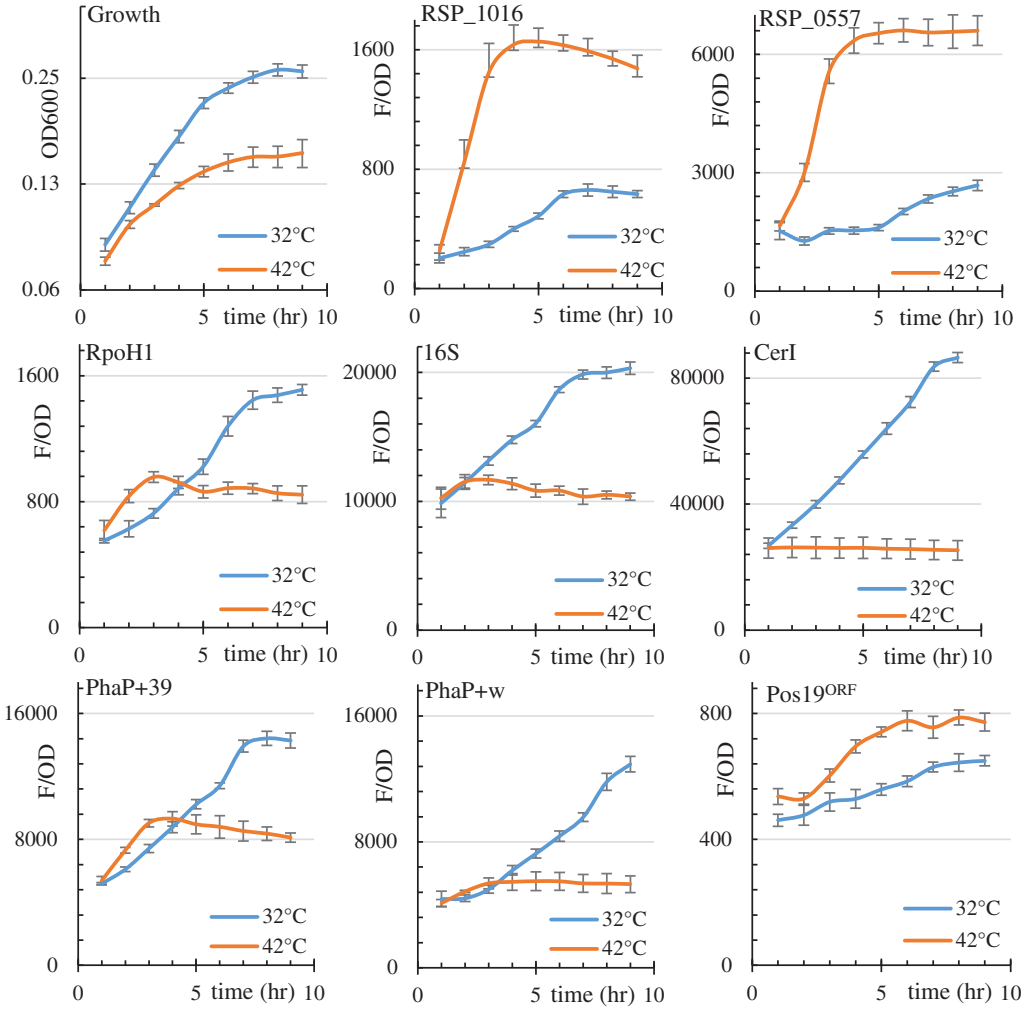

Fig. 7. mVenus production upon heat shock. Translational fusions between selected genes and the gene for mVenus carried on a low copy plasmid were measured at 32°C and 42°C, as indicated, using a Tecan reader with automated measurements every hour. Fold change in normalized fluorescence signal (F/OD) was plotted against time. In each 42°C data set, the first time point was measured at 32°C, while all following time points were measured at 42°C. Error bars represent standard deviation from at least four independent cultures grown in a 96-well plate. Top left panel represents growth (OD measured at 600 nm) of the wild-type *R. sphaeroides* 2.4.1 carrying an empty vector control.

and PcerI::UTR0557::mVenus) was then compared with that from the original *cerI* fusion (PcerI::UTRcerI::mVenus). Figure 8 shows that all three UTRs showed comparable translation activity at 32°C. At 42°C, however, the UTRs of RSP_1016 and RSP_0557 were more effective at expressing mVenus than the UTR of *cerI*. This result confirms the importance of the UTR for translation under heat shock.

Leaderless transcripts are preferably translated at 42°C

How do genes with leaderless transcripts respond to heat shock? The two uncharacterized sigma factor genes, RSP_3095 and RSP_4081, produce leaderless transcripts, as judged by viewing the previously published differential RNA-seq data (Remes *et al*., 2017) via the Integrated Genome Browser (Nicol *et al*., 2009). The transcript from RSP_3095, for example, contains only three nucleotides upstream of the ATG translation start (see Table 1 for sequence). The situation for RSP_4081 is more complicated because the RNA-seq data show a transcription start 53 nucleotides downstream from the annotated ATG and one nucleotide upstream of a second in-frame ATG. This situation indicates that the second ATG is the correct translation start, and that it is leaderless. Therefore, the fusion construct began with the native promoter of RSP_4081, included the first ATG and extended through to the second ATG which was fused with mVenus. Interestingly, the mVenus fusions revealed

a clear response to 42°C (Fig. 9, top row). Although neither of the proteins were detected via MS analysis, a Flag-tagged copy of RSP_3095 was detected via western blot analysis and slightly increased in abundance at 42°C (Fig. 5B), suggesting that this sigma factor is indeed part of the heat shock response. Based on our data above, which highlights the importance of the UTR for protein expression (Fig. 8), we asked whether the lack of a UTR might somehow lead to increased expression under heat stress. One way to test this idea is to modify the sequence of an mVenus fusion construct by deleting the entire UTR. *cerI* and *phaP* were suitable candidates because their transcripts increased by a $\log_2$fc of 3.7 and 5.6, respectively. Therefore, we generated leaderless fusions in which the translation start of mVenus coincided with the transcription starts from the promoters of *cerI* and *phaP*. Remarkably, these fusions showed clear activation at 42°C compared to 32°C (Fig. 9, bottom row), consistent with the idea that leaderless transcripts are preferably translated at 42°C. This novel result contrasts with their native translational fusions (see Fig. 7) and supports the idea that translation of leaderless transcripts is activated during heat shock. It is also noteworthy that the overall level of expression of mVenus from the leaderless fusions was 10- to 100-fold weaker than those with a UTR, which explains why proteins translated from leaderless transcripts were not obvious in the [^{35}S]-methionine analysis.

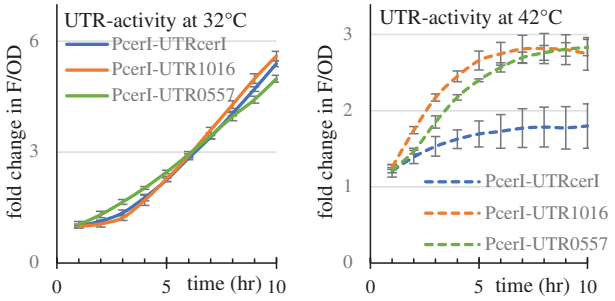

Fig. 8. The UTR influences translation during heat shock. DNA carrying the promoter of *cerI* along with its own UTR, or UTRs of RSP_1016 and RSP_0557, was fused to the gene for mVenus. Fluorescence from these fusion constructs was monitored during growth of *R. sphaeroides* at 32°C and 42°C and represents the average from eight independent cultures. Using the first measurement ($t = 0$) as a reference, all subsequent measurements of normalized fluorescence signal (F/OD) were derived as fold change (fold change = (F/OD)/(F/OD$_{t=0}$)) and plotted against time.

Discussion

Our study revealed that the heat shock transcriptome response of *R. sphaeroides* involves 459 genes with significantly (defined as a $\log_2$fc ≥ 2) increased transcript levels, and a similar number of genes with significantly decreased transcripts. Heat shock studies on a range of model organisms from bacteria and archaea to human cell lines have typically reported higher levels of mRNA from 50 to 200 genes (Richter *et al*., 2010). This comparison suggests that the *R. sphaeroides* transcription response to heat shock is more extensive, although we cannot rule out the influence of improved transcript

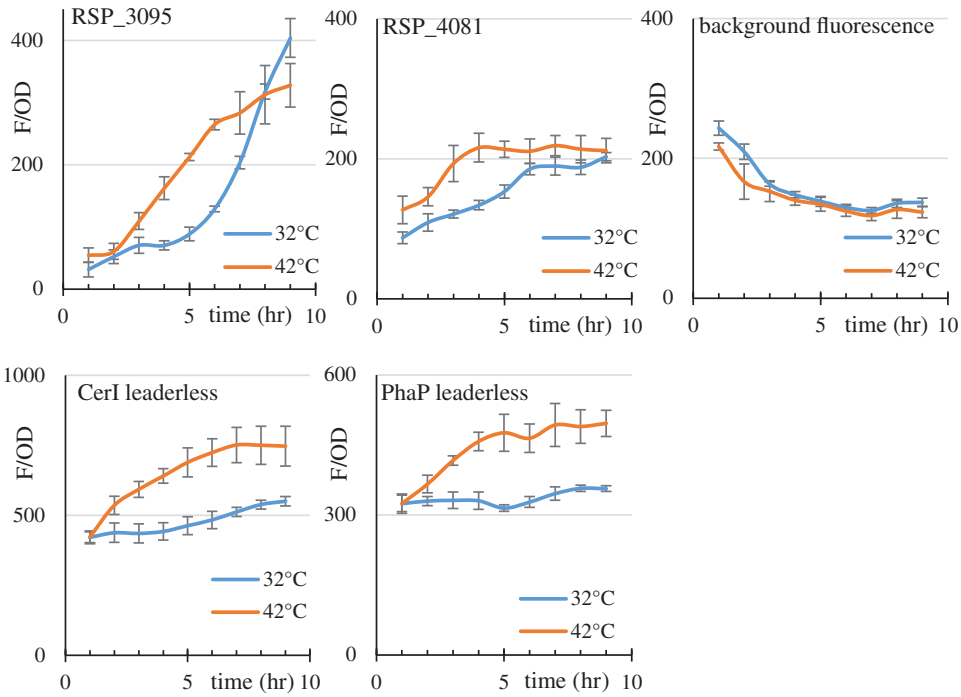

Fig. 9. Activation of mVenus production from leaderless transcripts upon heat shock. Fusions of promoter regions of leaderless genes RSP_3095 and RSP_4081 to the gene for mVenus revealed activation of mVenus production upon heat shock. In each construct, the translation start of mVenus either coincided with the transcription start of the promoter or was located close (within three nucleotides) to the transcription start. Experimental conditions were comparable to those outlined in Figs. 7 and 8. Error bars represent standard deviation from eight independent cultures grown in a 96-well plate. Panel on the far right shows the background fluorescence/OD from cultures lacking the mVenus reporter.

detection via RNA sequencing (e.g., RNA sequencing is more sensitive than DNA microarrays).

A previous study noted a relatively high correlation coefficient of 0.51–0.53 between the transcription response of *R. sphaeroides* to singlet oxygen and the transcription response during recovery in fresh medium (also referred to as outgrowth) after an extended (72 h) stationary phase (Remes *et al*., 2017). This result indicates that outgrowth after prolonged stationary phase and exposure to singlet oxygen stimulate relatively similar transcription responses. In contrast, the heat shock response of *R. sphaeroides* documented in this study correlates poorly with the singlet oxygen stress response (0.24–0.32) but to a greater extent with the response during outgrowth (0.47–0.48).

Low correlation between transcripts and proteins

The fact that the vast majority of transcripts with increased levels under heat shock does not correspond to proteins with increased levels is a remarkable result that caught us by surprise. It is possible that protein production can be off-set by stress-induced protein degradation, i.e., that the rate of production equals the rate of degradation and therefore no net change. Degradation of specific proteins is known to increase during heat shock (Rodriguez *et al*., 2008), and some degradation was apparent in our western blot analysis (Fig. 5B). However, SDS-PAGE analysis excluded the possibility that large-scale degradation of protein occurred during the first 150 min of heat stress (Fig. 5C).

Interestingly, *in vivo* ^{35}S-methionine labelling matched the mass spec data, confirming that protein synthesis was mostly limited to that of RSP_1016 and RSP_0557 (Fig. 6). Western blot analysis and the mVenus fusions also supported this conclusion (Figs. 5 and 7). Thus, our data indeed indicates that most of the strong increases in transcript abundance do not result in a significant change in protein abundance.

Comparison of heat stress response to adaptation to stationary phase

The appearance of a general shutdown of translation in *R. sphaeroides* was not because of cell death. While the cells grew poorly under heat stress, they were still viable, as reflected by the large increases in the amounts of RSP_1016 and RSP_0557. Poor growth is also typical of cultures transitioning from exponential growth to starvation conditions. During this transition, the *R. sphaeroides* transcriptome involves very few changes, while the proteome undergoes stronger changes (Bathke *et al*., 2019). This is precisely the opposite of the heat stress response observed in this study. Interestingly, correlation between transcript levels and protein levels was low in both the transition to stationary phase (0.1–0.2) (Bathke *et al*., 2019) and heat stress (0.2–0.3, this study). Higher correlation (0.64) between the proteome and transcriptome was observed upon singlet oxygen stress (Berghoff *et al*., 2013). This situation suggests that the changes in the transcriptome and proteome that we have detected represent the impressive ability of the bacterium to use checkpoints at both the level of transcription and translation to adjust gene expression in response to environmental changes.

Comparison to heat shock responses of other organisms

We wondered whether this appearance of a general shutdown of translation during heat stress was unique to *R. sphaeroides*; therefore, we included two other bacteria, *E. coli* and *A. tumefaciens*, in the [^{35}S]-methionine labelling. Here, it is important to recognize that the [^{35}S]-methionine labelling method captures only the most abundantly expressed proteins; the full number could be several fold higher. Thus, the differences detected here (2 bands for *R. sphaeroides*, 6 for *A. tumefaciens*, and about 13 for *E. coli*, see Fig. 6) are likely representative of significant differences between the bacteria. The additional labelling of *E. coli* at 45°C was performed because we expected *E. coli* to have a higher tolerance to heat than the alphaproteobacteria. *E. coli* is routinely cultured at 37°C in the laboratory while the alphaproteobacteria are grown at 28–32°C. Indeed, growth curves of all three bacteria (Fig. 5A) revealed that *E. coli* grows better than *R. sphaeroides* and *A. tumefaciens* at 42°C but did not grow further at 45°C. We do not know why *R. sphaeroides* produces fewer proteins than *E. coli* in the heat stress response. It is noteworthy that the alphaproteobacterial responses are more similar to each other than to *E.coli* and may represent a fundamental difference between the gamma- and alphaproteobacteria. For example, the *E. coli* response involves more than 20 heat shock proteins including DnaK, DnaJ, GrpE, GroEL and GroES, and proteases ClpP and ClpX (Arsène *et al*., 2000). However, none of their orthologues showed increased levels during the heat shock response of *R. sphaeroides* despite transcript increases in *grpE* ($\log_2$fc = 2.59) and *clpP* ($\log_2$fc = 2.68) (see Supporting Information Table S1).

Transcript/protein correlation is not high in *R. sphaeroides*, even under favourable (32°C) conditions (Bathke *et al*., 2019). Notably, this study shows that the correlation decreased under heat stress. One possibility is that *R. sphaeroides* has lower correlation than other bacteria. However, this low correlation is not an isolated case. Multiple studies have reported low correlation upon heat shock. For example, low correlation between total transcripts and ribosome-associated transcripts upon

heat shock was observed in *E. coli* (Zhang *et al*., 2017) and low correlation between the transcriptome and translatome was observed in the actinomycete *S. coelicolor* (Bucca *et al*., 2018). Increased levels of only 56 proteins was reported in the heat shock response of *A. tumefaciens* using protein 2D gels (Rosen *et al*., 2001), and about 60 proteins using MS analysis in the heat shock response of the lactic acid bacterium *Lactobacillus casei* (Adu *et al*., 2018). In *Bacillus licheniformis*, a shift from 37°C to 54°C resulted in more than 1000 genes with either increased or decreased transcripts, but only 60 proteins with increased levels were identified (Voigt *et al*., 2013). The heat shock response of the fungus *Aspergillus flavus* also involves a surprisingly low correlation (0.13) between transcript and protein abundance (Bai *et al*., 2015). Altogether, judging by the literature on this topic, it is possible that low correlation between transcript and protein levels upon heat shock may be widespread or even a general feature of the heat shock response. This situation suggests that the heat shock response generally involves a high degree of post-transcriptional regulation compared to non-stress conditions.

Mechanisms regulating translation

Some of the mechanisms regulating translation are known. For example, the carbon storage regulator or repressor of stationary phase metabolites system (Csr or Rsm) involves a well-studied posttranscriptional regulatory RNA-binding protein, CsrA/RsmA which is common to the gammaproteobacteria. Generally, CsrA represses stationary phase metabolism and stress responses, and this repression is released when the cell encounters certain forms of stress or nutrient limitation (reviewed by Pourciau *et al*., 2020). However, such a regulatory protein would not explain the narrow protein expression response in *R. sphaeroides*. Rather than a release of repression upon stress, our data show stronger repression of protein expression. Most of the transcripts with increased levels are not translated into protein upon heat stress, suggesting some kind of stress-initiated repression. Furthermore, a CsrA/RsmA homologue is absent in *R. sphaeroides*, as is the case with almost all the alphaproteobacteria, with a single known exception (Agaras *et al*., 2013).

One possible contribution to the differences between the transcript and protein abundance under heat shock in eukaryotes is the temporary and reversible formation of cytoplasmic stress granules consisting of RNA and protein which selectively sequester some mRNAs to prevent their translation (Verghese *et al*., 2012; Wallace *et al*., 2015). Formation of stress granules may cause the appearance of a wide-scale inhibition of translation. A limited proteolysis-mass spectrometry approach on the *E. coli* heat shock response found relatively few changes in protein abundance upon heat shock (compared to osmotic shock) but massive, large-scale changes in protein structure, possibly due to the formation of stress granules (Cappelletti *et al*., 2021). The same study also investigated the heat shock response of yeast and found only 26 proteins (from a total of 2600 detected proteins) with levels increased by ≥2-fold.

Another possible factor that could influence protein abundance is the S1 protein of the 30S subunit of the ribosome, which functions as an RNA chaperone under physiologically optimal conditions. It influences the translation rate by altering RNA structural folds around the RBS (Duval *et al*., 2013). S1 is considered to have a weak and reversible association with the ribosome, is essential for growth, and is necessary for the translation of bulk mRNA in *E. coli* (Sorensen *et al*., 1998; Briani *et al*., 2008; Delvillani *et al*., 2011). However, leaderless transcripts can be translated by ribosomes lacking the S1 protein (Moll *et al*., 2002). In this context, it is interesting to note that translation from the leaderless transcripts of RSP_3095 and RSP_4051 is higher during heat shock (Fig. 9). Furthermore, translation from *phaP* and *cerI* transcripts during heat shock was only activated when these were rendered leaderless (see Fig. 9). When carrying their native RBS, however, these were inactive at 42°C (see Fig. 7). One intriguing possibility is that the RBS may be involved in the stalling or pausing of the ribosome lacking the S1 protein.

RBS as a pause site for the ribosome

Ribosome pausing was previously detected in *E. coli* by Zhang *et al*. (2017). That study showed that ribosome pauses induced by heat stress were concentrated around the translation start. We speculate that the RBS may be involved in the pausing. In such a scenario, translation of leaderless mRNA, which lacks a RBS, could not pause. Indeed, when the 70S ribosomes are stressed, translation of bulk mRNA ceases while translation of leaderless mRNA continues (Moll *et al*., 2004). Our results indicate that translation of leaderless transcripts continues at 42°C, possibly via increased transcript abundance, and thus forms a part of the heat shock response.

Zhang *et al*. (2017) postulated that ribosome pausing occurs via an unknown mechanism in the starting regions of open reading frames (i.e., not the RBS). However, our use of mVenus translational fusions excludes this possibility, particularly in cases where only the translation start (ATG) was included in the fusion (e.g., *cerI*::mVenus). Moreover, the cessation of mVenus expression from the *cerI*::mVenus translational fusion at 42°C was not due to a decrease in *cerI* promoter activity, since this promoter

was sufficient for increased mVenus production from a leaderless mVenus gene as well as from the fusions between the *cerI* promoter and the UTRs of RSP_1016 and RSP_0557. Therefore, collectively, our data points to the UTR as a checkpoint for translation under heat shock with ribosomal pausing at the RBS being one possible mechanism. However, this does not exclude other mechanisms involving the UTR as a source of control, e.g., structural changes to the RNA induced by specific RNA–RNA, RNA–metabolite (e.g., riboswitches) and RNA–protein interactions, and removal of the UTR by RNases of toxin–antitoxin systems such as RelE and MazF (reviewed by Gerdes *et al*., 2005).

Specialized UTRs that de-repress translation under heat shock

Our results using the translational fusions to mVenus showed that if the UTR is lacking, as in the case of leaderless transcripts, then translation can be activated under heat stress. If the UTR is present then translation will either weaken or cease. Exceptions occur if the UTR or the intercistronic region of an mRNA forms specialized structures such as temperature-sensing RNA thermometers, which decreases expression at physiological optimal temperatures by excluding ribosomal access to the RBS (Krajewski and Narberhaus, 2014; Righetti *et al*., 2016). Our results indicate that expression of RSP_1016, a heat shock protein, may be controlled by a temperature-sensing RNA thermometer in its UTR, based on similarities to a previous study (Nocker *et al*., 2001). No such similarity could be found for RSP_0557. It is possible that a specialized element in the UTR of RSP_0557 remains to be discovered.

Roles of heat shock proteins

RSP_1016 protein is an Hsp20 family chaperone. Hsp20 proteins can confer oxidative stress resistance in *E. coli* (Singh *et al*., 2014) and are involved in cyst desiccation resistance in *Azotobacter vinelandii* (Cocotl-Yañez *et al*., 2014). An Hsp20 protein of *Deinococcus radiodurans* was shown to form 36mers that dissociate into smaller oligomeric assemblies that stably bind substrate proteins and cooperate with ATP-dependent chaperones in protein refolding (Bepperling *et al*., 2012).

The role of the DUF1127 protein RSP_0557 is unknown. Interestingly, similarity has been found between RSP_0557 and the primosomal protein DnaI from *Roseobacter* and the RNA-binding protein Smaug (Müller *et al*., 2016). We speculate that RSP_0557 may function as an RNA chaperone. A function for another DUF1127 protein, CcaF1, was recently established as an RNA-binding protein involved in sRNA maturation and RNA turnover in *R. sphaeroides* (Grützner *et al*., 2021). DUF1127 proteins from *Agrobacterium tumefaciens* are important for the expression of a large number of genes as well as cell aggregation, growth and biofilm formation, but their mechanisms of function are unknown (Kraus *et al*., 2020).

Conclusions

Analysis of the changes in the transcriptome and proteome of *R. sphaeroides* reveals that the correlation between the abundance of transcripts and their respective proteins is low during heat stress. The cells respond to heat stress by producing an extensive range of transcripts that are mostly not translated. We conclude that regulation of translation is indeed a major checkpoint for gene expression under heat stress. Why do bacteria produce a tremendously large heat stress response if indeed very few of these transcripts are translated into proteins? While it is impossible at this stage to know exactly how this could provide a survival advantage, we propose that the transcription response represents a state of high alert. *R. sphaeroides* is capable of harnessing solar radiation, which can raise the temperature in some aqueous habitats. In an environment where bacteria have reached high population density, death caused by heat stress offers an opportunity for growth and reproduction by the surviving members of the population once the heat has dissipated. In this context, cells, which have a transcriptional response to the heat stress, might respond better to favourable conditions and thus have a reproductive advantage. This could explain, for example, why the heat stress transcription response overlaps to a high degree with that of outgrowth (i.e., the response to growth-favourable conditions). Furthermore, we propose that the *R. sphaeroides* transcriptional response represents preparations by the bacterium to not only survive heat stress but also rapidly adapt to any changes which could follow. In contrast, regulation at the level of translation represents a committed response by the cell directed at coping with heat stress. In this way, a bacterium manages the heat stress response by using transcription and translation as two distinctly regulated processes which complement each other to enhance survival.

Materials and methods

Bacteria and growth conditions

R. sphaeroides 2.4.1 (van Niel, 1944), recently reclassified as *Cereibacter sphaeroides* (Hördt *et al*., 2020), was grown in malate minimal medium (Remes *et al*., 2014) under chemotrophic and microaerobic conditions (25 μM dissolved oxygen) at 32°C or

42°C. For the S35-labelling experiments, *E. coli* (strain JM109) was grown in 3-(N-morpholino)propanesulfonic acid (MOPS) minimal medium (Neidhardt *et al*., 1974), which was modified by adding the vitamins thiamine (10 μM), niacin (13 μM), nicotinamide (13 μM) and biotin (0.3 μM). *A. tumefaciens* (strain C58, also known as *Agrobacterium fabrum* str. C58 or *Rhizobium radiobacter* str. C58) was grown in another MOPS buffered minimal medium adapted for rhizobia (Zhan *et al*., 1991). For 96-well plate cultures, 0.1 ml of inoculated culture medium was added to each well of a transparent Greiner bio-one plate with condensation rings. The plate was covered with a transparent Greiner bio-one lid, constantly shaken in a Tecan Infinity plate reader, and incubated at 32°C or 42°C. Automated measurements of optical density (OD660 nm) and fluorescence (for mVenus, ex 515 nm, em 548 nm) were taken at 1 h intervals for up to 24 h.

Primers and plasmid constructs

For the construction of mVenus fusions, primers (Supporting Information Table S2) were used to amplify 300–400 bp fragments carrying the promoter, UTR and translation start of each target gene, plus some of the coding region (as indicated in Table 1). This fragment was fused to the gene for mVenus in the plasmid pPHU231 as previously described (Charoenpanich *et al*., 2013). Plasmid pPHU231 is one of a series of low copy broad host range plasmids created for use in *R. capsulatus* (Hübner *et al*., 1991) and previously used in *R. sphaeroides* (McIntosh *et al*., 2019). For the fusion of the Flag tag to the C-terminus of target proteins, 500–800 bp which included the target gene, except for the stop codon, plus the upstream region, were amplified using the primers listed in the Supporting Information Table S2. These fragments were cloned into the suicide vector pK18mobII (Schäfer *et al*., 1994) which carried the 3x Flag coding sequence. The plasmid was conjugated into *R. sphaeroides* using the *E. coli* strain S17-1 (Simon *et al*., 1983). Following homologous recombination (single cross-over) between the cloned fragment in the suicide vector and the genomic copy of the target gene, the modified *R. sphaeroides* chromosome carried two copies of the target gene, one of which was fused to the 3x Flag-coding sequence at the 3' end. All plasmids used in this study are listed in the Supporting Information Table S3.

Protein extracts from whole cells for SDS-PAGE

To minimize protein loss, the sample preparation was kept simple; cells were harvested from malate minimal medium and normalized according to OD 660 nm. Cells were then pelleted, the supernatant removed, and the cells were resuspended in SDS sample buffer and boiled for 10 min. After vigorously mixing for 1 min on the vortex, the sample was centrifuged for 13 k, 10 min, and then the supernatant was loaded into the well of the SDS-PAGE gel. Gels were either stained with Coomassie Blue or treated according to the standard western blot protocol. Flag tag was detected via a monoclonal ANTI-FLAG M2-Peroxidase (HRP) antibody (Sigma-Aldrich) produced in mouse, diluted by 1:1000. Blots were developed with the SuperSignalTM West Pico PLUS Chemiluminescent Substrate kit (Thermo Scientific).

RNA isolation and northern blot analysis

RNA isolation (Förstner *et al*., 2018) and northern blot analysis (Berghoff *et al*., 2009) were performed as previously described. The oligodeoxynucleotide used for hybridization with the RSP_0557 mRNA was CAGA GACCGATGTCGTCGAG, and for Pos19 mRNA was GAGATAGCTCATCGGTCAGGTCC. α-32[P]-ATP (SRP-301; Hartmann Analytic) and T4 polynucleotide kinase (#EK0031; Fermentas) were used for the end-labelling reaction.

Quantitative real-time RT-PCR

RNA from *R. sphaeroides* cells was isolated after 0, 10 and 20 min of heat stress using the hot phenol technique. For precipitation 3 M sodium acetate (1/10 x vol.) and 96% ethanol (2.5 x vol.) was used. A 1 ng of *sinI* RNA from *S. meliloti* was added as a spike-in RNA control (Gruetzner *et al*., 2021) for normalization. All qRT-PCRs were performed as biological triplicates and technical duplicates and the primers used are listed in the Supporting Information Table S2.

Library construction and RNA sequencing and bioinformatical analysis

RNA-Seq data used for this study were analysed and published previously (Förstner *et al*., 2018) and placed under the NCBI GEO accession number GSE71844.

Proteome preparation and mass spectrometry analysis

Cells were harvested from cultures incubated at 32°C and at 60 and 90 min following the shift to 42°C. Cells were prepared for mass spectrometry as previously described (McIntosh *et al*., 2019). Briefly, peptides were separated using a UHPLC system (EASY-nLC 1000; ThermoFisher Scientific) and C18 silica columns (1.9 μm C18 beads, Dr. Maisch GmbH) coupled to a Q-Exactive HF orbitrap mass spectrometer (ThermoFisher Scientific)

using an electrospray ionization source. MS raw data were processed using the *R. sphaeroides* Uniprot database containing 17 687 entries (July 2016). For protein identification, peptides with a minimum of seven amino acids and at least one unique peptide were considered. Only proteins with at least two peptides and at least one unique peptide were considered as identified and were used for further data analysis. For protein quantification, the label free quantitation function of MaxQuant (Cox and Mann, 2008) was used.

Radioactive labelling of proteins with [^{35}S]-methionine

L-[^{35}S]-methionine (15 μCi, Hartmann Analytic) was added to 4.5 ml of exponentially growing cultures of wild-type *R. sphaeroides* 2.4.1. The cells were harvested 10 min after the addition of [^{35}S]-methionine by centrifugation at 8000 rpm for 10 min at 4°C. For heat shock samples, [^{35}S]-methionine was added at 10 min after the shift to 42°C. Samples were normalized according to OD 660 nm by suspending cell pellets in appropriate volumes of SDS-PAGE loading buffer and stored at −20°C until SDS-PAGE analysis.

Data analysis

Log_2 ratios calculated from the normalized RPKM (Mortazavi *et al*., 2008) values were used for the differential gene expression analysis at the transcriptome level. Expression analysis on the proteome level was performed using the normalized ratios from the MS experiments. The statistical analyses as well as the graphical representation of the results were performed with the R software environment (R Development Core Team, 2008). The transcript-level expression analysis was conducted via the Bioconductor/R analysis pipeline DESeq2 (Love *et al*., 2014). For each of the differential expression analyses, incomplete data rows were discarded. Threshold values for the log_2 ratios were defined individually for each experiment, depending on the desired cutoff. Additionally, targets had to exhibit *P*-values less than 0.05. The *P*-values were derived from the results of the RNASeq analysis performed with the DESeq2 software package (Love *et al*., 2014). The programme generates as part of its output a series of *P*-values, corrected by the Benjamini–Hochberg method, to minimize the number of false-positive genes in the results. The presentation of these *P*-values in form of their negative decadic logarithm is intended to graphically convey the information in a more intuitively understandable fashion, where higher values represent an increase in significance.

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Author Contributions

M.M. and G.K. contributed to the conception and design of the experiments; M.M., A.K., A.L. and S.H. participated in data acquisition and interpretation; T.K. was responsible for the bioinformatics analysis; M.M., T.K. and G.K. were involved in the analysis and interpretation of the data; M.M. wrote the manuscript, with contributions from G.K. and T.K. G.K. was responsible for funding acquisition; G.K. and M.M. were responsible for project management. All authors have approved the submitted version.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Supporting Information.

Table S1.