Expression of rpoS, ompA *and* hfq *genes of* Cronobacter sakazakii *strain Yrt2a during stress and viable but nonculturable state*

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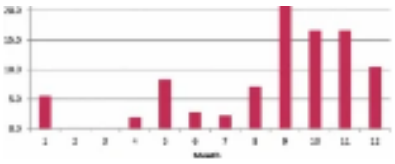

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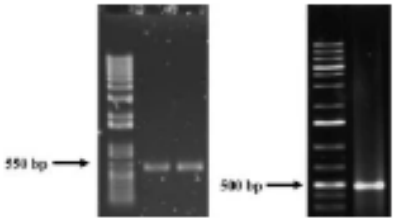

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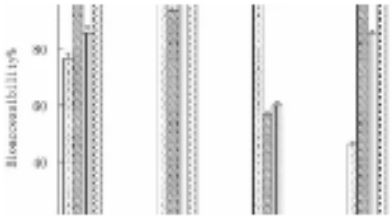

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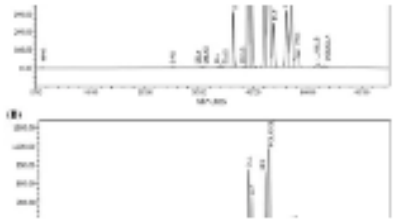

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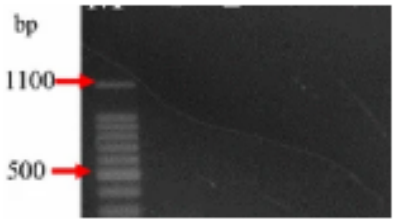

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Expression of *rpo*S, *omp*A and *hfq* genes of *Cronobacter sakazakii* strain Yrt2a during stress and viable but nonculturable state

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Abstract *Cronobacter* spp. in powdered infant formula has been etiologically linked to meningitis and necrotizing enterocolitis in certain groups of infants. This study aimed to determine whether *C. sakazakii* Yrt2a strain experiencing desiccation stress could enter viable but nonculturable (VBNC) state as well as to examine the expression of genes associated with stress and virulence during the above states. Stress and VBNC conditions were determined based on viability and culturability assays. Expression of genes related to stress (*rpo*S) and virulence (*hfq* and *omp*A) was evaluated by real-time PCR. The results showed that *C. sakazakii* Yrt2a entered VBNC 24 days post exposure to 2 h of desiccation treatment. The expression of *rpo*S, *hfq* and *omp*A genes was up-regulated during stress conditions, suggesting that *Cronobacter* successfully managed stress to maintain its culturability while maintaining its virulence. The expression of the target genes decreased at VBNC state but remained higher than that of a normal state. These findings reinforce the assumption that *C. sakazakii* undergoing VBNC state maintains its pathogenicity.

Keywords *Cronobacter sakazakii* · *rpo*S · *omp*A · *hfq* · VBNC state

Introduction

Various technologies have been developed to reduce, kill, or control microorganisms in order to achieve safe food. However, this process could cause bacteria to enter a starvation mode of metabolism and transform into viable but nonculturable (VBNC) state [1]. The VBNC state raises its own problems, especially when conventional detection was done and showed negative results due to the absence of typical colonies in the test medium. In addition, other publications reported that VBNC cells could still be harmful and express their virulence genes [2].

Cronobacter spp. has been isolated from various dried food samples in Indonesia, such as powdered infant formula (PIF), weaning foods, and cornstarch [3, 4]. In addition, *Cronobacter sakazakii* has been isolated from dry foods in several developed and developing countries [5, 6]. Many *C. sakazakii* demonstrate their ability to grow and survive under stress conditions better than other species in *Enterobacteriaceae* family [7]. Another study showed that *C. sakazakii* has persisted in dried food (Aw 0.25–0.30) longer than in other foods (Aw 0.69–0.82), and showed resilience at low temperature (4 °C) [8].

As described earlier, general difficult conditions in food processing may cause bacteria to experience stress conditions which may result in bacteria entering VBNC state. The occurrences of VBNC cells in *C. sakazakii* were less reported. Recently laboratory studies on *C. sakazakii*

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showed that these bacteria could enter VBNC during biofilm formation in a nutrient-limited medium on stainless steel surfaces [9]. Stress and VBNC conditions in bacteria have been studied at the genomic level, transcriptomic study, and gene expression [1]. However, studies on the gene expression of *C. sakazakii* during stress and VBNC states have not been reported. The *rpo*S gene is known as the main signal regulating the general stress responses in *C. sakazakii* [7] and as the controlling gene for activating 18 of 22 genes [10]. Meanwhile, *hfq,* an RNA chaperone gene, plays important role in stress adaptation and virulence of *C. sakazakii* [11]. The gene also regulates the expression at the post-transcriptional level [12]. OmpA protein has been associated with the ability of *C. sakazakii* to adhere and invade the host cells [13]. The protein is required by *Cronobacter* sp. cells for invasion to human brain microvascular endothelial cells [14]. This research aims to find out whether desiccation stress in *C. sakazakii* could lead to VBNC state and affect the expression of stress gene (*rpo*S) as well as virulence (*omp*A and *hfq*) genes.

Materials and methods

Bacterial strains

C. sakazakii (Yrt2a strain) was previously isolated from PIF [3]. The frozen stock of *C. sakazakii* Yrt2a was revived by placing one loop of suspension into the Brain Heart Infusion (BHI) broth followed by 18 h incubation at 35 °C. After incubation, one loop was streaked onto a Druggan-Forsythye-Iversen (DFI) agar plate and confirmed as *C. sakazakii* as blue-green pigmented colonies on DFI agar medium. The culture was made by inoculating one colony from DFI agar medium into 10 ml BHI broth (Oxoid, UK) and incubating it for 18 h at 35 °C to reach ca. 10^9 CFU/ml.

Detection of *rpo*S, *omp*A, and *hfq* genes

Detection of the target genes began with selection of specific primers to be used in this study (Table 1). The primers used in this study were obtained from the primer BLAST on NCBI website. The extracted genomic DNA was used as a template for PCR reaction to confirm the presence of the target genes and specificity of the primers used. A primer is considered specific when it show a single positive band with a specific size of the target gene amplicon. Isolation of the bacterial DNA was carried out according to previous studies [4].

The PCR reaction mixture (20 µl) was made of 2 µl DNA extract (100 ng/µl), 10 µL PCR master mix (2X) buffer (Thermo Fisher Scientific, Massachusetts, USA), 1 µl of each of the primers (0.5 µM final concentration), and an appropriate volume of nuclease-free water (NFW) (Thermo Fisher Scientific, Massachusetts, USA). Thermal cycling was performed on a PCR *Thermal Cycler* 2720 (Applied Biosystems, California, USA) via an initial denaturation step at 94 °C for 3 min followed by 30 cycles at 95 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 5 min. The amplification products were analyzed with electrophoresis on 2% agarose gel and examined under UV light (Bio-Rad Laboratories Pte. Ltd, Singapore).

Desiccation stress

Desiccated *C. sakazakii* cell suspensions were obtained as described by previous study [15] with minor modification. One ml of late log phase (18 h) culture of *C. sakazakii* was diluted to obtain a culture concentration of 10^7 CFU/ml. The diluted culture (1 ml) was each spread onto several empty Petri dishes and let dry. The Petri dishes were kept without lid and placed in 40 °C incubator with silica gel to facilitate desiccation. After 2 h of desiccation, the plates were covered and further stored at room temperature. Before and after desiccation treatment as well as at day 2, 4, 6, 10, 14, 19, 24 of storage, samples were taken for viability and culturability assays. Observations were carried out until no colony was found on the culturability assay.

Viability and culturability assays

At timed intervals, i.e. before and after 2 h of desiccation and 2, 4, 6, 10, 14, 19, 24 days of storage post desiccation, cell viability and culturability were evaluated. Cell viability was observed by direct viable count (DVC) [16] using a microscope fluorescence while culturability assay was observed by total plate count (TPC) spread plate method [17]. The DVC was conducted by incubating the desiccated cells on Petri dishes in submersion solution for 18 h (dark conditions). The submersion solution was 2 ml mixture (1:1) of yeast extract (0.025%) (Oxoid Ltd., UK) and nalidixic acid (0.002%) (Sigma-Aldrich, USA). After 18 h, the Petri dish was rinsed with saline solution (SS or NaCl 0.85%) and 2 ml of acridine orange (0.0026%) (Sigma-Aldrich) staining was added. The Petri dish was then incubated at room temperature for 5 min (dark conditions) and then washed again with SS. Cell viability was observed using a fluorescence microscope (Olympus CH3O Binocular Quadruple, Olympus Corporation, Center Valley, USA) and the number of viable bacteria (green fluorescence cells) was calculated by counting a minimum of 10 fields in triplicate. The viable cells, expressed as number of cells/cm^2, was converted into log cells/ml.

Table 1 Description of primers used in this study

Target gene	Sequences (5′ → 3′)	Gene position	Amplicon (bp)[a]	Tm of amplicon (°C)
16s rRNA	*f:* CGGACGGGTGAGTAATGTCT	105-124	194	84
	r: CTCAGACCAGCTAGGGATCG	298-279		
hfq	*f:* TGCAAGATCCGTTCCTCAAC	20-39	209	82
	r: CACCCGCGTTGTTACTATGG	229-210		
*omp*A	*f:* TGAGCAACCTGGATCCGAAA	737-756	155	83
	r: GGAGATCTTGTTGGACGGGA	891-872		
*rpo*S	*f:* TCGAACGGGCAATCATGAAC	461-480	136	81
	r: GCAATCTCTTCTGCACTCGG	596-577		

[a]Gene bank of NCBI

The TPC was observed by enumerating colonies on TSA (Oxoid Ltd., UK). As much as 2 ml of a BPW-Na hexametaphosphate (Oxoid Ltd) was added onto each Petri dish in order to collect the desiccated cells and mixed with 8 ml BPW-Na hexametaphosphate. The aliquots of appropriate dilutions of the samples were plated in duplicate on TSA followed by incubation at 37 °C. At the end of the incubation, the surviving colonies were enumerated and the number of colonies per dish area was converted into log CFU/ml. For the purpose of this study, stress condition is defined when the number of cells observed by DVC is higher than the number of the colonies on TPC assay. Meanwhile, VBNC is defined as a condition in which there are alive cells observed by DVC assay but no colony was recovered by TPC assay.

RNA extraction and reverse transcription-polymerase chain reaction

The RNA was extracted from the cells on Petri dishes samples taken before and after 2 h of dessication as well as 4, 10, 24 days after storage at room temperature. Culture suspensions for RNA extraction were prepared with similar method for the above culturability assay. The total RNA was extracted using silica based column according to the manual guidance of peqGOLD Bacterial RNA extraction Kit (PEQLAB Biotechnologie, Erlangen, Germany). At the end of extraction step, total RNA was treated with DNAse I RNase free (Thermo Fisher Scientific, Massachusetts, USA) referring to manual guidance. The expression levels of 16s rRNA, *rpo*S, *omp*A, and *hfq* genes were determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR) based on two-step protocol by RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific).

The cDNA synthesis protocol was performed based on the kit manual guidance (Thermo Fisher Scientific). Two µl of total RNA (100 ng/µl) and 1 µl Random Hexamer primer (10 µM) were homogenized with NFW up to 12 µl total volume reaction, followed by incubation at 65 °C for 10 min. After incubation, 4 µl buffer reaction (5 ×), 1 µl RiboLock RNAse Inhibitor (20 U/µl), 2 µl dNTP 10 mM, and 1 µl RevertAid M-Mulv RT (200 U/µl) were added into PCR tube. The reaction mixture was incubated at 42 °C for 60 min and stopped by heating at 70 °C for 5 min. Reaction was performed in PCR Thermal Cycler 2720, while quantity and quality of RNA and cDNA were measured with NanoDrop ND-1000 spectrometer.

The qPCR assay was performed by real time PCR AB 7500 (Applied Biosystems, California, USA). A reaction mixture of 20 µl total volume was composed of 2 µl cDNA, 1 µl of each primer (10 µM), 10 µl KAPA SYBR FAST qPCR master mix (2 ×) universal, 0.4 µl ROX low reference dye (50X), and 5.6 µl of NFW. The PCR protocol consisted of pre-denaturation (95 °C, 3 min) and 40 amplification cycles (denaturation at 95 °C for 30 s, annealing at 50 °C for 32 s).

The expression of the target genes was calculated relative to the reference gene or housekeeping gene (16s rRNA). The relative expression ratio of each target gene was calculated by using Pfaffl method shown in Eq. 1 [18]. Statistical parameters such as averages and standard errors mean were calculated using Microsoft Excel.

$$\text{Gene expression ratio} = \frac{E_{GOI}^{\Delta CT(\text{control}-\text{treatment})}}{E_{HKG}^{\Delta CT(\text{control}-\text{treatment})}} \quad (1)$$

Results and discussion

Detection of target genes

Detection of *rpo*S, *omp*A, and *hfq* genes was conducted to ensure that the genes were present in *C. sakazakii* Yrt2a and the primers successfully amplified the specific target genes. The results suggested that the designed primers were

specific to the target genes as indicated by the presence of a single band for each gene. Each primer successfully detected the 16s rRNA, *rpo*S, *omp*A, and *hfq* genes of *C. sakazakii* Yrt2a. Based on visualization of the agarose gel, the sizes of the DNA bands were similar to the output prediction of BLAST Primer NCBI i.e. 194 bp for 16s rRNA; 136 bp for *rpo*S; 155 bp for *omp*A; and 210 bp for *hfq* (Fig. 1).

Desiccation-induced changes in cell culturability and viability

Both culturability and viability assays were conducted by calculating the total plate count on TSA medium and DVC, respectively. Observation of cell culturability and viability was conducted before and after 2 h of desiccation treatment as well as at 2, 4, 6, 10, 14, 19, and 24 days storage at room temperature. The TPC of *C. sakazakii* before and after desiccation were 7.37 ± 0.08 and 4.16 ± 0.19 log CFU/ml, respectively. Exposure to desiccation at 40 °C has caused a significant reduction (3 log CFU/ml) in the number of culturable cells. Significant reduction in the number of culturable cells as a result of desiccation has been suggested by Shaker et al. [19], who reported that desiccation stress decreased the culturability of *Cronobacter* spp. Meanwhile, the DVC revealed a population of 7.17 ± 0.03 log cell/ml (before desiccation) and 6.88 ± 0.05 log cell/ml (after desiccation) suggesting a lower decrease as compared to that of the culturable cells. Subsequent storage of the desiccated cells at room temperature revealed a slower reduction of culturable cells.

Changes in the number of culturable cells and viable cells of *C. sakazakii* Yrt2a post desiccation during storage were shown in Fig. 2. Prior to day 24, we observed a decline in the culturable cells as well as viable cells. At 10 days of storage, the culturability assay showed 1.59 ± 0.02 log CFU/ml, while viability assay revealed a

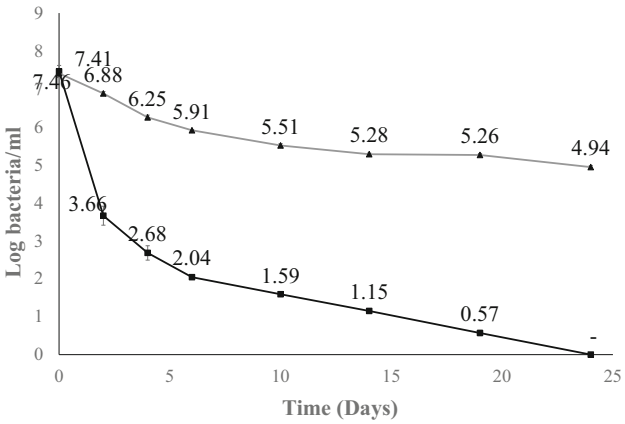

Fig. 2 Viability (log cell/ml) and culturability (log CFU/ml) of *C. sakazakii* Yrt2a. Viable count shown by triangle (▲) and culturable count shown by circle (●). Error bars show a standar error mean

population of 5.51 ± 0.05 log cell/ml suggesting that the bacteria were experiencing stress. At 24 days of storage, *C. sakazakii* Yrt2a cells was no longer detected on the culture plate, although the viable cells number were 4.94 ± 0.04 log cells/ml indicating that the bacteria entered VBNC state.

Based on microscope fluorescence observation, *C. sakazakii* Yrt2a undergoing stress and at VBNC state showed differences in size (Fig. 3). Some cells were small, normal, and elongated. In line with other research, size conversion of bacterial cells in various environmental stresses was thought to be a mechanism of adaptation for

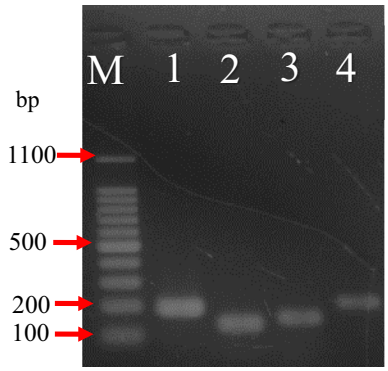

Fig. 1 Detection of target genes in agarose gel 2%. M, 100-bp DNA ladder size marker; 1, *16s rRNA* gene; 2, *rpo*S gene; 3, *omp*A gene; 4, *hfq* gene

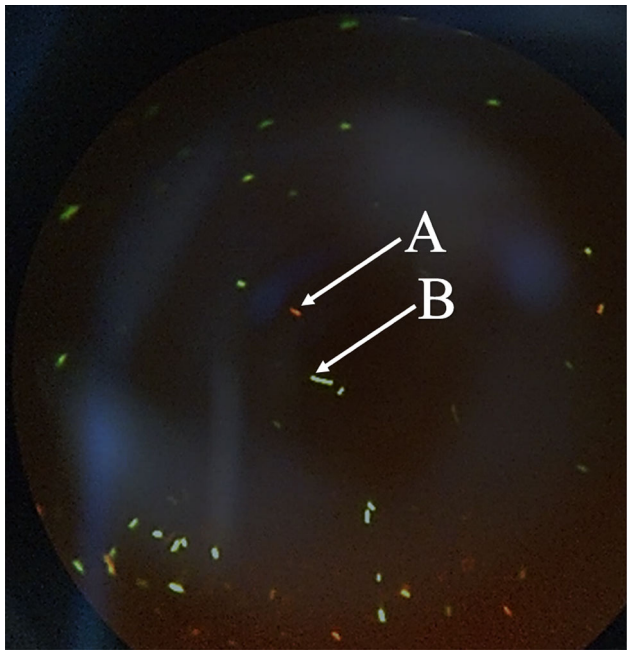

Fig. 3 *Cronobacter sakazakii* Yrt2a inVBNC state observed by microscope fluorescence. Dead cells are shown by orange (A), viable cells are shown by green (B). (Color figure online)

the bacterial survival [20]. Adams et al. [21] stated that cells in VBNC condition appeared to be elongated, while Zeng et al. [22] stated that VBNC cell has a smaller size than a normal cell. In relation to this, other report explained that elongation does not occur in cells that are sensitive to antibiotics [23]. It is possible that within the bacterial cells population studied, some were sensitive while others were resistant to the presence of antibiotic used (nalidixic acid). *Cronobacter* sp. isolated from desiccated rice powder were susceptible to nalidixic acid [24]. Nalidixic acid is an antibiotic capable of inhibiting DNA synthesis and prevent cell division, while yeast extract is an additional nutrient for bacteria [25].

Expression of *rpo*S, *omp*A, and *hfq* genes

The expression of the target genes is shown in Fig. 4. All target genes of *C. sakazakii* Yrt2a exhibited a down-regulation after 2 h desiccation at 40 °C. A decrease in the gene expression was associated with the decline in the number of culturable cells. After 4 days of storage post desiccation, *C. sakazakii* Yrt2a showed an up-regulation of gene expression up to 5.27 log of *rpo*S gene; while *omp*A and *hfq* were up to 0.55 and 0.68 log, respectively. Furthermore, the highest expression level was observed at the 10th day of incubation as shown by increase in *rpo*S gene up to 11.01 log, *omp*A gene up to 6.38 log, and *hfq* gene up to 3.27 log. At this point, the bacteria were still capable of growing on TSA medium (1.59 ± 0.02 log CFU/ml). At day 24 of storage, the cells lost their culturability as no colonies appeared on TSA plates and the gene expression decreased by 2 log for *hfq*, 5 log for *omp*A and 10 log for *rpo*S as compared to those at day 10. However, these numbers still showed an up-regulation as compared to normal condition at exponential phase.

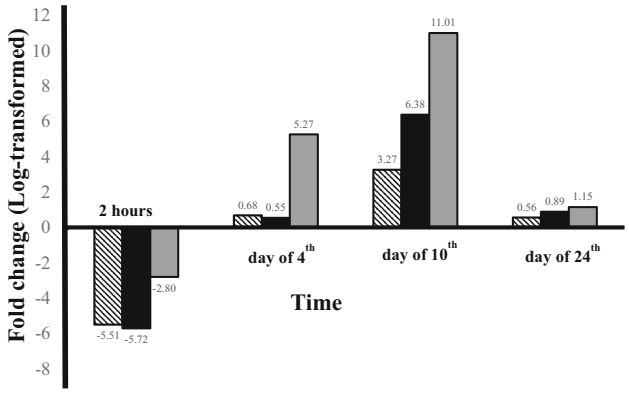

Fig. 4 Log fold change ratio of gene expression *C. sakazakii* Yrt2a. Expression of *rpo*S gene ( ), *omp*A gene ( ), and *hfq* gene ( )

In this study, the expression of the three target genes under desiccation stress followed by storage condition has a similar pattern. After desiccation, all of the genes showed down-regulation, possibly due to heat exposure (40 °C) that caused nucleic acid damage. Wesche et al. [26] reported DNA, RNA, enzymes and protein damage in bacteria due to heat treatment. The activities of glycosylase and endonuclease enzyme that play an important role in DNA repair, are unstable at 40–50 °C [27].

After a down-regulation following 2 h desiccation at 40 °C, *C. sakazakii* showed an up-regulation during storage at 25 °C. This condition was observed at 4th and 10th days of incubation, in which the *rpo*S gene showed a higher expression. Increase in *rpo*S gene expression usually suggests that *C. sakazakii* is coping with stress. Kusumoto et al. [28] demonstrated that *rpo*S regulates the rate of bacteria to enter the VBNC state. At 24 days of storage when *C. sakazakii* entered VBNC state, the expression of *rpo*S decreased. This is similar to report claiming that bacterial culturability decreases with a low level of *rpo*S gene expression [29].

The *omp*A gene plays an important role in the invasion and adhesion of *C. sakazakii,* in which invasion ability of *C. sakazakii* decreased by 87% in the absence of the *omp*A gene [13]. Another report also showed that expression of the outer membrane protein in stress-sensitive strains was higher than that in the stress-resistant strains of *E. coli* [30]. Our study showed that the expression of *omp*A increased during stress, especially at the 10th day, but decreased at 24th day when the bacteria enter VBNC state. The expression of most membrane proteins was repressed (down-regulated) at VBNC state [31], which may explain the reason for the decrease in expression of the *omp*A gene at day 24.

The *hfq* gene has been extensively studied pertaining to its primary role in regulating post-transcription of various genes. In this study, the *hfq* gene expressions showed a similar pattern with *omp*A gene. *C. sakazakii* virulence was associated with the presence of *hfq* gene [11]. At 10 days of storage, *hfq* gene showed the highest gene expression. In VBNC state, expression of *hfq* gene seems to have a similar pattern with *omp*A and *rpo*S.

Adaptation process of another bacteria such as *Escherichia coli* in long-term stress is reversible and not affected by genetic changes in the cells [32]. This study showed that desiccation induced *C. sakazakii* strain Yrt2a to experience stress and finally enter VBNC state. During stress, the expression of *rpo*S, *omp*A, and *hfq* was extremely high at day 10. Nevertheless, at VBNC state, the expression of *rpo*S, *omp*A, and *hfq* showed a significant reduction, i.e. only 1 log-fold as compared to that of normal condition.

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