The stringent response of Staphylococcus aureus and its impact on survival after phagocytosis through the induction of intracellular PSMs expression

GEIGER, Tobias, et al.

Abstract

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Introduction

In most bacteria, nutrient limitations provoke the so-called stringent response, which is initiated by the rapid synthesis of the alarmones pppGpp and/or ppGpp, here referred to as (p)ppGpp. Under stringent conditions, (p)ppGpp results in the shut-down of proliferation-related activities, including the transcriptional repression of genes coding for major components of the protein synthesis apparatus (rRNA, ribosomal proteins and translation factors) as well as the inhibition of replication [1,2,3]. Typically genes that are presumed to be important for maintenance and stress-defence are activated under stringent conditions. However, the stringent phenotype resulting from (p)ppGpp synthesis seems to be bacteria species specific and may be mediated by fundamentally different molecular mechanisms [3].

The molecular mechanisms leading to the profound reprogramming of the bacterial cellular machinery under stringent conditions were mostly studied in Escherichia coli. Here, (p)ppGpp can be synthesized by either one of the two homologous enzymes: RelA and SpoT. The RelA-synthase is activated by sensing uncharged tRNAs that are bound to the ribosome. SpoT is a bifunctional enzyme that not only produces (p)ppGpp in response to diverse signals but also contains a (p)ppGpp hydrolase domain important for (p)ppGpp turnover. In E. coli, (p)ppGpp binds, with the help of the DksA protein, directly to the RNA polymerase (RNAP). However, even in this model organism, there is still much debate concerning how (p)ppGpp eventually leads to different promoter activities, how (p)ppGpp influences the stability of open complex formation at the initial phase of transcription and which of the promoters are indirectly regulated via secondary regulatory circuits such as alternative sigma factors or other transcription factors [1].

In other organisms, such as the gram-positive Bacillus subtilis, (p)ppGpp probably does not interact with the RNAP, and no

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* E-mail: christiane.wolz@med.uni-tuebingen.de

Current address: Department of Surgery and Cancer, Biomolecular Medicine, Faculty of Medicine, Imperial College London, London, United Kingdom
Stringent Response of *Staphylococcus aureus*

**Author Summary**

The stringent response is a bacterial response to a multitude of different environmental stress conditions which is characterized by the synthesis of the messenger molecules (p)ppGpp. There is now growing evidence that these molecules also play a key role for pathogens to switch between specific phenotypic states within the host. This seems crucial for the adaptation to different micro-environments encountered during infection for instance after uptake by phagocytes. Killing of phagocytes as well as survival within these cells was proposed as major mechanisms for the success of the human pathogen *Staphylococcus aureus* to spread within the body. In the current study we demonstrate the effect of the stringent response on global gene expression in *S. aureus* and its impact on intracellular survival in human neutrophils. We reveal that a stringent response is induced after uptake of *S. aureus* in neutrophils and RSH activity is crucial for intracellular induction of *psm* expression, coding for cytotoxic phenol-soluble modulins (PSMs). Finally we show that this in turn mediates bacterial survival and escape after phagocytosis. These findings contribute to the understanding of how and where PSMs can act as potent cytolitic molecules and emphasise the importance of (p)ppGpp as an intracellular signalling molecule.

DksA homologue is present. Here (p)ppGpp has been proposed to affect promoter activities only indirectly via changes of the intracellular nucleotide pool [4,5,6]. In this model, the nature of the initiation nucleotide (iNTP) determines whether genes are under positive or negative stringent response [4,5,7,8]. (p)ppGpp synthesis is usually accompanied by a decrease in intracellular GTP concentration. rRNA promoters in *B. subtilis* initiate with GTP and a change of this base at position +1 results in a loss of regulation by (p)ppGpp and GTP. Furthermore, GTP can act as a co-factor for the repressor CodY, and thus the lower GTP levels imposed by the stringent response result in the de-repression of CodY target genes at least in some firmicutes, e.g., *B. subtilis* [9] or *Listeria monocytogenes* [10].

In most firmicutes, three genes coding for putative (p)ppGpp synthases are present. The bifunctional RSH (RelA/SpoT homologue) enzymes are typically composed of a C-terminal sensing domain and an N-terminal enzymatic domain with hydrolyse and synthase functions. RelQ and RelP (also named Sas1 and Sas2, for single small alarmone synthase) are small hydrolase and synthase functions. RelQ and RelP (also named Sas1 and Sas2, for single small alarmone synthase) enzymes are typically composed of a C-terminal synthase domain and an N-terminal enzymatic domain with hydrolyse and synthase functions. RelQ and RelP (also named Sas1 and Sas2, for single small alarmone synthase) are small hydrolase and synthase functions. RelQ and RelP (also named Sas1 and Sas2, for single small alarmone synthase) enzymes.

Little is known about the stringent response in the human pathogen *Staphylococcus aureus*, most likely because of the essentiality of the major (p)ppGpp synthase/hydrolyse enzyme RSH [13,14]. Previously, we have shown that, at least under nutrient rich conditions, mutants defective only in the synthase domain of RSH (rshSyn) are not impaired in growth [13]. Furthermore, RSH is the only enzyme responsible for (p)ppGpp synthesis in response to amino acid starvation triggered by leucine/valine deprivation or mupirocin treatment. Preliminary characterisation of the rshSyn mutant revealed that part of its phenotype mainly the regulation of genes involved in amino-acid metabolism can be explained by the (p)ppGpp induced de-repression of the CodY regulon. CodY of *S. aureus* was previously shown to be a major regulator of virulence gene expression [15,16]. Thus, the CodY regulon seems to be an integral part of the stringent response in *S. aureus*, linking metabolic circuits and virulence. However, whether, and to what extent, other direct or indirect regulatory circuits are involved in RSH mediated stringent response is unclear. A CodY independent contribution of the stringent response to virulence could, so far, not be indicated. However, for many other pathogenic bacteria, a contribution of (p)ppGpp to bacterial virulence could be shown [17]. In particular, for many intracellular bacteria, (p)ppGpp is essential to survive and replicate in diverse host cells, e.g., epithelial and endothelial cell types and professional phagocytes.

Here, we delineate three (category I–III) main consequences of RSH-dependent stringent response in *S. aureus*. (I): Similar to other organisms, a severe down-regulation of the protein synthesis machinery was observed, and thus, the inhibitory effects of (p)ppGpp on gene expression seem to be highly conserved in bacterial species. (II): In contrast, genes that are activated upon (p)ppGpp induction are highly species specific. In *S. aureus*, they are mostly regulated indirectly via the de-repression of CodY. (III): Interestingly, seven genes, including those coding for the toxic phenol-soluble modulins (PSMs), were found to be up-regulated independently of CodY. We demonstrate that a stringent response is induced after uptake of wild-type bacteria in human neutrophils. Moreover, RSH activity is required for *psm*-1-4 and *psm*-12 expression in neutrophils which in turn promotes survival after phagocytosis.

**Results**

Experimental setup to characterize RSH-mediated stringent response in *S. aureus*

*S. aureus* is able to mount a stringent response upon amino acid starvation, which is characterized by the generation of (p)ppGpp [18,19]. However, little is known about the impact of (p)ppGpp synthesis on global physiological processes in *S. aureus*. Mupirocin treatment, an antimicrobial agent that inhibits the bacterial isoleucyl tRNA synthetase and therefore mimics isoleucine starvation, results in profound reprogramming of gene expression [20,21]. Parts of these effects are mediated by (p)ppGpp. However, at least some of them were also observed in an RSH-mutant, which is devoid of (p)ppGpp synthesis after mupirocin treatment [13].

Here, we aimed to delineate the impact of (p)ppGpp synthesis induced by amino acid starvation on gene expression in *S. aureus* under defined nutrient limitation rather than after the addition of inhibitors such as mupirocin. Previously we could show that transferring *S. aureus* into a chemical defined medium (CDM) lacking the two branched-chain amino acids leucine and valine results in growth inhibition [13]. A similar experimental setup was chosen here for a further in-depth analysis including transcriptional profiling by microarray: Bacteria were grown to mid-exponential growth phase in complete CDM, filtered and shifted into a medium lacking leucine and valine (¬leu/val) (Fig. 1A, open arrow). After 30 minutes of incubation in CDM ¬leu/val the bacteria were harvested for RNA purification (Fig. 1A, filled arrow). At this time all strains showed a slight growth inhibition compared to bacteria grown in complete CDM, which is accompanied by a clear induction of the stringent response indicated by RSH dependent (p)ppGpp synthesis and reduction of the GTP pool [13].

The rshSyn mutant showed no accumulation of (p)ppGpp under these conditions which indicates that a contribution of the two putative (p)ppGpp synthases RelP and RelQ is negligible.

To analyze the effects mediated by (p)ppGpp synthesis we compared the transcriptional profile of wild-type strain HG001 with that of the rshSyn mutant under the indicated stringent conditions. Previously a close link between the stringent response and the activity of the repressor CodY was demonstrated in different firmicutes [10,13,22,23]. Thus, to delineate which and
how many [PPP]Gpp regulated genes are influenced through CodY we also analyzed the RSH mediated stringent response in codY-negative S. aureus.

In accordance to previous results [13] we could confirm by northern blot analysis that under the selected conditions of leucine/valine starvation the wild-type strain showed a significant repression of genes of the translational apparatus, such as rpB (ribosomal protein S2), infB (translation initiation factor B) and tf (translation stable factor), whereas no repression occurred in the rshS mutant (data not shown). By contrast, typical CodY target genes like bmQF coding for an amino acid transporter were induced in the wild-type strain (Fig. 1B).

For the analysis of the stringent response in CodY positive strains we performed microarray analysis of wild-type S. aureus HG001, rshS, codY and rshS, codY double mutants after leucine/valine depletion (Fig. 1A). Three major categories (I–III) of stringently regulated genes were observed: I) genes that are negatively regulated by [PPP]Gpp independent of CodY. II) genes that are positively influenced by [PPP]Gpp through CodY de-repression and III) genes positively influenced by [PPP]Gpp independent of CodY.

Category I: Genes negatively regulated during the stringent response

Microarray analysis revealed that 102 genes were significantly (p-value cut-off, 0.05) down-regulated in the wild-type compared to the rshS mutant in the codY positive (WT>rshS>codY) as well as in the codY negative background (codY<rshS,codY) (Fig. 2A, overlap blue Venn diagram). This indicates that these genes are negatively regulated by [PPP]Gpp independent of CodY. Since in total 161 genes were significantly down-regulated in the codY positive background, we also verified the 59 missing genes in the codY negative background. All of these genes appeared also to be down-regulated, although the difference did not reach the significance level.

Most of these CodY-independent negatively regulated genes are hallmark genes of the stringent response in other bacteria [24,25,26]. They are mostly involved in the transcription (Fig. 2B), such as coding for ribosomal proteins (ps, pfl), for translation initiation factor (infB) and translation elongation factor (inf) (Fig. 2C). The rRNA processing machinery is also influenced since genes coding for ribonuclease P (rnpA) and rmlM (coding for a processing protein) are repressed in an RSH-dependent manner (Fig. 2C). In addition, genes like DNA helicases (dnhB, C, D), part of the so called “replicon”, are negatively influenced by [PPP]Gpp, and genes of the recombination/repair system (ruvAB and dinP) were also repressed under amino acid starvation due to [PPP]Gpp (Fig. 2B,C). In accordance with published data from E. coli and B. subtilis [24,25], genes of other physiological processes that are typical for dividing cells (nucleotide, lipid and coenzyme biosynthesis and transport, inorganic ion transport and protein modification) are also part of the negative stringent response in S. aureus (Fig. 2B,C).

In contrast to B. subtilis [24], E. coli [25], or S. pneumoniae [26], genes involved in cell division and cell wall biosynthesis were not found to be significantly influenced by the stringent response in S. aureus. However, we can not exclude that such genes are affected at later time points.

Category II: Genes positively influenced during stringent response through interaction with CodY

We aimed to obtain a comprehensive overview of genes that are part of the predicted regulatory overlap between stringent response and CodY repression. Under stringent conditions most of the up-regulated genes were expressed only in the codY positive background (Fig. 2A). An additional introduction of a codY mutation into this analysis (codY vs. rshS, codY) abrogated the up-regulation of these genes (Fig. 2C). Thus, under amino acid

bmQF in the rshS mutant however, could be restored by additional mutation of codY, indicating that the rshS mutation affects bmQF transcription through CodY (Fig. 1B). To analyze whether this observation is due to a general response to amino acid limitation, we induced serine starvation by addition of serine hydroxamate (SHX). The same transcriptional pattern was observed when compared to conditions of leucine/valine starvation (–leu/val) (Fig. 1B).

Based on the results of the northern blot hybridizations, we performed microarray analysis of wild-type S. aureus HG001, rshS, codY and rshS, codY double mutants after leucine/valine depletion (Fig. 1A). Three major categories (I–III) of stringently regulated genes were observed: I) genes that are negatively regulated by [PPP]Gpp independent of CodY. II) genes that are positively influenced by [PPP]Gpp through CodY de-repression and III) genes positively influenced by [PPP]Gpp independent of CodY.

Figure 1. The experimental setup and the interaction of the stringent response with CodY. (A) Growth inhibition in medium lacking leucine and valine (–leu/val). S. aureus wild-type strain HG001 (square), rshS mutant (triangle), codY mutant (circle) and rshS, codY double mutant (diamond) were grown in complete CDM to an optical density of OD600 = 0.5 (open arrow), filtered, washed twice and re-suspended in an equal volume of CDM medium without leucine/valine (open symbols) or CDM with leucine (wild-type only, filled square). Aliquots for microarray analysis were harvested after 30 minutes (filled arrow). (B) The interaction of the two regulons CodY and stringent response under leucine/valine starvation (–leu/val) and after serine starvation induced in the wild-type strain (Fig. 1B). The low expression of CodY we also analyzed the RSH mediated stringent response in codY negative S. aureus.
deprivation many genes are derepressed in the wild-type bacteria (WT > rshSyn) through the relief of CodY repression, but stay constitutively repressed via CodY in the rshSyn mutant. These genes are part of the recently described CodY regulon [15,16] mainly involved in amino acid metabolism and transport (Fig. 2B). Accordingly, most of them possess CodY binding motifs as described elsewhere [16] and as indicated in Fig. 2C (three asterisks). It was shown previously that CodY represses also several virulence genes in part via inhibition of the quorum sensing system agr. Of note, none of these codY influenced virulence genes were found to be significantly down-regulated in the rshSyn mutants.

Category III: Genes positively regulated during stringent response independent of CodY

Next, we investigated if there are genes activated by (p)ppGpp with no contribution of CodY. Microarray analysis revealed that only seven genes were expressed significantly lower in the rshSyn mutants in both, a codY-positive (codY+) and codY-negative background (codY−) (Fig. 2A, table 1). One gene encoding a putative ribosome-associated protein Y was described in E. coli to bind to the small ribosomal subunit and to stabilize ribosomes against dissociation when bacteria experience environmental stress [27]. Also inA was expressed significantly lower in the rshSyn mutants. The function of TraP remains unknown since a proposed involvement of this protein in the activation of the agr system was recently disproved [28,29,30]. Surprisingly, two genes, part of the staphylococcal defence mechanism, coding for β1 and β2 phenol-soluble modulins (psm β1/β2) were transcribed significantly lower in both rshSyn mutants. This result was of special interest because no other obvious virulence gene appeared to be part of the stringent response in S. aureus.

RSH-dependent induction of psmS during the stringent response is not mediated by increased agr expression

The cytolytic activity of β-type PSMs was described to be minor, and their role in virulence is less pronounced compared with α-type PSMs [31]. Since no probes for α-type PSMs are present on the microarray chip, we performed northern blot hybridisation to analyse the RSH-dependent expression of these important molecules (Fig. 3). To exclude strain specific effects, rshSyn mutants of the prototypic S. aureus strain Newman were also included in the analysis. After leu/val starvation, there is no or very little, α- and β-type psm transcription detectable in the rshSyn mutants of strain HG001 and strain Newman (Fig. 3A) in contrast to the wild-type strain, which shows strong induction of both psm classes (Fig. 3B). Introducing full-length rsh chromosomally into the rshSyn mutant strains could restore their deficiencies to induce psm expression (Fig. 3A). An involvement of CodY could be excluded since the rshSyn, codY double mutant shows the same low transcription of psmS as the rshSyn single mutant.

Table 1. Genes positively regulated under stringent response independent of CodY.

| Annotation       | Common Description                                    | codY− (WT/rshSyn) | codY+ (codY/rshSyn, codY−) | Category                                      |
|------------------|-------------------------------------------------------|-------------------|----------------------------|-----------------------------------------------|
| SAOUHSC_000401   | hypothetical protein                                 | 5.3               | 2.15                       | Function unknown                             |
| SAOUHSC_00767    | putative ribosome-associated protein Y               | 7.14              | 4.55                       | Translation, ribosomal structure              |
| SAOUHSC_01135    | psm β2 antibacterial protein (phenol-soluble modulin)| 8.33              | 3.85                       | Virulence/Defense mechanisms                 |
| SAOUHSC_01136    | psm β1 antibacterial protein (phenol-soluble modulin)| 10                | 4.55                       | Virulence/Defense mechanisms                 |
| SAOUHSC_01819    | putative universal stress protein                    | 3.52              | 2.47                       | General function predicted                   |
| SAOUHSC_01907    | oxidoreductase                                       | 2.17              | 2.63                       | General function predicted                   |
| SAOUHSC_01964    | traP RNAIII-activating protein TRAP                   | 4.76              | 5                          | General function predicted                   |

1: Fold changes are indicated for each comparison and displayed for genes showing statistically significant differential expression. Values corresponds to expression ratios, i.e. averaged expression levels from three independent replicate experiments (p<0.05).

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Figure 2. Transcriptome analysis of (p)ppGpp dependent genes during stringent response. Transcriptional changes of S. aureus in response to leucine/valine starvation (~leu/val) and the overlap of the two regulons CodY and stringent response. (A) Venn diagrams showing genes which are down- (blue) and up-regulated (red) during RSH mediated stringent response. A contribution of CodY to the regulation was determined by analyzing the transcriptional changes in a codY-positive background (WT strains vs. rshSyn, mutant) indicated as codY+ in the figure and in a codY-negative background (codY− mutant vs. rshSyn, codY− double mutant) indicated as codY−. (B) Functional classes of genes which are down- (blue) and up-regulated (red) during RSH mediated stringent response. (C) Heat maps of gene expression ratios for indicated functional classes prominently altered in their expression. The ratios were analyzed in a codY+ (positive) and a codY− (negative) background as described for the Venn diagrams. ** saturated signal *** genes with annotated CodY binding motifs [16].

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RSH-dependent expression of psmS is not mediated by the initiation nucleotide (iNTP)

Aside from the requirement of functional agr, little is known about psm regulation in *S. aureus*. The mechanism by which stringent response leads to increased transcription of certain genes is much under debate. In *B. subtilis*, (p)pGpp does not appear to interact directly with RNA polymerase (RNAP) [5]. The nature of the iNTP during transcription was proposed to determine whether genes are under positive or negative stringent response. In this model, positive stringent controlled genes of *B. subtilis* are characterized by iATP, and these genes are presumably activated because of an increased ATP pool [4]. Previously, the transcriptional starting points of the α- and β-psms were identified as an adenine ribonucleotide [32]. To analyze whether induction of phenol-soluble modulins in the wild-type strain correlates with an increased ATP pool, the intracellular ATP concentration was measured (Fig. 4). A significant increase of the intracellular ATP pool was detectable upon amino acid deprivation in all strains. However, no significant difference was found between the strains analyzed (Fig. 4). Thus, the ATP levels cannot explain the observed differences in psm expression between wild-type and rshSyn mutant.

RSH-dependent survival in polymorphonuclear leukocytes (PMNs)

Since α-type PSMs are strongly involved in the survival of *S. aureus* upon PMN treatment [31,33], we analyzed whether there is an association between phagocytosis and the RSH-dependent stringent response. Comparison of published data revealed that the transcription pattern during neutrophil phagocytosis (Voyich et al., 2005) is similar to that observed after amino acid starvation and/or mupirocin treatment [13,20,21], namely down-regulation of the translational machinery and de-repression of CodY regulated amino acid transporters and biosynthesis genes. Hence, we hypothesized that phagocytosis may induce an RSH-dependent stringent response, similar to the depletion of amino acids. Under these conditions, the (p)pGpp accumulation should result in increased PSMs synthesis. Thus, an rshSyn mutant, unable to transcribe psmS, might be more sensitive to phagosomal killing. Indeed, the PMN bactericidal killing assay revealed that the rshSyn mutant is significantly less able to survive after phagocytosis compared to the wild-type strains (Fig. 5A). This effect could be reversed by the introduction of full-length rsh into the chromosome of the rshSyn mutants. A comparison of the two strains (HG001 and Newman) with their respective mutant derivatives revealed similar results. Also an assumed contribution of relP and relQ could be excluded, since a relP/Q double mutant showed no decreased survival phenotype compared to the wild-type strain (data not shown).

To further analyze whether reduced intracellular psm expression solely accounted for the rshSyn defect in survival, we constructed a psmα1-4, psmβ1-2, double mutant and an agr mutant, known to be defective in psm expression. The phenotype of the psmα, psmβ double mutant and the agr mutant closely resemble that of the rshSyn mutant concerning intracellular survival after phagocytosis (Fig. 5B). Of note, there were no significant differences between the psmα, psmβ double and the rshSyn psmα, psmβ triple mutant, as well as between the agr single and the rshSyn agr double mutant, indicating that there are no additive effects of these mutations. We speculate that survival of phagocytosis is mediated by intracellular psm expression mediated by RSH dependent (p)pGpp synthesis.

To address this assumption, we used a plasmid, in which psmα1-4 and psmβ1-2 were cloned behind a tetracycline inducible promoter, to induce psmα or psmβ expression in the rshSyn mutant after phagosomal uptake. The intracellular induction of psmα and psmβ could significantly complement the reduced survival of the
rsh\textsubscript{Syn} mutant (Fig. 5C). Interestingly, the survival of the rsh\textsubscript{Syn} \textit{agr} double mutant was not complementable, neither by \textit{psm}a nor by \textit{psm}b expression.

We also performed PMN lysis assays by measuring the amount of released lactate dehydrogenase (LDH). Accordingly the mutants which are defective in \textit{psms} expression were shown to be less toxic compared to the wild type strain (Fig. 5D).

These results indicate that the RSH-mediated stringent response in \textit{S. aureus} plays a major role in survival PMN phagocytosis most probably through regulation of intracellular \textit{psm} expression.

**Stringent response during phagocytosis**

To analyze whether an RSH-dependent stringent response is induced during phagocytosis, we performed quantitative RT-PCRs from bacterial RNA obtained after 60 and 90 minutes of phagocytosis. At this stage, typically all of the bacteria are internalized [34]. We analyzed transcripts of typical genes of each of the three regulatory categories relative to the constitutive \textit{gyrB} transcript (Fig. 6). For category I, the transcripts of \textit{rpsB}, coding for ribosomal protein S2, and \textit{infB}, coding for initiation factor 2 were chosen. As expected, the \textit{rpsB} and \textit{infB} transcription is significantly lower in the wild-type and complemented strain than in the rsh\textsubscript{Syn} mutants (rsh\textsubscript{Syn} single and rsh\textsubscript{Syn} \textit{codY} double mutant) (Fig. 6). In contrast, \textit{ilvC} transcription (category II gene) is significantly higher in the wild-type compared to the rsh\textsubscript{Syn} mutant. Here, comparable to results of the microarray analysis, an additional \textit{codY} mutation in the rsh\textsubscript{Syn} mutant could restore \textit{ilvC} transcription. For category III, \textit{psm}1-4 and \textit{psm}b1,2 transcripts were analyzed. The wild-type and the complemented rsh\textsubscript{Syn} strain showed significantly higher transcription compared to rsh\textsubscript{Syn} mutants after incubation for 60 or 90 min with phagocytes. These results are consistent with the assumption that an RSH-mediated stringent response is induced after phagocytosis in wild-type bacteria. Of note, low expression of \textit{psms} within the PMNs was also observed in the rsh\textsubscript{Syn} \textit{codY} double mutant, indicating that intracellular \textit{psm} regulation occurs independently of \textit{CodY}.

One may speculate that (p)ppGpp affect somehow the activity of the response regulator AgrA in the phagolysosomes thereby activating \textit{psm} expression. Thus, we analyzed the intracellular expression of the prototypic AgrA target transcript RNAIII. However, no significant alteration of RNAIII transcription could be detected in the rsh\textsubscript{Syn} mutants after 60 and 90 minutes of incubation (Fig. 6). In fact, the rsh\textsubscript{Syn} \textit{codY} double mutant showed a slight increase of the RNAIII transcription compared to wild-type strain, which is in line with published data showing that \textit{agr} transcription is slightly repressed by \textit{CodY} [15,35]. These results indicate that stringent response is induced during phagocytosis which is required for \textit{psm} expression. The reduced transcription of \textit{psm}1-4 in the rsh\textsubscript{Syn} mutant and rsh\textsubscript{Syn} \textit{codY} double mutant after phagocytosis is clearly not caused by diminished AgrA activity.

**Discussion**

A conserved “core regulon” is repressed during stringent response

To adapt to changing environmental conditions, bacteria rely on sensory and regulatory systems to modulate complex physiological processes. The stringent response is a highly conserved regulatory mechanism that is provoked by nutrient limitation. It is effective in most bacteria and is mediated by the rapid synthesis of the alarmones ppGpp or pppGpp. Previously, it was shown that, in \textit{S. aureus}, the RSH enzyme alone is responsible for (p)ppGpp accumulation upon amino acid deprivation [13]. Here, we...
Figure 5. Phagocytosis killing assays and a PMN lysis assay with different *S. aureus* strains. (A) and (B) Survival of wild-type *S. aureus* strain HG001 and strain Newman (WT), and their isogenic rsh<sup>syn</sup> mutants, complementation strains (compl), psm<sub>a</sub>, psm<sub>b</sub> double mutants (HG001), rsh<sup>syn</sup>, psm<sub>a</sub>, psm<sub>b</sub> triple mutants (HG001), agr mutant (HG001) and rsh<sup>syn</sup>, agr double mutant (HG001) were determined after phagosomal uptake by counting colony-forming units after 60 min incubation. Bacterial cells used for the experiment were harvested at similar points of growth at an OD<sub>600</sub> of 1.0. The levels of significance were determined by the two-tailed Student t test (p<0.05). (C) Complementation of survival of the rsh<sup>syn</sup> mutant with tetracycline inducible psm<sub>a</sub> and psm<sub>b</sub>. Therefore membrane permeable anhydrotetracycline (ATc) 0.1 µg/ml was used to induce psm expression after phagosomal uptake of the bacteria. The levels of significance were determined by the two-tailed Student t test (p<0.05). (D) Lysis of PMNs, as measured by release of lactate dehydrogenase (LDH) activity. PMN lysis was determined after a phagocytic interaction of 60 min with *S. aureus*.
characterized the stringent response of *S. aureus* in a more comprehensive way. So far, no microarray studies, in which (p)ppGpp synthase mutants were compared with wild-type strains under stringent conditions, are available for *S. aureus*. For other bacteria only a few microarrays of this type were published [24,25,26,36,37]. We identified 161 genes that were significantly repressed via RSH. Most of these genes are hallmarks of the stringent response also appearing in other bacteria that have been analyzed. This conserved “core-regulon” mainly encompasses genes of the translational machinery, including initiation and elongation factors. Furthermore, genes coding enzymes of physiological processes used by dividing cells, like biosynthesis and nucleotide transport, are typically under negative stringent response. Thus, negative regulation by (p)ppGpp seems to be an evolutionarily conserved mechanism. However, the molecular mechanism by which (p)ppGpp leads to the inhibition of these genes remains mostly unclear but seems to be organism dependent [4,5,30,39]. For firmicutes it was proposed that genes starting with an iGTP are repressed due to the lowering of the GTP pool [4]. We have shown previously that also in *S. aureus* stringent condition lead to lowering of the GTP level [13]. We have mapped the transcriptional start site of two rRNA operons of *S. aureus* (data not shown) and could confirm that the primary promoters initiate with iGTP which is in line with the mechanism proposed by Krasny et al., 2008 [4].

**CodY - RSH interaction**

Induction of the stringent response also leads to the activation of genes proposed to be necessary for survival and maintenance. For *E. coli*, it was recently shown that most genes that are positively influenced during the stringent response are, depending on the (p)ppGpp concentration, either part of the Lrp (transcriptional regulator) or RpoS (alternative sigma factor) regulon [40]. So far, there is little indication that alternative sigma factors are involved in the stringent response in gram-positive bacteria. Instead in *S. aureus*, most of the genes that are less expressed in the *rshSyn* mutant

**Figure 6. Quantitative RT-PCRs of phagocytosed bacteria.** Transcript analyses of *rpsB*, *infB* (category I), *ilvC* (category II), *psmA1-a4*, *psmB1-2* (category III) and RNAIII genes after phagocytosis of the *S. aureus* strain HG001 and the corresponding *rshSyn* mutant, *rshSyn*/*codY* double mutant and the complemented *rshSyn* mutant (compl). The transcripts were relatively quantified in reference of *gyrB* and are shown in a log10 scale relative to the wild-type strain (HG001). RNA was isolated after a 60 and 90-minute interaction of *S. aureus* with PMNs. Values from four separate experiments were used to calculate the mean expression. The levels of significance compared to the wild-type strain were determined by the two-tailed Student t test (p<0.05).

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compared with the wild-type strain are part of the previously described CodY regulon [15,16]. For these genes, introduction of a codY mutation into the rshS~mutant leads to an expression pattern that is similar to that of the wild-type strain. Thus, these genes are de-repressed upon the stringent response. An obvious link between the stringent response and CodY is the GTP pool. Amino acid deprivation leads to a lowering of the GTP pool [13], which in turn may inactivate the CodY repressor. A similar link between the stringent response and CodY was also shown for Listeria monocytogenes [10] and B. subtilis [24]. In contrast, in Streptococci, CodY and the stringent response seem to act independently of each other [23,41]. This discrepancy is most likely due to species specific differences in GTP affinity of CodY [42]. In lactococci and streptococci branched-chain amino acids (e.g., isoleucine), act as the only known ligand that mediates the repressive function of CodY.

Notably, not all genes of the known CodY regulon seem to be affected under the induced stringent condition, analyzed here. Interestingly, virulence genes like the capsular gene cluster or the agr operon, which were found to be repressed via CodY [15,16], did not appear to be affected during the stringent response. One explanation might be that the deactivation of the CodY repressor in the wild-type strain due to amino acid limitation is not as effective as knocking out the complete codY gene. Alternatively, the different CodY target genes may differ with regard to their sensitivity to CodY activity. One may assume that some of the CodY target genes are still repressed even under low GTP conditions as long as the primary CodY ligand isoleucine is present. This assumption is inline with recent findings that in B. subtilis, CodY targets are differentially sensitive to alteration in the GTP pool: the ibC promoters but not the boaP promoter was derepressed in a mutant not able to synthesize GTP [43].

Regulation of PSMs

Only seven genes were positively regulated under the stringent response independently of CodY, including those coding for the cytotoxic phenol-soluble modulins (PSMs). So far, psm transcription was shown to be directly activated via the response regulator AgrA [32]. We could show that AgrA is still essential for the stringent controlled psmX1-4 and psmB1,2 transcription, since no psm transcripts were detectable in an agr mutant strain. However, the induction appears not to be due to altered agrA activity, since agrA expression was not found to be affected during the stringent response. Also after phagocytosis only psm transcripts but not the Agr effector molecule RNAIII are diminished in the rshS~mutant. How (p)pGpp regulates the transcription of PSMs remains unknown. Additional regulatory elements upstream of the promoter region of psm genes [32] could possibly take part in the (p)pGpp regulation, presumably mediated by unknown factor(s). In our microarray analysis, no apparent regulatory systems appeared to be affected, and thus, no candidate molecule that may indirectly be involved in (p)pGpp dependent activation could be identified. Small molecules like (p)pGpp or GTP may interact directly with proteins altering their enzymatic activity or binding affinities to DNA similar to the proposed GTP-CodY interaction. One may speculate that such an interaction may also alter the activity of other regulatory molecules such as the transcriptional regulator AgrA (Fig. 7).

Alternatively, it was postulated that under stringent conditions, the iNTP determines the transcription of the gene followed [4]. psm genes were shown to start with an iATP [32]. In B. subtilis, genes starting with iATP were predicted to be up-regulated after amino acid limitation through an increase of the intracellular ATP pool [4,7]. However, this increase in ATP is obviously indepen-
dent of the RSH-dependent (p)pGpp synthesis, since we found a similar increase in the rshS~mutant which is in line with results obtained in B. subtilis (Tojo et al., 2000). Thus, increasing the ATP pool is not sufficient to trigger induction of the PSMs since no enhanced psm transcription was observed in the rshS~mutant despite a similarly elevated ATP pool than the wild-type and rshS~complemented strain.

Stringent response during phagocytosis

Our results indicate, by taking the example of a gene coding for a ribosomal protein S2 (rpsB) and initiation factor 2 (infB), that the down-regulation of translational proteins after phagocytosis is mediated via rsh activity (Fig. 6). Together, with the lower expression of ibC, psmX1-4 and psmB1,2 in the rshS~mutants, these findings suggest that the stringent response is induced after phagocytosis (Fig. 7). This result is supported by the global transcriptional analyses of the S. aureus strains after uptake by professional [34] and non-professional [44] phagocytes. A transcription pattern very similar to those described, was found here especially down-regulation of typical hallmark genes of the stringent response, i.e., ribosomal proteins and up-regulation of amino acid biosynthesis genes.

However, the intracellular signal for an RSH mediated stringent response remains speculative. For S. aureus, so far, only amino acid limitation was shown to induce (p)pGpp synthesis via RSH. We also analyzed other conditions such as glucose starvation as potential signals for RSH dependent (p)pGpp synthesis but found no detectable (p)pGpp accumulation (data not shown). Thus, these findings suggest that amino acid limitation triggers the response within phagocytes. Moreover, in L. monocytogenes, a gram-positive intracellular pathogen, the amino acid limitation sensing RSH was shown to be essential for survival and replication in macrophages [10]. However, this finding is in contradiction to results obtained from gram-negative intracellular pathogens. Here, the authors concluded that no amino acid limitation occurs in infected host cells. This conclusion is based on the finding that RelA, the enzyme solely responsible for accumulation of (p)pGpp upon amino acid limitation in gram-negative bacteria, is not activated [17,45,46,47,48]. For these bacteria, the bifunctional (p)pGpp synthase SpoT seems to be more important for adaption to the intracellular environment. It was presumed that balancing the basal levels of (p)pGpp (due to the hydrolase and synthase activity of SpoT) is essential for intracellular adaptation [46]. There may be major differences between gram-negative and gram-positive bacteria concerning the mechanisms of signalling and responses to intracellular environments. Interestingly, treatment with azurophilic granule proteins [49] imposed transcriptional changes similar to those of the stringent response. It has to be elucidated whether parts of the reactive oxygen species (ROS) in phagolysosomes may be a trigger for the stringent response in S. aureus.

PSM expression contributing to survival in phagocytes

The predominant role of S. aureus PSMs in killing human neutrophils was previously shown [31]. The reduced survival of the rshS~mutant after phagocytosis is probably due to a decreased expression of PSMs within the phagolysosome. No other candidate virulence gene which could potentially account for this observation appeared in our microarray analysis to be down-regulated in the rshS~mutant. Comparing the survival of an rshS~mutant to a psmX, psmB double mutant or an agr mutant reveals a similar low survival rate which is in line with published data [33]. The distinct role of psm expression for survival could be demonstrated by complementation assays of the rshS~mutant with tetracycline.
induced \textit{psm} \textit{a} or \textit{psm} \textit{b}. The fact that the reduced survival of an \textit{rib} \textit{syn}, \textit{agr} double mutant was not complementable by \textit{psm} expression indicates that probably additional \textit{agr} dependent factors are needed to survive after phagocytosis.

Little is known about the intracellular behaviour of \textit{S. aureus}. Growing evidence suggests that \textit{S. aureus} can survive after phagocytosis and persists in neutrophils or macrophages to hide from the immune system, as well as to travel to and infect distant sites in the host [50,51,52]. However, the importance of single virulence factors contributing to intracellular survival seems to be dependent on the type of host cell and bacterial strains analyzed. For instance, \textit{S. aureus} can escape from the phagoendosomes of human epithelial and endothelial cells into the cytosol, which is mediated by \textit{\delta}-toxin, \textit{\beta}-toxin and \textit{\beta}-PSMs [53]. PSMs may similarly contribute to escape from the more toxic phagolysosomes of neutrophils and thereby allow intracellular survival. Whether they also contribute to a lysis of phagocytes from the inside remains to be shown. This was recently proposed based on observations that PSMs are efficiently inhibited by human serum and therefore lyse neutrophils rather from the inside than from the

Three postulated consequences:
1. Destruction of the phagosome, escape and survival in the cytosol
2. Phagosomal escape and lysis of the phagocytes from the inside
3. Induction of programmed necrosis of the phagocytes (55)

Figure 7. Overview of the presumed impact of the stringent response on survival after phagocytosis. The induction of the stringent response (pppGpp) in phagocytosed bacteria leads to increased \textit{psms} expression. The PSMs in turn mediate a pronounced survival after phagocytosis, most likely through phagosomal escape or intracellular lysis of the phagocytes.

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outside [54]. These authors could also show that psm promoter activity is strongly induced after phagosomal uptake. Moreover there could be an indirect impact of PSMS on the lysis of phagocytes. Previously it could be shown that different clinical isolates of strain USA300 induced a programmed necrosis of PMNs [53]. One may assume that intracellular expressed PSMS thereby could play an important role for this induction.

The results we obtained are significantly important for the question how and where PSMS can act as potent cytolytic molecules. The data of the current study support the conclusion that in phagolysosomes a stringent response is activated and that the activity of the (p)pGpp synthase RSH is essential for the intracellular induction of pmsA1-4 and psmA1-2 expression. These cytolytic peptides in turn are responsible for the ability of S. aureus to survive after phagocytosis (Fig. 7). The signalling mechanisms leading to the activation of the (p)pGpp synthases as well as the mechanism leading to gene activation through (p)pGpp after phagocytosis is largely unknown and needs further examination.

Materials and Methods

Strains and growth conditions

Strains and plasmids are listed in Table S1. S. aureus strains were grown in CYPG (10 g/l casein acids, 10 g/l yeast extract, 5 g/l NaCl, 0.5% glucose and 0.06 M phosphoglycerate) [56] or in a chemically defined medium (CDM) [15]. For strains carrying tetracycline, erythromycin or chloramphenicol resistance genes, antibiotics were used only in precultures at concentration of 5 μg/ml for tetracycline, 10 μg/ml for erythromycin and 25 μg/ml for chloramphenicol, respectively. Bacteria from an overnight culture were re-suspended in an equal volume of CDM and grown for another 30 minutes. For serine starvation experiments, bacteria were re-suspended in an equal volume of sterile phosphate buffered saline (PBS) and bacteria were re-diluted to an initial optical density at OD600 of 0.05 in 25 ml fresh medium containing serine hydroxamate (SHX, 1.5 mg/ml) and grown for 30 minutes.

Determination of haemolytic activities on sheep blood agar plates

The haemolysis test was performed as described previously [29]. Briefly, bacteria to be tested are streaked at a right angle to strain RN4220 and the plate was incubated overnight. β-hemolysin of strains to be tested form a zone of clear haemolysis (synergistic effect) on blood agar plates.

Construction of mutant strains and complementation

The rshsyn cod1 double mutant was obtained by transducing the cod1::tet(K) mutation into S. aureus strain Newman rshsyn [13] using Φ11 lysates of strains RN4220-21 [15]. Transductants were verified by PCR and PFGE. For complementation the full length rsh gene with a 900 bp upstream region was transduced into strain Newman rshsyn (Table S1) using Φ11 lysates of strain CXL316-199 [13]. The psmA and psmB mutants were obtained by replacing pmsA1-4 with a tetracycline resistance cassette and psmB1-2 with an erythromycin resistance cassette using a newly developed temperature-sensitive shuttle vector pBASE6. This vector is based on the expression of S. aureus secY regulatory unit. Since some of the restriction sites of the pBT2 multiple cloning site (MCS) are also present in the introduced pKOR1 part, the MCS was ligated into the EcolI-HindIII digested vector, resulting in pBASE6.

For gene replacements, two fragments flanking the pmsA1-4, psmB1-2 locus, the tetracycline and erythromycin resistant cassette were amplified and annealed by overlapping PCR to generate the pmsA1-4-tetM and psmB1-2-ermC mutagenesis vectors pCG307 and pCG308, respectively. The amplicons were cloned into the EcoRI/ SalI restriction sites of pBASE6. These plasmids were used to mutagenize strain RN4220 as described previously [13]. The obtained psm gene replacement mutant strains (RN4220-307 and RN4220-308) were verified by PCR. In the mutants the whole pmsA1-4 operon respectively psmB1-2 operon was replaced by the corresponding resistant cassette. The rshsyn, psmA, psmB triple mutant was obtained by transducing the psmB::erm(C) and psmA::tet(M) mutations into S. aureus strain HG001 rshsyn using lysates of strain RN4220-307 and RN4220-308.

RNA isolation

RNA isolation for microarray analysis and northern blot analysis was performed as described previously [59]. Briefly, bacteria were lysed in 1 ml of Trizol reagent (Invitrogen Life Technologies, Karlsruhe, Germany) with 0.5 ml zirconia-silica beads (0.1 mm in diameter) in a high-speed homogenizer (Savant Instruments, Farmingdale, NY). RNA was isolated as described in the instructions provided by the manufacturer of Trizol.

RNA isolation after phagocytosis was performed as described in the instructions provided by the manufacturer of the RNA isolation kit (ExpressArt RNAready, AmpTec) with the modification adding an inhibitor removal buffer (high pure viral nucleic acid kit, Roche diagnostics) in the first step. Then DNA digestion was performed as instructed by the RNA isolation kit.

Northern blot analysis

Northern blot analyses were performed as described previously [59]. Digoxigenin-labeled probes for the detection of specific transcripts were generated using a DIG-Labeling PCR Kit following the manufacturer’s instructions (Roche Biochemicals). Oligonucleotides were used for probe generation as described previously [13,15,60] or are listed in Table S2.

Reverse transcription and quantitative real-time PCR

Relative quantifications of α-type psm genes, β-type psm genes, rpsB, RNAIII and gyrB transcripts were performed using LightCycler instrument (Roche). Briefly, RNA isolated from cultures after phagocytosis (60 and 90 min) was transcribed into complementary DNA using SuperScriptIII Reverse Transcriptase (Invitrogen) and 200 ng of random hexamer primers (Fermentas). Complementary DNA was diluted 1:5 and quantitative real-time PCR was performed using the QuantFast SYBR Green PCR Kit (Qiagen). A standard curve for each gene was generated using 5-fold serial dilutions of wild-type HG001 cDNA at timepoint 0 h (oligonu-
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did see Table S2). Statistical analysis was performed with the Prism software package (version 5.0; GraphPad) using the Student t test two-tailed analysis (p<0.05).

**Quantitative measurement of the intracellular ATP pool**

*S. aureus* wild type HG001, isogenic mutants and the complemented strain were grown in CDM to OD_{600}=0.5. Cells were shifted to CDM with and without leu/val as described above. Samples for intracellular ATP analysis were harvested 30 min after the shift by fast filtration over a 0.22 μm sterile filter applying vacuum. Cells were washed, quenched and nucleotides were extracted as described recently [61,62]. The extracts were resuspended in 5 ml of 0.1 M Tris-acetate buffer (pH 7.75) and stored at −80 °C. The detection of ATP was performed by the Enlighten ATP assay system using luciferase and luciferin (Promega). Therefore nucleotide extracts were diluted 1:100 in Tris-acetate buffer and 10 μl mixed with 90 μl of the ATP assay reagents. Luminescence was measured with a luminescence detector reader (Infinite M200Pro, Tecan, Austria). The standard curve was generated by using known concentrations of ATP. The results of the intracellular ATP measurements represent the mean of 2 biological replicates measured in triplicates. Statistical analysis was performed with the Prism software package (version 5.0; GraphPad) using the Student t test two-tailed analysis (p<0.05).

**Microarray manufacturing and microarray design**

The microarray was manufactured by in situ synthesis of 60-base-long oligonucleotide probes (Agilent, Palo Alto, CA), selected as previously described [63]. The array covers >98% of all open reading frames (ORFs) annotated in strains N315 and Mu50, MW2, COL, NCTC8325 and USA300, and MRSA252 and MSSA476, as well as Newman, including their respective plasmids.

**Preparation of labeled nucleic acids for expression microarrays**

Total RNA was purified from strain HG001 WT, rshS{n} mutant, codT mutant and rshS{n} codT double mutant grown in CDM to an OD_{600} of 0.5. For each strain RNA of three independently grown cultures was analyzed. After additional DNase treatment, the absence of remaining DNA traces was confirmed by quantitative PCR (SDS 7700; Applied Biosystems, Framingham, MA) with assays specific for 16S rRNA [15]. Batches of 5 μg of total *S. aureus* RNA were labeled by Cy3-dCTP using SuperScript II (Invitrogen, Basel, Switzerland) following the manufacturer’s instructions. Labeled products were then purified onto QiaQuick columns (Qiagen). Purified genomic DNA from the different sequenced strains used for the design of the microarray was extracted (DNeasy; Qiagen), labeled with Cy5-dCTP using the Klenow fragment of DNA polymerase I (BioPrime, Invitrogen, Carlsbad, CA), and used for the normalization process [64] Cy3-labeled DNA (500 ng) and a Cy3-labeled cDNA mixture were diluted in 50 μl of Agilent hybridization buffer and hybridized at a temperature of 60 °C for 17 h in a dedicated hybridization oven (Robbins Scientific, Sunnyvale, CA). Slides were washed, dried under nitrogen flow, and scanned (Agilent, Palo Alto, CA) using 100% photon multiplier tube power for both wavelengths.

**Microarray analysis**

Fluorescence intensities were extracted using Feature Extraction software (version 9; Agilent). Local background-subtracted signals were corrected for unequal dye incorporation or unequal load of the labeled product. The algorithm consisted of a rank consistency filter and a curve fit using the default LOWESS (locally weighted linear regression) method. Data consisting of three independent biological experiments were expressed as log 10 ratios and analyzed using GeneSpring, version 8.0 (Silicon Genetics, Redwood City, CA). A filter was applied to select oligonucleotides mapping ORFs in the Newman genome, yielding approximately 95% coverage. Statistical significance of differentially expressed genes was calculated by analysis of variance [65] using GeneSpring, including the Benjamini and Hochberg false discovery rate correction of 5% (p value cutoff, 0.05) and an arbitrary cutoff of twofold for expression ratios.

**Microarray data accession number**

The complete microarray data set has been posted on the Gene Expression Omnibus database [http://www.ncbi.nlm.nih.gov/geo/] under accession number GSE99340 for the platform design and GPL7137 for the original data set.

**PMN bactericidal activity**

Killing by human neutrophils was performed as described previously [66]. Briefly, bacteria of the logarithmic phase were washed and adjusted in potassium phosphate buffer (10 mM K_{2}PO_{4}). Neutrophils were isolated from peripheral blood of healthy volunteers by ficoll/histopaque gradient centrifugation as described previously [67] and resuspended in HBSS-HSA (hank’s balanced salt solution, Sigma, containing 0.05% human serum albumin). Bacteria were opsonized by addition of pooled human serum (Sigma) to a final concentration of 10%. Opsonized bacteria (10^7/ml) and neutrophils (10^6/ml) were combined to a volume of 500 μl and samples were shaken at 37 °C. For the psm complementation assays, neutrophils and bacteria were preincubated for 30 minutes without anhydrotetracycline (ATc) followed by addition of 0.1 μg/ml ACT for another 30 minutes. Aliquots were diluted in ice-cold water and vortexed vigorously to disrupt the neutrophils and halt bacterial killing. Appropriate dilutions were plated on tryptic soy agar plates and incubated at 37 °C for the following day for enumeration of CFU. The percent of bacterial survival was calculated with the equation CFU_{PMN} at t_{0}/CFU_{PMN} at t_{60}. Statistical analysis was performed with the Prism software package (version 5.0; GraphPad) using the Student t test two-tailed analysis (p<0.05).

**S. aureus-induced PMN lysis**

Following phagocytosis of *S. aureus*, lysis of human neutrophils was determined with a standard assay for release of lactate dehydrogenase (LDH) as described by the manufacturer (Cytotoxicity Detection Kit; Roche Applied Sciences). Statistics were performed with the Prism software package (version 5.0; GraphPad) using the Student t test two-tailed analysis (p<0.05).

**Supporting Information**

Table S1 Strains and plasmids used in the study. (DOC)

Table S2 Oligonucleotides used for the construction of hybridisation probes and for qRT-PCR. (DOC)

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Author Contributions
Conceived and designed the experiments: T. Geiger, C. Goerke, C. Wolz. Performed the experiments: T. Geiger, P. Francois, M. Liebeck. Analyzed the data: T. Geiger, P. Francois, M. Liebeck. Contributed reagents/materials/analysis tools: T. Geiger, P. Francois, M. Liebeck, M. Fraunholz, B. Krismer, J. Schrenzel, M. Lalk. Wrote the paper: T. Geiger, C. Wolz.

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