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Original

Investigation into the Physiological State of Heat Stressed *Escherichia coli* Used in the Evaluation Testing of an Intrinsic Fluorescence-Based RMM.

KANAMI IRIE[1], ALLISON SCOTT[2], AND NORIO HASEGAWA[1]*

[1]*Azbil Corporation, 1-12-2, Kawana, Fujisawa-shi, Kanagawa, 251-8522, Japan*
[2]*Azbil North America Research and Development, Inc., 2005 W. Ruthrauff Road, Suite 151, Tucson AZ, USA 85705*

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Rapid microbiological methods (RMMs) have been used as novel quality control technologies in industry. The ability of RMMs to detect stressed bacteria, in particular, is of continued interest due to the limitations of the conventional method in stressed bacteria detection. Accordingly, there is a need to better characterize an RMM's ability to detect stressed microorganisms. Previously we reported on the detection ability of an intrinsic fluorescence-based RMM using a 50% injured (determined based on colony-forming ability) bacterial cell group after heat treatment at 55°C for 8 min. In this study, we added further information about the physiological state of the heat treated *Escherichia coli*, besides proliferation ability, by investigating respiratory activity using CTC fluorescent staining and expression of DnaK, a heat shock protein. It was found that 89% of cells (control 96%) retained respiratory activity, but only 20% (control 41%) retained proliferation ability after heat treatment. The difference between the percentage of cells with respiratory activity versus that of cells still capable of proliferation further supports the existence of viable but non-culturable stressed cells in the test sample. Also, we suggest such analysis would be one approach to confirming the use of stressed as opposed to dead cells when evaluating an RMM's ability to detect stressed microorganisms.

Key words : Heat shock response / Heat stress / VBNC / DnaK / RMM (Rapid Microbiological Method).

INTRODUCTION

Rapid microbiological methods (RMMs) have been developed (Gray et al., 2011; Gordon et al., 2011; Shintani et al., 2011) and used as novel quality control technologies in the pharmaceutical, personal care, and food and beverage industries. These systems have been available on the market for decades (Peris-Vicente et al., 2015). As RMM adoption becomes more prevalent, validation (Peris-Vicente et al., 2015) and implementation (Friedman et al., 2015; Miyashita et al., 2015), in particular, can introduce unique challenges. RMMs can provide the benefit of rapid and enhanced detection over the traditional culture based method. Bacteria in a VBNC (viable but non-culturable) state (Oliver, 2005) is one detection target which the routine culture method cannot detect. According to Oliver (Oliver, 2000 and 2005), the definition of VBNC is the state in which bacteria fail to grow on the routine bacteriological media on which they would normally grow and develop into colonies, but are alive and capable of renewed metabolic activity. Stressed bacteria are also important detection targets because it is known that microorganisms in natural or industrial environments are stressed by a variety of factors in the environment (Wesche et al., 2009). According to the above mentioned literal definition of VBNC, stressed bacteria may also show a VBNC-like state as a result of physiological condition (Gunasekera et al., 2002). There is previous research that evaluated the differences between VBNC state and other stress responses by protein profile investigation (Heim et al., 2002). It is known that the VBNC state is induced by various environmental stresses, especially cold stress

**Corresponding author. Tel: +81-466-52-7151, Fax: +81-466-20-2431, E-mail : n.hasegawa.om(a)azbil.com*

(Oliver, 2010).

In any case, common issues with stressed bacteria and VBNC state bacteria include the impossibility of detection by the traditional culture method with routine bacteriological media and culture conditions (i.e. time and temperature); and the possibility of resuscitation with virulence, which brings potential health risks (Ding et al., 2017). Consequently, it is important to investigate an RMM's detection ability for VBNC state and stressed bacteria to provide a more complete understanding of microbes present in the manufacturing environment and changes therein in the food and pharmaceutical industries.

Previously, we investigated one of the RMMs, the BioVigilant IMD-A® system with detection based on cellular intrinsic fluorescence, for its ability to detect stressed bacteria (Irie et al., 2014). Heat was selected as the stress factor because it is easy to control and is utilized for sterilization processes in a variety of industries. This 2014 report discussed heat stress levels of bacteria within the parameters of colony-forming ability and time of the extended lag phase ("growth delay") (Irie et al., 2014; Stephens et al., 1997; Takano and Tsuchido, 1982; Tsuchido et al., 1989; Wesche et al., 2009), both of which are culture based method results. For example, the stress level was defined by the colony ratio between the stressed and non-stressed sample (e.g. colony number of stressed is 50 cfu: non-stressed 100 cfu = 50% stress level) (Irie et al., 2014). However, with this method, it's impossible to explain clearly what the bacteria state was. For example, the bacteria were in a stressed physiological state and/or the culture conditions were simply inadequate. In the previous research, validity of the method was shown by using some initial density tests for growth delay (Irie et al., 2014).

We believe it is possible to more specifically describe the bacterial physiological state and through this study, results of previous research can be better understood and the state of bacteria used to assess RMM detection confirmed. Some possible methods to explain the bacterial state are to evaluate the metabolic activity of each cell after stress exposure treatment, and to measure the expression of specific proteins, which relate to stress. In this study, the physiological state of heat stressed samples, for use in the evaluation of an RMM, was investigated with advanced research that measures respiratory activity as a metabolic activity by fluorescent staining methods, and heat shock response by a promoter assay.

MATERIALS AND METHODS

Reagents and Chemicals

Soybean casein digest broth (SCDB), also known as tryptic soy broth (TSB) (Becton Dickinson, Franklin Lakes, NJ), and its agar plate (TSA) (Eiken Chemical, Tokyo, Japan) were used for bacterial culture. Fluorescent staining of bacterial cells was performed with -*Bacstain*- CTC (5-cyano-2,3-ditolyl tetrazolium chloride) rapid staining kit and DAPI (4', 6-diamidino-2-phenylindole, dihydrochloride) solution (DOJINDO LABORATORIES, Kumamoto, Japan). Restriction enzymes, *Bam*HI, *Eco*RI and *Nde*I, and ligase were obtained from Takara Bio Inc. (Shiga, Japan). Phosphate buffered saline (PBS) and Nalidixic acid sodium salt were obtained from Sigma-Aldrich (St. Louis, MO).

Bacterial Strains and Culture Condition

Escherichia coli ATCC 13706 was cultured and prepared in the same way as previously (Irie et al., 2014). After cultivation, cells were harvested in 5 ml of sterile filtered distilled water (DW) with a sterilized disposable loop, and suspended by gentle vortexing. The cells were then washed by centrifugation at 2100g for 3 minutes (Model 2410, Kubota Corporation, Tokyo, Japan). The supernatant was removed, and the pellet was re-suspended in 5 ml of sterile filtered DW by mild pipetting. The optical density at 600 nm (OD_{600}) was then measured using a spectrometer (GeneQuant Pro, GE Healthcare, Chalfont, UK) to estimate bacterial cell concentration. The suspension was diluted with sterile filtered DW to achieve a target concentration with OD_{600}=1, then used for heat treatment followed by CTC-DAPI staining.

E. coli DH5α (pNHKI1α), described as follows, was used for promoter assay. Bacterial preparation for heat treatment was the same as ATCC 13706.

Construction of Plasmids and Transformant

In order to reevaluate whether the heat shock was loaded to the bacterial sample as expected and also what kind of cell state the bacterial sample had in our previous study (Irie et al., 2014), in this study we investigated the expression of a heat shock protein in a bacterial sample with the same heat loading experimental condition as in the previous study. We used DnaK as a marker, which is a terminal molecule of heat shock cascades. The promoter assay was performed using the *dnaK* promoter conjugated with a red fluorescent protein, FresnoRFP, in *E. coli* to quantify DnaK expression (Cha et al., 1999). The conjugate was introduced on a plasmid, and was expressed under the regulation of σ factor originating from genome DNA (Figure 1).

The *dnaK* promoter, P_{dnaK}, obtained by Nested PCR on the genome of *E. coli* ATCC 13706, and the FresnoRFP encoding gene with the *rrnB* terminator obtained by PCR from the Ref: FPB-54-441 DNA vector (DNA2.0

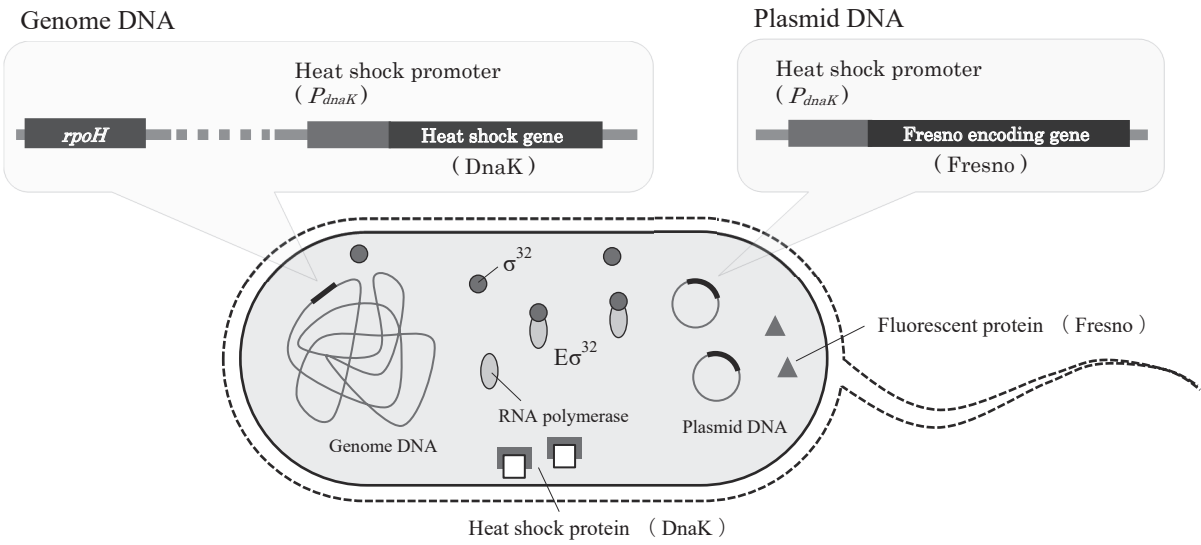


FIG. 1. Illustration of *E. coli* expressing FresnoRFP under *dnaK* promotor. Eσ^{32} means a complex with RNA polymerase and σ^{32}.

TABLE 1. Strains, primers and plasmids.

Bacteria and DNA	Description and Sequence	Reference or Source
E. coli ATCC 13706	Prototroph, P_{dnaK} and P_{rpoH} source	ATCC
E. coli DH5α	Host strain for plasmid construction	(Stock of a contracted company)
E. coli DH5α (pNHKI1α)	*E. coli* DH5α transformed by pNHKI1α	This study
Primers[a]		
dnaKnest FWD[1]	GAATGCCTTGGCTGCGATTCATTCT,	This study
dnaKnest Rev[2]	TCGCTACACAAGAGTTGGTAGTACCC,	This study
dnaK FWD BamHI-AI[3]	AAAAAggaTCCTGCCATATCGCGAA,	This study
dnaK Rev NdeI-A[4]	AAAAAAACATatgAACGTCTCCACTATATA,	This study
Fresno FWD NdeI[5]	GAGGTAAcAtATGAATAGCCTGATTAAAG,	This study
rrnB Rev EcoRI[6]	TCTCATGAGCGaATtCATATTTGAATG,	This study
Plasmids		
pUC18	Cloning vector, Amp^r	Messing, 1983
pBR322	Cloning vector, Amp^r, Tet^r	Bolivar et al., 1977
Paintbox™ vector Ref: FPB-54-441	Km^r, FresnoRFP encoding gene source	DNA2.0 Inc.
pNHKI1	1.2 kbp *Bam*HI-*Eco*RI fragment cloned into pUC18 carrying P_{dnaK}, FresnoRFP encoding gene and *rrnB* terminator	This study
pNHKI1α	1.2 kbp *Bam*HI-*Eco*RI fragment cloned into pBR322 carrying P_{dnaK}, FresnoRFP encoding gene and *rrnB* terminator	This study

[a] The number of superscript on the primer name corresponds to primer number in FIG 2. Underline of the sequence indicates mutated restriction enzyme sites and small letters indicate replaced nucleotides.

Vectors, IP-Free © Fluorescent ProteinPaintbox™ –*E.coli*, DNA2.0 Inc., CA, USA) were conjugated and inserted into pUC18 with constructed restriction sites to generate pNHKI1 and its DNA sequence was checked (Table 1 and Figure 2). *E. coli* DH5α was transformed by pNHKI1. It was expected that a red fluorescent protein by the FresnoRFP encoding gene would be expressed only in the case of exposure to heat

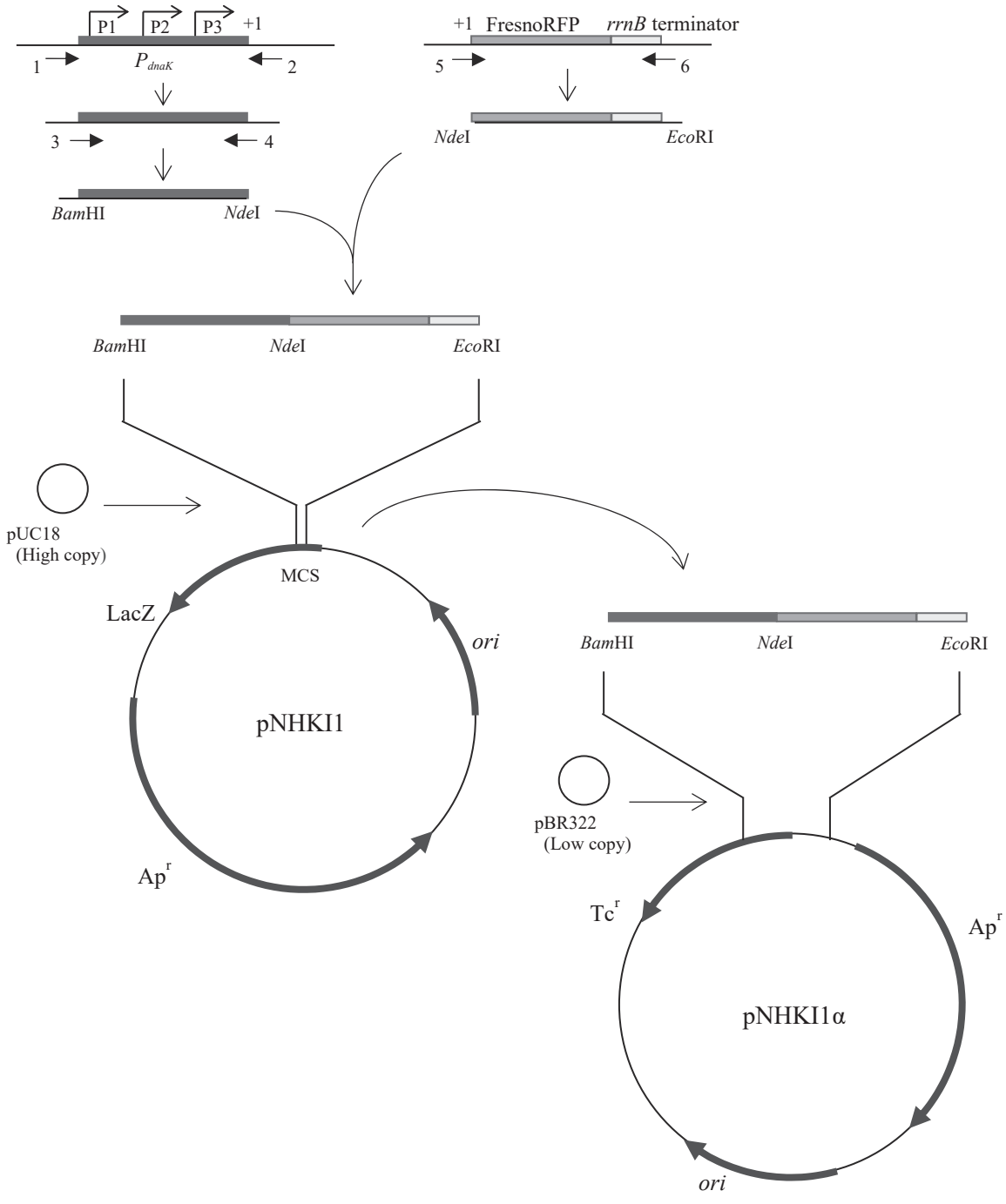


FIG. 2. Schematic explanation of plasmid construction. Numbers of primers correspond to those of Table 1. MCS means multiple cloning site. A P_{dnaK} (*dnaK* promoter) including fragment added *Bam*HI site and *Nde*I site at 5'- and 3'-end respectively was amplified using Primer3 and 4. And a FresnoRFP coding gene and *rrnB* including fragment added *Nde*I and *Eco*RI site at 5'- and 3'-end respectively was amplified using Primer5 and 6. Those two fragments were ligated at *Nde*I and the conjugate was inserted to pUC18 at multiple cloning site to generate pNHKI1. The insert was sub-cloned again into pBR322 to generate pNHKI1α.

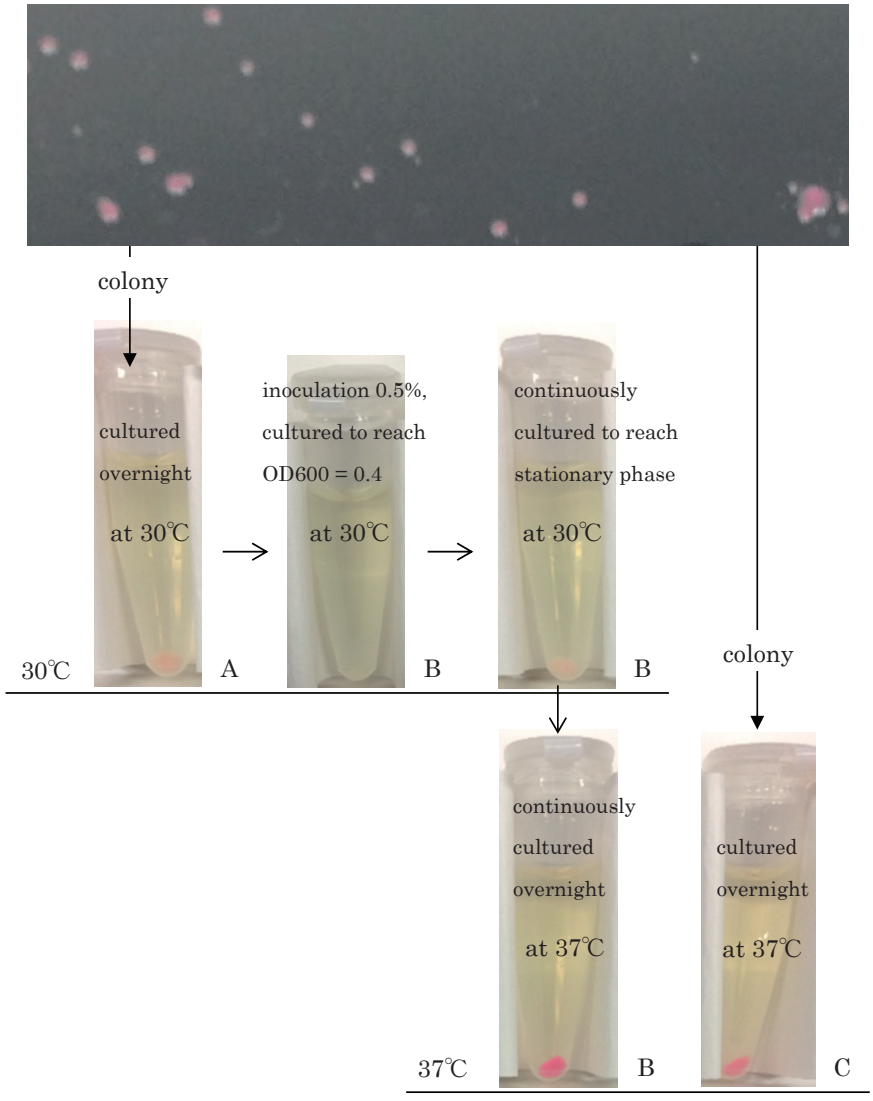


FIG. 3. Photographs of Fresno protein expressed *E.coli*. Tube A was inoculated directly from the cultured sample. Tube B contains a 0.5% inoculation from tube A and was then cultured at 30°C to reach the stationary phase, followed by an overnight incubation at 37°C. Tube C was inoculated directly from the cultured sample and then cultured overnight at 37°C.

stress. However, the red colonies appeared when the transformants were incubated at 37°C; and also when incubated at 30°C, showing a less intense red color at the lower temperature (Figure 3). It was realized that this result was, as a matter-of-course, caused by the fact that the *rpoH* of *E. coli* is required for growth at high temperatures (>35°C) (Yura et al., 1984). Accordingly, the [P_{dnaK} – FresnoRFP encoding gene] fragment was transferred to pBR322 (which is a low copy plasmid) to generate pNHKI1α instead of pUC18 (which is a high copy plasmid) in order to avoid excess expression of FresnoRFP in the background state. *E. coli* DH5α was transformed by pNHKI1α and the white-to-red (pink) colored colonies were picked up as FresnoRFP-expressing transformants. For the transformation, *E. coli* was cultivated on a Luria-Burtani (LB) agar plate with carbenicillin (final 50 µg/ml) at 37°C. Genetic recombination and the experiments using transformants were performed in the laboratory of a contracted company with their protocols and materials.

Loading Heat Stress on *E. coli* for CTC-DAPI staining

After a bacterial suspension was prepared as described above, 1 ml was allocated to each of four centrifugal tubes (size 2 ml). Two of the centrifuge tubes were kept at room temperature (22°C) as the control

samples. The other two centrifuge tubes were heated at 55°C for 8 minutes as the heat stressed samples. This heating condition was intended to produce an injury-level of approximately 50% injured bacteria based on the previous study (Irie et al., 2014). After the suspension was heated, the tubes were cooled down to room temperature by putting them in room temperature DW for 20 minutes.

CTC-DAPI staining

Each 2 ml centrifugal tube sample was placed in a 1.5 ml centrifugal tube for further processing. Then, they were centrifuged at 2100g for 10 minutes. The supernatant of each tube was removed, and the pellet was resuspended in 1 ml of SCDB by mild pipetting. Each pair of control and heat stressed samples were recombined into a single centrifugal tube, respectively. The optical density at 600 nm (OD_{600}) was then measured as described above. The suspension was diluted with SCDB to achieve a target concentration with OD_{600}=0.5. Nalidixic acid was added to achieve final 50 µg/ml to disturb cell proliferation, not to change total cell number during incubation.

400 µl of each control and heat stressed sample was allocated to another centrifugal tube, and 8 µl of CTC solution and 2 µl of enhancer solution were added to each of these tubes according to the product manual. CTC reagent is a fluorescent dye for direct epifluorescent microscopic enumeration of respiring bacteria (Rodriguez et al., 1992). These samples were then cultured at 37°C for 40 minutes to allow for metabolic activity to reduce intracellular CTC to CTF (CTC-formazan). After that, 0.4 µl of DAPI, which specifically binds to double-stranded DNA, was added to each of the centrifuge tubes and left to stand for approximately 5 minutes at room temperature for counterstaining (King and Parker, 1988). In order to induce cell elongation, aerobic cultivation (120 rpm, lean 30°) was immediately invoked on the remainder of the control and heat stressed samples, which weren't fluorescent stained. After 5 hours of this incubation, the samples were fluorescent stained as described above.

Heat Stress Treatment for promoter assay

The aliquot of transformed *E. coli* was inoculated from the stock in the LB liquid media with carbenicillin (final 50 µg/ml) and cultivated aerobically at 30°C overnight (preculture). These cells were then streaked on LB agar plates with carbenicillin and cultivated at 30°C overnight. After cultivation, cells were harvested in 5 ml of sterile DW with a sterilized loop, and suspended by gentle vortexing (two identical samples were prepared for the next step). The cells were then washed by centrifugation at 2100g for 3 minutes. The supernatant was removed from each of the two samples, with one pellet resuspended in 5 ml of LB liquid media with carbenicillin, and the other pellet in 5 ml of PBS with mild pipetting. The optical density of each sample was then measured at 600 nm (OD_{600}) using a SP-300 spectrometer (OPTIMA, Tokyo, Japan) to estimate bacterial cell concentration. The suspension of each sample was diluted to achieve a target concentration with OD_{600}=0.4. Each 1 ml sample (LB with carbenicillin and PBS) was transferred to a Spitz tube and heated by water bath at 55°C for 8 minutes. Then, the tubes were cooled down to room temperature by putting them in room temperature DW for 12 minutes. Concurrently, each unheated 1 ml sample was transferred to a Spitz tube in the same manner and kept at room temperature as a control (two of the same samples were prepared for the next step). Aliquots of both heated and unheated samples (unheated as control) were treated with 4 % paraformaldehyde fixation (a 100 µl cell suspension + 100 µl 8% paraformaldehyde, room temperature 10 minutes, centrifugal washing 3 times). The remaining heated samples were incubated at 30°C aerobically (both LB with carbenicillin and PBS), while the rest of the control samples were incubated at 30°C and 37°C also (both LB with carbenicillin and PBS). After 1, 4 and 22 hours incubation, an aliquot of all incubated samples was treated with 4% paraformaldehyde fixation as aforementioned.

Fluorescence Microscopy

For CTC and DAPI staining, 10 µl of each of the samples were placed on a glass plate and covered with a glass cover slip. Transmitted light and fluorescence images were obtained with a fluorescence microscope system (BX51, OLYMPUS, Tokyo, Japan). A WIB (Ex 460 – 490 nm, Em 510 nm) and a NU (Ex 360 – 370 nm, Em 420 nm) filter cubes were used for CTF and DAPI, respectively.

For promoter assay, 10 µl of each of the samples were placed on a glass plate and covered with a glass cover slip, and fluorescence images with a WIG filter cube (Ex 530 – 550 nm, Em 590 nm) for Fresno red fluorescence were obtained.

Image Analysis

Obtained images were analyzed with the following methods using software (Image-Pro Plus Ver 7.0.1., Media Cybernetics, Inc., Washington DC, MD, USA) in order to convert into numerical values and normalize background.

CTC fluorescent staining ratio:

1) Convert an image of transmitted light, taken of each of three heat treated and control CTC fluorescent stained samples, to 8 bit gray scale.

2) Get an average of the background values of each arbitrary upper left, center and lower right site by making a histogram.
3) Calculate the background value in gray scale of the image by averaging the three background values obtained in step 2), and then multiply by the coefficient, 1.05, in order to cancel the outlier background pixels with a higher value.
4) Count the bacteria regions (bright spots compared to the background) based on the minimum required brightness value determined in step 3) to 255 in gray scale.
5) Exclude from this count the obviously non-bacterial artifacts.
6) Let the value (A) obtained in step 5) represent the total number of bacteria in the image.
7) Compare the transmitted light image and fluorescence WIB filtered image.
8) Visually count the non-fluorescent particles from the comparison of the transmitted light image and fluorescence WIB filtered image and set it as the number of non-fluorescent bacteria (B).
9) Let the number of fluorescent bacteria = A – B.
10) Let the CTC fluorescent staining ratio (%) = (A – B)/A ×100.

Cell elongation ratio:

1) Observe bacteria that were just stressed but not yet cultured (culture time = 0) as their cell elongation ratio remains 0. Then, analyze the bacterial samples after they've been cultured for 5 hours.
2) Convert images of transmitted light to 8 bit gray scale.
3) Get an average of the background values of each arbitrary upper left, center and lower right site by making a histogram.
4) Calculate the total background value of the image by averaging the three background values obtained in step 3), and then multiply by the coefficient, 1.05, in order to cancel the outlier background pixels with a higher value.
5) Count the number of bacteria (bright part compared to background) based on the minimum required brightness value determined in step 4) to 255 in gray scale.
6) Exclude from the count the obviously non-bacterial artifacts.
7) Measure the "radius (maximum)" value of all counted objects.
8) Consider an object with "radius (maximum)" > 15 (pixel) as an elongated cell (D).
9) Let the cell elongation ratio (%) = D/A×100. A is calculated above in the CTC fluorescent staining ratio step 6).

Fresno fluorescence brightness (intensity):

1) Convert image to 8 bit gray scale.
2) Within the "Count / Size" dialog, smooth the background (settings: dark / width of object 10) once.
3) Get an average of the background values of each arbitrary upper left, center and lower right site by making a histogram.
4) Calculate the total background value of the image by averaging the three background values obtained in step 3).
5) Normalize the brightness by subtracting the background value of the image calculated in step 4) from the image once.
6) Count the bacteria (bright spots compared to the background) based on the range 30 to 255 in gray scale.
7) Measure the "density (average)" value (i.e. the average brightness) of each counted object.
8) Calculate the average of the "density (average)" values from all objects.

The value obtained in step 8) is regarded as the average Fresno brightness of the cells in the image.

RESULTS

Respiratory Activity

In order to stain cells by CTC fluorescence, proper time (e.g. 30 minutes to a few hours) and substrate (oxidizable carbon supplementation) are required to reduce intracellular CTC to CTF (Rodriguez et al., 1992), and proper calefactory temperatures are needed for cell metabolic activity. In addition, continuous observation is necessary to evaluate recovery, by incubation, from stress exposure. Even short incubation times change the number of cells in the sample suspension (cell density) because the bacteria proliferation speed is rapid (the doubling time of *E. coli* is around 20 minutes under proper incubation conditions). That is, some cells which aren't greatly affected by stress exposure may proliferate actively, distorting the number of other cells which can't proliferate well at that time due to stress exposure. To avoid this, Nalidixic acid, a quinolone antibiotic, was used (Crumplin and Smith, 1975; Goss et al., 1964; Roszak and Colwell, 1987) for the purpose of keeping the number of cells in the sample suspension constant. Nalidixic acid inhibits only the synthesis of DNA; it neither inhibits respiration, the synthesis of RNA or protein, nor has any direct effect on the integrity of the cell wall or membrane (Goss et al., 1964). If the cells are proliferative, those treated with Nalidixic acid elongate instead of dividing (Joux and LeBaron, 1997; Roszak and Colwell, 1987; Yokomaku et al., 2000). CTC fluorescent staining using cells treated with Nalidixic acid allows investigation of cell proliferation ability and

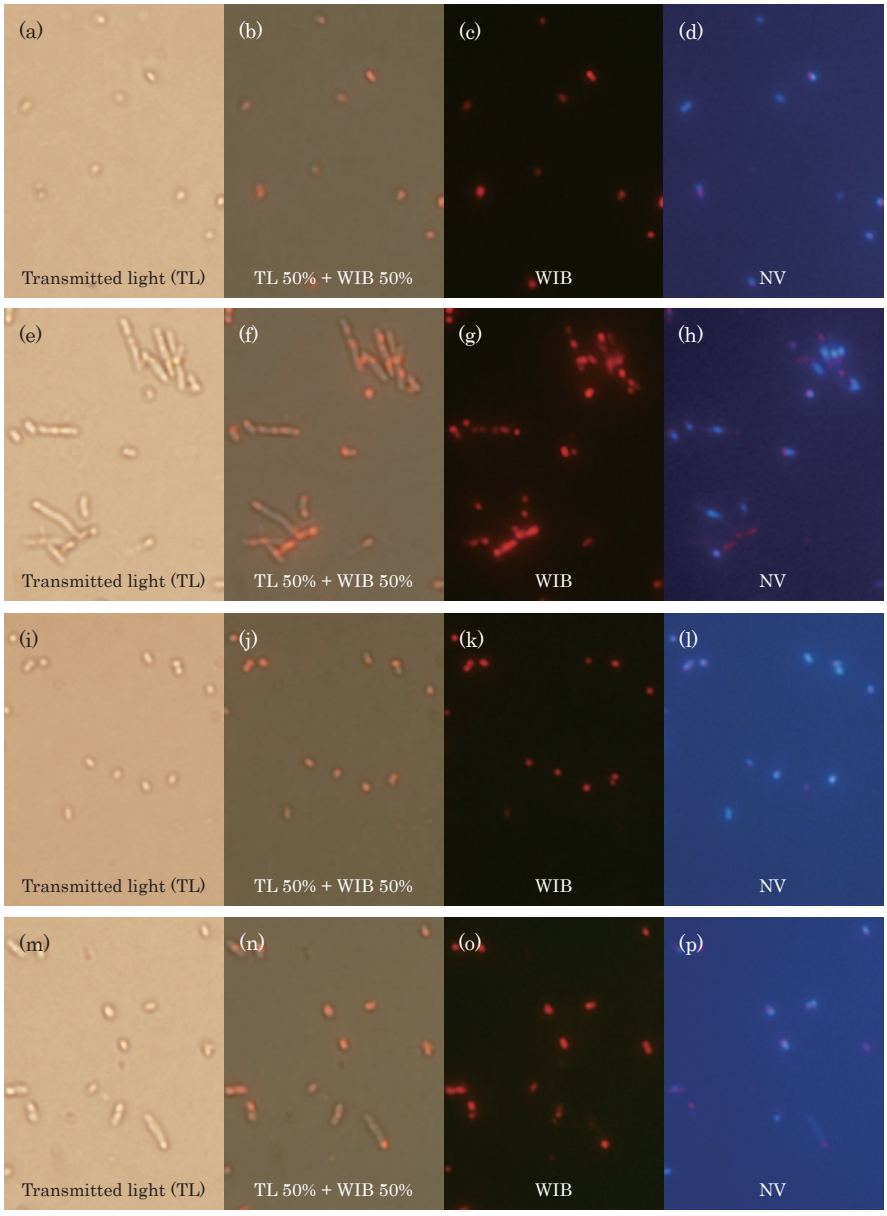


FIG. 4. Microscopic images of *E. coli* treated with Nalidixic acid, DAPI and CTC. (a)-(d): control sample, incubation time 0 h, (e)-(h): control sample, incubation time 5 h, (i)-(l): heat stressed sample, incubation time 0 h, (m)-(p): heat stressed sample, incubation time 5 h. Images (b), (f), (j), and (n) were created by superimposing the transmitted light (TL) (50%) and WIB filter (50%) images.

metabolic (respiratory) activity without the number of cells changing in the suspension.

Figure 4 shows the microscopic fluorescent image results obtained for *E. coli* treated with Nalidixic acid and CTC-DAPI fluorescent staining. Here, cells showing red fluorescence of CTF were recognized as viable and elongated cells showed that they maintain proliferation ability. The resulting microscopic observations were similar to those of a previous study (Nielsen et al., 2003). Figure 5 shows the result of quantitative analysis based on the images in Figure 4, by the procedure described in Materials and Methods. According to the bar chart in Figure 5, compared to that of the control, the resulting number of heat-stressed *E. coli* that could proliferate was half as approximately 20% of heat-stressed *E. coli* showed cell elongation after 5 hours of incubation as compared to approximately 41% of the control sample showing elongation. However, according to the dot

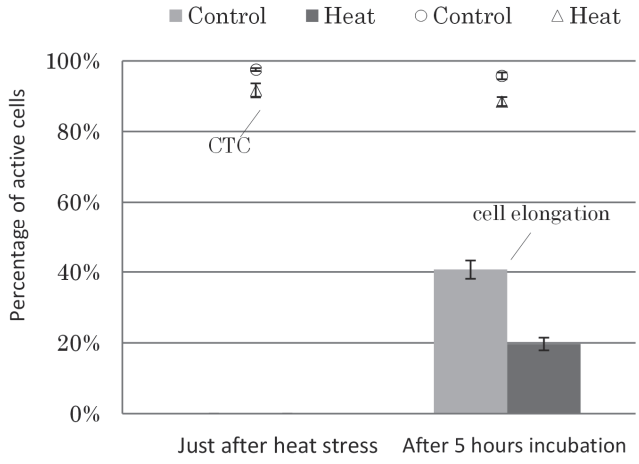


FIG. 5. Comparison of respiratorily active and elongated cells after heat treatment in *E. coli.* Percentage of active cells to total cells are shown. Nine images were taken of each sample, with three replicate images taken on three separate days for each sample/data point on the graph. For the CTC dot chart, each data point represents the average CTC fluorescent staining ratio of the nine images taken and the error bars the standard deviation. Similarly, for the cell elongation bar chart, each bar represents the average cell elongation ratio of the nine images taken and the error bars the associated standard deviation. The cell elongation ratio is zero just after heat stress and before incubation occurs.

chart, most of the entire population kept CTC reducing activity, even after heat exposure (CTF florescence, heat-stressed 89% : control 96%). This indicates the existence of the stressed cells which maintain respiratory activity, but not proliferating ability.

Heat Shock Response

Heat shock proteins (HSPs) are induced by heat stress, called "heat shock response", in the cell (Richter et al., 2010; Sherman and Goldberg, 1993; Yamamori and Yura, 1980; Young and Hartl, 2003). They are also reported to be induced by other stressors as well (Grossman et al., 1984; Grossman et al., 1985; Grossman et al., 1987; Krueger and Walker, 1984; Richter et al., 2010). DnaK is one of the most researched Hsp 70 family chaperones induced under stress and is constitutively expressed. (Calloni et al., 2012; Itikawa and Ryu, 1979; Sherman and Goldberg, 1993; Zylicz et al., 1983; Zylicz and Georgopoulos, 1984). Heat shock response in *E. coli* is summarized in Figure 6. It is possible to recognize the stress response in cytoplasm and in periplasm separately. (Mecsas et al., 1993; Young and Hartl, 2003).

In the cytoplasm, DnaK expression is regulated by σ^{32}, *rpoH* product, in the same manner as DnaJ and GrpE, etc (Grossman et al., 1987; Morita et al., 1999; Yura et al., 1984). The expressed DnaK also affects σ^{32} activity (Young and Hartl, 2003). In addition, mRNA secondary structures of *rpoH* cannot be translated because of folding by intramolecular hydrogen bonds; but this structure melts partially at high temperature, facilitating ribosome binding and enhancing σ^{32} synthesis (Morita et al., 1999; Yura et al., 2000). This mechanism functions as a kind of direct temperature sensor by the mRNA structure. HSPs' synthesis reactions are transitory during HSPs accumulation up to a certain upper limit at which point it stops. HSP gene expression is negatively regulated by DnaK-DnaJ-GrpE chaperones via the control of the synthesis and stability of σ^{32} (Blaszczak et al., 1995; Straus et al., 1990), which maintain the amount of HSPs at certain constant levels (Rodriguez et al., 2008).

While, in the periplasm, the transcription factor of the periplasmic stress response is σ^{E}, (Erickson and Gross, 1989; Wang and Kaguni, 1989). There are at least four promoters for transcription of *rpoH*. Three of them (P1, P4, P5) are identified by σ^{70}, and are active largely under normal conditions. However, the P3 promoter is identified by σ^{E} and activated only at relatively high temperatures (45 to 50°C) (Yura et al., 2000). The regulation of σ^{E} is also controlled by some membrane proteins (Ades, 2008; Young and Hartl, 2003). Thus, expression and activation of HSPs are controlled by a complicated regulation mechanism that maintains intracellular homeostasis.

Fluorescent microscopic images of *E. coli* DH5α (pNHKI1α) after heat treatment of 55°C for 8 min are shown in Figure 7. In contrast to the CTC staining (Figure 4), red fluorescence (attributed to FresnoRFP) was observed in the entire cell. The degree of brightness (intensity) of each cell corresponds to the expression level of FresnoRFP. The fluorescence intensity of the microscopic image was influenced by the cell number within the field of view, increasing the background level. In order to account for the difference in background across each image, image analysis was carried out as described in Materials and Methods. Figure 8 shows the fluorescence intensity originating from the expressed FresnoRFP which is quantified by that image analysis method. The treated condition is abbreviated such as [Heat-LB-37] indicating heat stressed, incubated LB at 37°C, and [Cont-PBS-30] indicating control (non-heated), incubated in PBS at 30°C. FresnoRFP expression was observed even without heat treatment followed by 37°C incubation. Heat shock response might be induced in such a condition. Furthermore, a higher intensity of [Cont-LB-37] than [Cont-PBS-37] indicates that nutrition is needed for FresnoRFP synthesis. Focusing on the endpoint of [Heat-LB-30] and

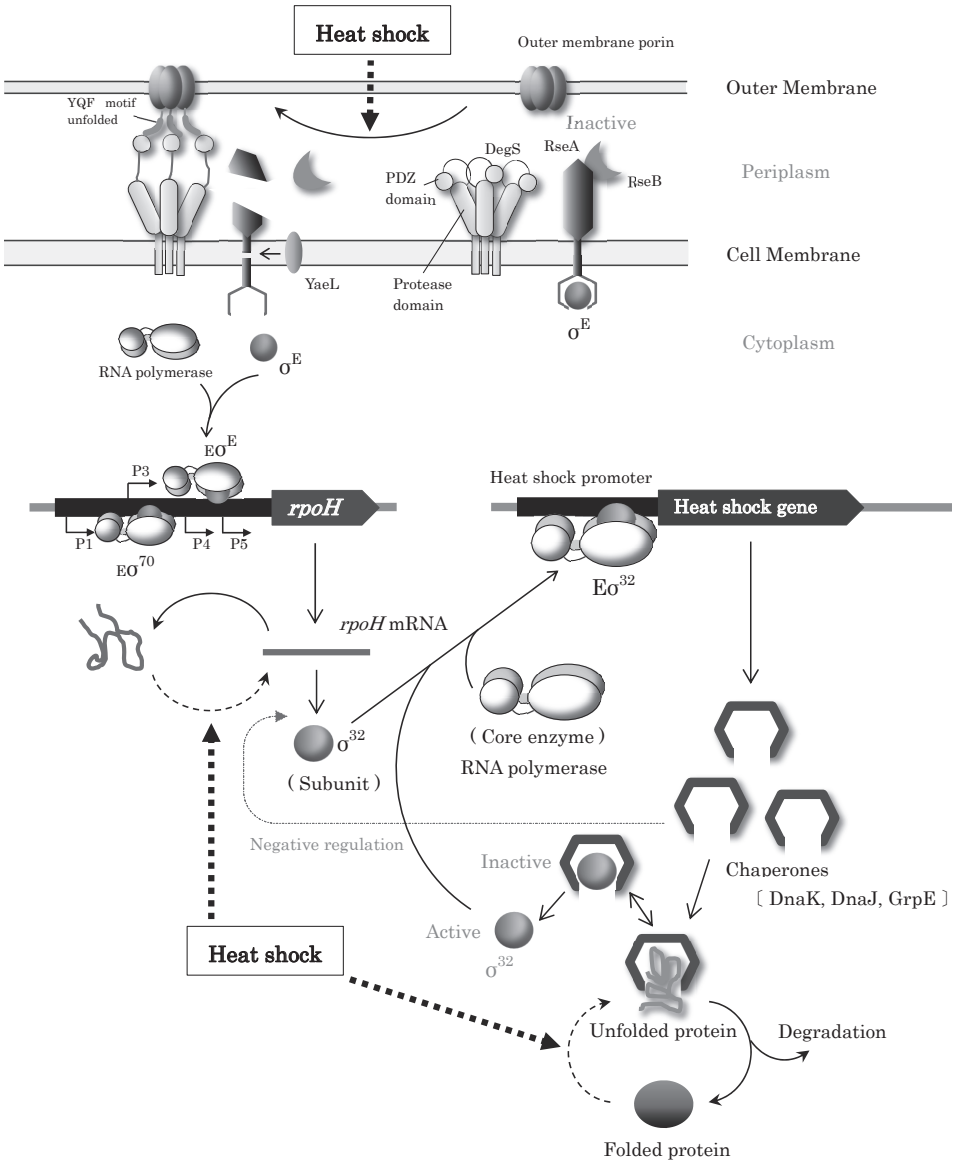


FIG. 6. Schematic explanation of regulation of the σ^{32} mediated heat shock response including σ^{E} regulation. This figure was drawn by integrating the information shown in Fig. 1 (Young and Hartl, 2003), Fig. 1 (Ades, 2008), Fig. 1 and Fig. 2 (a) (Yura et al., 2000), and Fig. 4 (Tsuchido 2006) of references. Eσ^{32}, Eσ^{70} and Eσ^{E} indicate the complex of RNA polymerase and each sigma factor.

[Cont-LB-30], heat treatment of 55°C for 8 min, which is utilized in our previous study (Irie et al., 2014) would have induced a heat stress response to the cells.

DISCUSSION

Confirming the propriety of a new detection system and the evaluation method itself, are important parts of understanding the ability of the technology in the intended environment. Based on such opinion, respiratory activity and proliferation ability, on the routine bacteriological media, of non-heat stressed and heat stressed *E. coli* were compared in order to confirm the bacterial state of a sample used to evaluate the ability of an RMM to detect stressed microorganisms (Irie et al., 2014). As shown in Figure 5, almost the entire subpopulation of non-heat stressed *E. coli* (96%) had respiratory activity, but just under half (41%) had proliferation ability on the routine bacteriological media. On the other hand, heat stress reduced the subpopulation of *E. coli* that

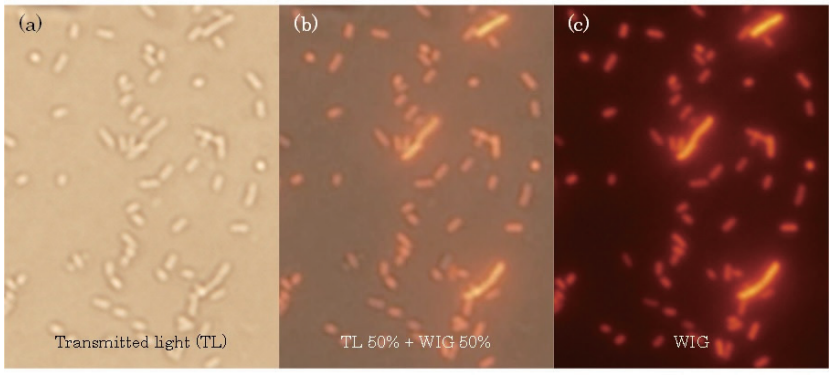


FIG. 7. Microscopic images of Fresno fluorescence of *E. coli* DH5α (pNHKI1α). Image (b) was created by superimposing the transmitted light (50%) and WIG filter (50%) images.

could proliferate on the routine bacteriological media by half (from 41% to 20% of the total subpopulation); this, in spite of a large majority of the subpopulation (89%) maintaining their respiratory activity, regardless of their ability to proliferate.

Mechanisms of bacterial thermal injury are complicated (Grau, 1978) and, for the recovery process, respiratory activity may be important and is generated preferentially (Allwood and Russell, 1970). The traditional culture method using routine bacteriological media can underestimate the viable bacteria number, especially when bacteria are under stress.

Based on these results, it is concluded that the heat stressed *E. coli* prepared previously (Irie et al., 2014) and in this study were, in fact, bacteria that were stressed, injured, and with a high non-colony-forming subpopulation indicating a VBNC state on the routine bacteriological media. It is possible to say that heat stressed *E. coli* and other bacteria remain viable and has the potential to re-divide under adequate conditions (McDonald et al., 1983; Shintani, 2006). These results support that the bacterial sample used in the previous research was stressed and in a VBNC-like state, and therefore that an RMM based on intrinsic fluorescence detection was able to detect heat stressed bacteria. Since it is not possible to artificially generate a stressed bacterial sample with the same physiological cell state as naturally occurring bioaerosols, it is important to understand as much as possible about the state of the laboratory prepared stressed bacteria used in the RMM evaluation test and the detection performance of the tested RMM in order to best understand its potential detection ability in the actual environment.

The amount of DnaK expression was quantified by promoter assay in order to clarify whether the observed approx. 50% non-colony forming result in the previous study (Irie et al., 2014) was attributed to heat stress

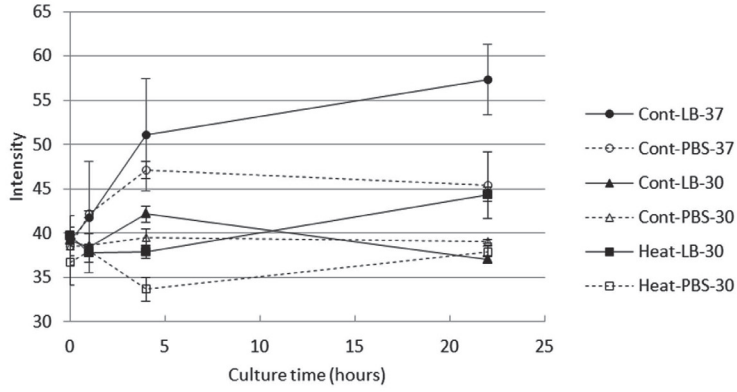


FIG. 8. Intensity of Fresno fluorescence protein at different points during the culture time. The images were processed and quantified according to the image analysis scheme described in MATERIALS AND METHODS. FresnoRFP intensity values were calculated for each of the three images captured for each sample. Treated condition is abbreviated such as [Heat-LB-37] indicating heat stressed, incubated LB at 37°C, [Cont-PBS-30] indicating control (non-heated), incubated in PBS at 30°C.

and whether the stressed bacterial sample responded for recovery (heat shock response). The results shown in Figure 8 imply that DnaK was induced under the utilized heat shock conditions. DnaK expression is directly dependent on σ^{32}, and the transcription of σ^{32} is regulated by σ^{E} against heat shock into periplasm. It is said that σ^{32} and HSPs synthesis increase almost immediately with a modest temperature increase (e.g. 30 to 42°C), reaching a maximum induction of 10- to 15-fold within 5 minutes (Herendeen et al., 1979; Lemaux et al., 1978; Yamamori and Yura, 1980; Yura et al., 2000), at which point, HSPs represent over 20% of

total protein synthesis (Yura et al., 2000). After such a rapid increase, the quantity of σ^{32} and HSPs decrease gradually during an adaptation phase to a new steady-state level (e.g. 2- to 3 fold of the pre-shift level) over the course of 20 to 30 minutes (Yura et al., 2000). In the case of extreme heat shock involving lethal temperatures (e.g. 50°C), production of most normal proteins stops, likely as a result of the inactivation of σ^{70}. However, in the case of HSPs, production continues to increase as long as the cell is able due to σ^{32} and σ^{E} (Blaszczak et al.,1995; Yura et al., 2000). Furthermore, transcription of *rpoH*, which depends upon σ^{E}, is required in order to synthesize σ^{32} at relatively high temperatures (Erickson and Gross, 1989).

According to the results in Figure 8, the DnaK expression of [Cont-LB-37] keeps increasing with elapsed incubation time. We believe that this increase in DnaK expression was induced for cell-growth (Bukau and Walker, 1989) at 37°C, (greater than 35°C (Yura et al., 1984)), because the DnaK expression of [Cont-PBS-37] doesn't continue to increase after 4 hours incubation. Needless to say, cells can't grow without nutrition. Some DnaK expression during the first 0 to 1 hour of incubation may have been induced by the heat shock response at modest temperatures (37°C), which is a cytoplasmic stress response regulated by σ^{32}. [Cont-LB-30] and [Cont-PBS-30] did not show an increase in DnaK expression explicitly. It is believed that this is because these were not exposed to heat stress higher than 30°C. This result is consistent with a previous study that DnaK is dispensable for de novo folding at 30°C (Hesterkamp and Bukau, 1998). The result of [Heat-LB-30] indicated explicit increases in DnaK expression around 4-to-22 hours into incubation. The heating temperature used as heat stress in both the previous and this study was 55°C. So, it is inferable that this increase in DnaK expression was initially induced by the periplasmic stress response regulated by σ^{E}. It has been reported that heat stress around 55°C causes damage mainly to periplasm (Katsui et al., 1982; Tsuchido et al., 1985; Tsuchido et al., 1992), and the results of this study are consistent with previous reports. [Heat-PBS-30] results indicated that bacteria cannot synthesize proteins efficiently without nutrition, but through small efforts might continue to recover. From another perspective, it is possible to say that an increase in DnaK expression in this study was merely caused by growth recoveries because it has been reported that σ^{32} is required for cell proliferation and DnaK is essential for cell growth at relatively high temperatures (Bukau and Walker, 1989; Hesterkamp and Bukau, 1998; Itikawa and Ryu, 1979; Kusukawa and Yura, 1988; Kregel, 2002; Paek and Walker, 1987; Tsuchido et al., 1986; Zhou et al. 1988). For example, [Heat-LB-30] looks as if there was a growth-delay compared to [Cont-LB-37]. However, if so, [Cont-LB-30] should have indicated a continuous increase in DnaK expression as did [Cont-LB-37], or, it should have indicated an earlier increase in DnaK expression than [Heat-LB-30]. Consequently, it is believed that the first inference stated above has more validity. Additional experimentation is needed to confirm this inference.

In this study, the state of heat-stressed bacteria as experimental samples from a biochemical/molecular biological perspective was investigated. It was found that our previous heat treatment condition (Irie et al., 2014) generated a VBNC state, but maintained respiratory activity. Furthermore, the heat shock response was investigated as it pertains to cell injury recovery by observing DnaK expression, which is a typical heat shock protein. Although the expression of a reporter protein, FresnoFRP, implies the heat shock response via σ^{32}, the periplasmic heat shock response system via σ^{E} may be induced more powerfully due to the relatively higher heat conditions and the blebbing and vesiculation of the outer membrane by heat stress (Katsui et al., 1982).

When stress derived from environments in the manufacture of foods and pharmaceuticals (e.g. the sterilization process) is discussed, it is necessary to comprehensively understand metabolic conditions and stress responses for both semi-lethal and lethal stress conditions, while considering other kinds of cell damage and additional recovery systems. Furthermore, it is important to understand what types of RMM technologies can detect bacteria under what kinds of conditions. For example, which RMM technology is capable of stressed bacterial detection, in what type of detection environment (e.g. density of microbes and presence of other interfering materials), in order to provide proper new technologies capable of the better qualification of varied manufacturing environments. Given the limited ability of the traditional culture based method to detect organisms that are non-culturable but maintain metabolic activity, for example respiratory activity, identifying complementary RMM technologies that can help fill this detection gap will better ensure an understanding of the environment and changes within in the food and pharmaceutical industries.

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AUTHORS' CONTRIBUTIONS

K. Irie planed this study, mainly carried out the experiments, and wrote the initial draft of the manuscript. A. Scott contributed to the accurate interpretation of data, evaluation of the relevance of discussion and helped to prepare the manuscript. N. Hasegawa designed DNA experiments, contributed to discuss the data and assist to prepare the manuscript.

CONFLICT OF INTEREST DECLARATION

The authors Kanami Irie and Norio Hasegawa are employed by Azbil Corporation, and Allison Scott is an employee of an affiliate of the Azbil Corporation, Azbil North America Research and Development, Inc. Azbil Corporation owns the intellectual property related to the IMD-A product. Although none of the data in this article is from the IMD-A system, the IMD-A system is mentioned in the article.

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