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DegP and Related Genes as Stress-Markers for *E.Coli*-Viability – Ultra-Sensitive RT-Real-Time PCR

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Summary. The study was done to set up an on-line viability test system based on the stress-response of pathogenic *E.coli*. Fully automated and computerized pathogen detection was achieved via *RNA* expression monitoring using an high-speed real-time PCR-system with single molecule sensitivity. The device was developed in a collaboration of SAUR, France, the TU Delft (Kluyver Laboratorium – Analytical Biotechnology, Netherlands) and Attophotonics Bioscience, Austria.

The automated viability assay is fully hands-off, using a continuous concentration process of the pathogens from litres sample down to tens of millilitres, cell cracking to *DNA*/*RNA* solution and further concentration down to a few hundred microlitres. RT-PCR and real-time-PCR are done with a robotic system.

DegP found from *E.coli* to human was selectively induced within the device via heat shock at 50°C. Up to 1000 fold induction was achieved from environmental to heat-shock level. It was proven that *degP* quantification *via* RT-real-time PCR provides an excellent basis for multi-organism viability detection.

Keywords. Oligonucleotide; *DNA*; *E.coli*; Stress response; Realtime PCR.

Introduction

Quantitative viability tests can be based on different physiological reactions and properties of the organism of interest. Among other techniques vital staining, mobility, enzymatic activity, or integrity under the microscope have been used to quantify the number of living cells within a population. The most reliable one is the *RNA* expression of a cell. Our aim was to find a gene that can be highly induced by

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a chemical or physical stimulus given by the automated pathogen detection unit (see Experimental). Furthermore, the gene has to be highly conserved throughout the prokaryotic and eukaryotic kingdoms but still needs significant variability to realize a feasible application of this viability test to screen for pathogenic organisms in the environment. The concentration process of the cells is based on Ca^{++} precipitation, adsorption, and inclusion as described in the experimental section. Due to the necessity of heating the system to 50°C it was appropriate to investigate the field of heat shock genes. Furthermore, to be able to distinguish between non-induced and induced cells in RT-real-time PCR it was necessary to have at least 20 times higher *RNA* expression levels in the induced state. It is also known that some heat shock genes are only induced within a very narrow time window (*e.g.* transcription factors) whereas our need was a gene which shows its highest expression level after 40 minutes because of the optimized time for the precipitation process before the cells get cracked. The overall goal was to achieve this induction without

Table 1. Features of putative genes for heat shock experiments

gene	*function*	*induction possible by*	*max. induction after* t/min
groEL[1, 2, 3]	chaperone, Hsp60	starvation, heat, phage infection, ethanol 4% v/v	40
degP[1, 2]	chaperone like protein at low temp, protease activity at raised temperature [5]	heat, starvation, ethanol 4% v/v	15
clpA[1, 2]	small heat shock protein	heat	15
dnaK	chaperone, Hsp70	starvation, heat, ethanol, *DTT* at 0.5 g/l ~ 12 m*M*	40
ibpA/B[1, 2]	small heat shock proteins	heat, ethanol 4% v/v, 1.2% paraquat	15
ibpA	small heat shock protein	heat, ethanol 4% v/v, 1.2% paraquat	15

Table 2. PCR features of the chosen genes

gene	*primer*	*length of fragment*/bp	*annealing temp.*/°C
groEL	groEL F/R	505	54
degP	degP F/R	410	53.2
clpA	clpEC F/R	411	52.9
dnaK	hspEC1 F/R	662	57.9
ibpA/B	ibpEC F/R	1356	57.1
ibpA	ibpAEC1 F/R	315	49

any external stimulus but only by the physical and chemical microenvironment within the unit. A set of putative inducible genes was selected due to published sequences and data (see Tables 1 and 2) and these genes were tested on their expression levels in a heat shock situation by realtime PCR analysis. Primers were taken from literature [1–3] or custom designed by the software program ''Oligo''.

Results and Discussion

Choice of the Appropriate Gene Target

Most of the genes exhibited a non-satisfying induction ratio between basic-level and induced state (data not shown). Especially the general heat shock response gene *groEL/Hsp60* as described by *Sheridan et al.* [4] was not applicable in the desired T-shock protocol. After extensive screening the heat shock protease *degP* [1, 2] (see Table 3 and Fig. 1) turned out to be superior to all the other candidates. *DegP* showed an immediate high induction potential of ~100 fold higher after 15 min which was supposed to be the optimum time for the highest expression level due to literature [1].

For automated robotic screening we extended the *RNA* induction period to 40 minutes. Surprisingly *degP* showed its highest *RNA* expression after 40 min and not after the previously described 15 min [1] (see Table 4 and Fig. 2). By taking the 40 min sample we increased the ratio between non-induced and induced state to 110 fold.

Table 3. *DegP mRNA* expression as response to heat shock from 30–50°C in LB media

X	*Sample taken after t*/min	*Threshold*
Sample 1 (neg. control)	X	0.000
Sample 2	0	19.675
Sample 3	5	17.428
Sample 4	15	13.263

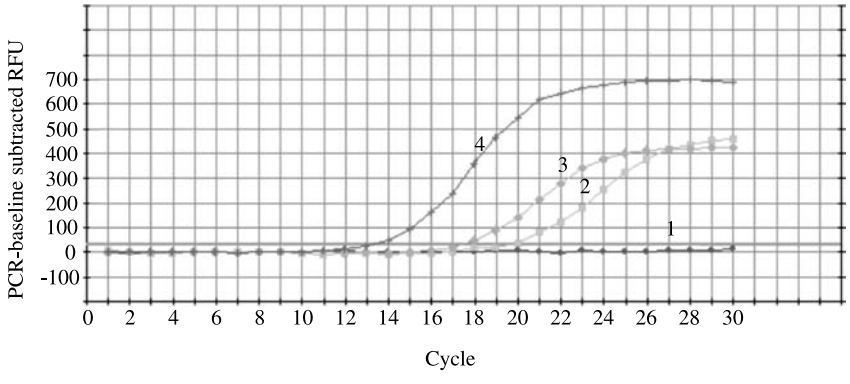

Fig. 1. *DegP mRNA* expression after heat shock from 30–50°C in LB media

Table 4. *DegP mRNA* expression as response to heat shock from 30–50°C in LB media

X	*Sample taken after t*/min	*Threshold*
Sample 1 (neg. control)	X	0.000
Sample 2	0	17.954
Sample 3	5	15.336
Sample 4	15	12.086
Sample 5	40	10.830

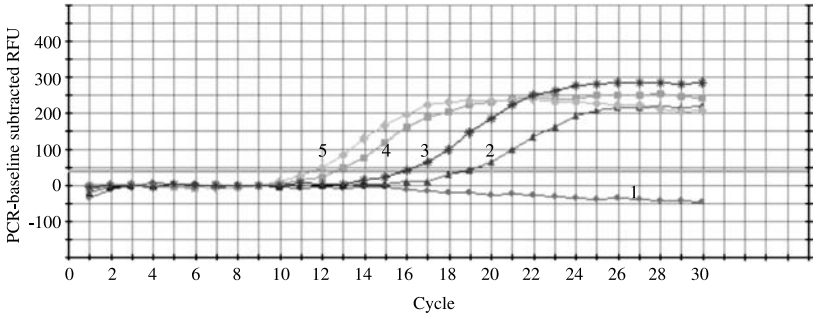

Fig. 2. *DegP mRNA* expression after heat shock from 30–50°C in LB Media (40 min included)

Optimisation of Induction-Ratio Between Non-Induced and Induced State

We further screened to optimise this ratio in between the non-induced and induced state. Surprisingly, this ratio increases when the cells are shocked from 4 to 50°C within one minute after keeping the cells at 4°C for two hours which is also proposed by *Spiess et al*. [5]. This very severe shock led to the unexpected RNA induction of 1000 fold (see Table 5 and Fig. 3).

Investigation of Co-Stimuli Along With Heat Shock

Furthermore, we investigated the inducibility of the *degP* gene with various other stimuli, *e.g*. UV light, chemical shock, and nutrition stress. *E.g*. growth without C-sources could lead to an unwanted activation of *degP* and reduce the signal ratio

Table 5. *DegP mRNA* expression as response to heat shock from 4–50°C in LB media

X	*Sample taken after t*/min	*Threshold*
Sample 1 (neg. control)	X	0.000
Sample 2	0	23.546
Sample 3	5	20.649
Sample 4	15	16.411
Sample 5	40	13.535

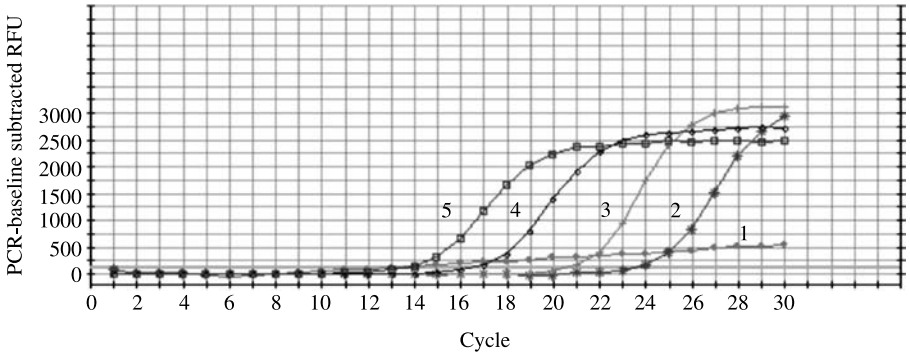

Fig. 3. *DegP mRNA* expression after heat shock from 4–50°C in LB Media

in a heat shock and the predictability of the signal. Neither UV light nor 4% ethanol had any impact on the expression of *degP* (data not shown). Contrary to that, nutrition stress showed a clear expression of *degP* after altering the media from LB to MMA [6, 7] (see Experimental – data not shown). Due to the fact that the water to be analysed by our unit will be more comparable to MMA media the described heat shock cannot be performed equally effective as in the lab experiment. We therefore sought for a way to stabilize the cells in a non-nutrition stress status before applying the heat shock. Adding 0.6% glucose [7] to the MMA-culture led to the same *RNA* expression ratio (see Table 6 and Fig. 4) as achieved in LB media (see Table 5 and Fig. 3).

Table 6. *DegP mRNA* expression as response to heat shock from 4–50°C in MMA media +0.6% glucose

X	*Sample taken after t*/min	*Threshold*
Sample 1 (neg. control)	X	0.000
Sample 2	0	22.464
Sample 3	40	12.969

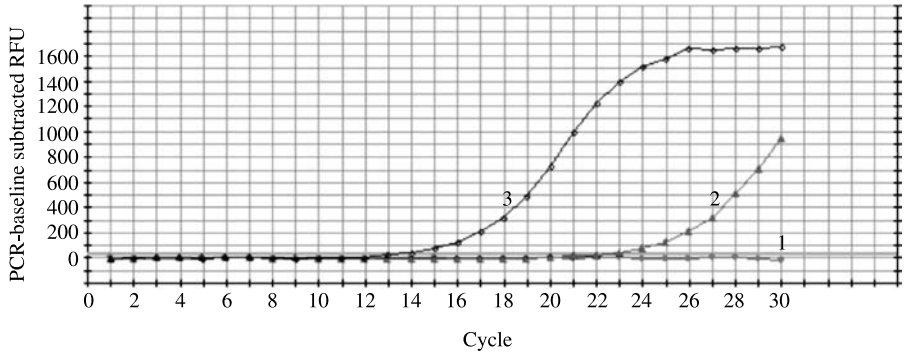

Fig. 4. *DegP mRNA* expression after heat shock from 4–50°C in MMA Media +0.6% glucose

Conclusion

Our goal to find an appropriate gene for performing a viability test was successful in finding the heat shock protease *degP/htrA*. This $\sigma 32$ independent [8], hardly described stress gene seems to play a pivotal role in the heat shock response of *E.coli* as investigated mainly by *Spiess et al*. [5] and others [8–11] and also a role in nutrition stress as proved by our experiments. It provides chaperone activity at low temperatures (below 22°C) and changes as proposed in the model by *Spiess et al*. [5] from chaperone activity *via* a conformational change to protease activity at high temperatures (above 42°C). *DegP* is wide spread over the procaryotic and eucaryotic kingdom which indicates a strong conservation (Table 7). It is as well present in many pathogenic organisms for waterindustry, medicine, and beyond, *e.g. Salmonella typhimorium*, *Klebsiella pneumoniae*, *Yersinia pestris*, *Vibrio cholerae*, *Haemophilus influenzae*, *Helicobacter pylori*, up to human [9] with relevance in medicine. In all organisms mentioned *degP* shares a sequence homology of more than 70% to *E.coli* (Table 8). Due to our results the gene seems to be completely inactivated in cells kept on cool temperatures and is strongly and reproducibly activated in a severe heat shock of 50°C (Fig. 5). It is a potential target for the design of an automated viability test due to our system. We could overcome the *degP* expression in nutrition stress by adding 0.6% glucose to the culture which was sufficient to lower the stress level of the cells. *DegP RNA* expression can be initialized by heat shock even in a media environment (MMA media [7]) which is more comparable to tap water than the LB media our experiments were first performed in. Furthermore, we have the goal to develop a cell arrest protocol to keep the cells in a steady state situation to provide a non growing culture to keep the cell number stable.

Table 7. PCR – fragment within sequence

Italic = in frame
Bold = amplified sequence
Underlined = Primer

```
  1 tatatcagcg gtatgaccga cctctatgcg tgggatgaat accgacgtct gatggccgta
 61 gaacaataac caggcttttg taaagacgaa caataaattt ttacctttg cagaaacttt
121 agttcggaac ttcaggctat aaaacgaatc tgaagaacac agcaattttg cgttatctgt
181 taatcgagac tgaaatacat gaaaaaaacc acattagcac tga gtcgact ggctctgagt
241 ttaggtttgg cgttatctcc gctctctgca acggcggctg agacttcttc agcaacgaca
301 gcccagcaga tgccaagcct tgcaccgatg ctcgaaaagg tgatgccttc agtggtcagc
361 attaacgtag aaggtagcac aaccgttaat acgccgcgta tgccgcgtaa tttccagcag
421 ttcttcggtg atgattctcc gttctgccag gaaggttctc cgttccagag ctctccgttc
481 tgccagggtg gccagggcgg taatggtggc ggccagcaac agaaattcat ggcgctgggt
541 tccggcgtca tcattgatgc cgataaaggc tatgtcgtca ccaacaacca cgttgttgat
601 aacgcgacgg tcattaaagt tcaactgagc gatggccgta agttcgacgc gaagatggtt
      ......
```

Translated sequence

T L A L S R L A L S L G L A L S P L S A T A A E T S S A T T A Q Q **Met** P S L A P **Met** L E K V **Met** P S V V S I N V E G S T T V N T P R **Met** P R N F Q Q F F G D D S P F C Q E G S P F Q S S P F C Q G G Q Q G G N G G G Q Q Q K F **Met** A L G S G V I I D A D K G Y V V T N N H V V D N A T V I K

Table 8. Sequence alignment

```
                                 **************************************************
Escherichia        1 ------MKKTTLALSALALSLGLALSPLSATAAETSS-ATT--------------------
Salmonella         1 ------MKKTTLAMSALALSLGLALSPLSATAAETSSSAMT--------------------
Yersinia           1 ------MKKTTLVLSALALSIGFAMGPVSSVVAAETA-ASS--------------------
Bos                1 ----------------------VCASNEPVCGSDAKTYTNLCQ--------------------
Homo              91 VPFGVPASATVRRRAQAGLCVCASSEPVCGSDANTYANLCQ--------------------
Helicobacter       1 -------------------------MSPLKTIRIYSYH-----------------------
Neisseria          1 -MFKKYQYLALAALCAASLAGCDKAGSFFGADKKEASFVER--------------------
Listeria          82 VEGVTPEGEKFAGATEDAAEASSTNAFFEEASNKESEPARP--------------------
Staphylococcus   301 NDINTPSLSKTDDDRKLDEKIHVEDKHKQNADSSETVGYQSQSSASHRITEKRNNAINDH

                                 ************************************************************
Escherichia       35 ----AQQMPSLAPMLEKVMPSVVSINVEGSTTVNTP----RMPRNFQQFFGDDSPFCQEG
Salmonella        36 ----AQQMPSLAPMLEKVMPSVVSINVEGSTTVNTP----RMPRNFQQFFGDDSPFCQDG
Yersinia          35 ----SQQLPSLAPMLEKVMPSVVSINVEGSAPVSSAGA-RGMPPQFQQFFGDNSPFCQDG
Bos               22 ----LRAASRRSERLHQPPVIVLQRGACGQGQEDPNSL-RHKYNFIADVVEKIAPAVVHI
Homo             132 ----LRAASRRSERLHRPPVIVLQRGACGQGQEDPNSL-RHKYNFIADVVEKIAPAVVHI
Helicobacter      14 ----DSIKDSIKAVVNISTEKKIKNNFIGGGVFNDP--------FFQQFFGDLGGMIPKE
Neisseria         41 ----IKHTKDDGSVSMLLPDFVQLVCSEGPAVVNIQAAPAPRTQNGSSNAETDSDPLADS
Listeria         123 ----APGPRRAGTTGGGAVPPTNRVNNGGSGNGNGEPP-KRGKHFIGYFLTALIGVIIGG
Staphylococcus   361 DKLNGQKPNAKTSANNNQKKATSKLNKGRATNNNYSDILKKFWMMMYWPKLVILMGIIILI

                                 ************************************************************
Escherichia       87 SPFQSSPFCQGGQGGNGG----------------------------------------------
Salmonella        88 SPFQNSPFCQGGGNGGNG----------------------------------------------
Yersinia          90 SPFQGSPMCQGDLGGLGQ----------------------------------------------
Bos               77 ELFRKLPFSKREVP------------------------------------------------
Homo             187 ELFRKLPFSKREVP------------------------------------------------
Helicobacter      62 RMER----------------------------------------------------------
Neisseria         97 DFFYEFFKRLVPNMPEIP--------------------------------------------
Listeria         178 LIIFFVAWDNGDNADTTSNSNNKATKVEKVSVDTTSDVTKAVDKVQDAVVSVLNYQSSSS
Staphylococcus   421 VILNAIFNNVNKNDRMNDNNDADAQKYTTTMKNANNTVKSVVTVEN---------ETSKD

                                 ****************************************
Escherichia      105 --GQQQK--FMALGSGVIIDAD--KGYVVTNNHVVDNATVIKVQLS-DGRKFDAKMVGKD
Salmonella       106 --GQQQK--FMALGSGVIIDAA--KGYVVTNNHVVDNASVIKVQLS-DGRKFDAKVVGKD
Yersinia         108 --GMPSKREFRSLGSGVIIDAG--KGYVVTNNHVVDNANKISVKLS-DGRSFDAKVIGKD
Bos               91 ----------VASGSGFIVSE---DGLIVTNAHVVTNKHRVKVELK-NGATYEAKIKDVD
Homo             201 ----------VASGSGFIVSE---DGLIVTNAHVVTNKHRVKVELK-NGATYEAKIKDVD
Helicobacter      66 ----------ALGSGVIISK---DGYIVTNNHVIDGADKIKVTIPGSNKEYSATLVGTD
Neisseria        115 --QEEADDGGLNFGSGFIISK---DGYILTNTHVVTGMGSIKVLLN-DKREYTAKLIGSD
Listeria         238 LDGTTTSEQEASSGSGVIYKKANGKAYIVTNNHVVADANKLEVTFT-NGKKSEAKLLGTD
Staphylococcus   472 SSLPKDKASQDEVGSGVVYKKSGDTLYIVTNAHVVGDKENQKITFS-NNKSVVGKVLGKD
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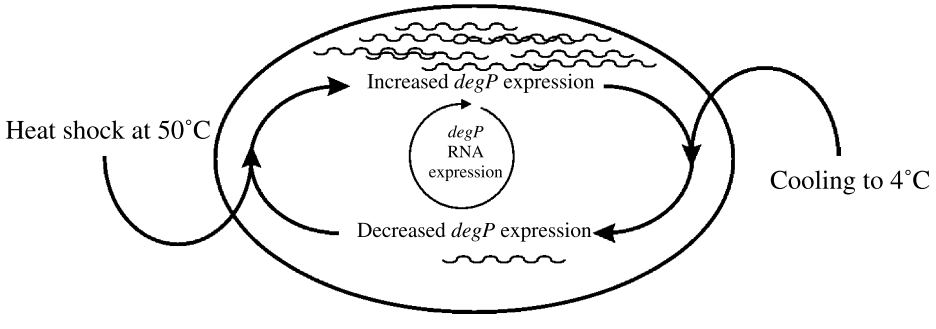

Fig. 5. Schematic *degP RNA* expression in cooled and heated environment

Experimental

Robotic System

The novel automated pathogen analyser is a device able to automatically detect a set of organisms, some of them highly pathogenic, *e.g. Legionella spec*., some of them indicator organisms, *e.g. E.coli*. The device is applied in technical water supply activities, drinking water supplies, water quality control, customer claims, search for and control of pathogenic micro organisms in drinking water, and screening for pathogenic micro-organisms in cooling water, in energy production, and in nuclear power plants. The robot system in use is described in a pending patent (Schalkhammer, Huau; France (2001), Europe (2002)). The diagram below summarizes the major steps of the process (Fig. 6).

Specific features are:

- Procedures to concentrate pathogens from water without using filtration or centrifuges
- Procedures to concentrate genetic material from pathogens from ml down to μl
- Molecular methods based on real-time *DNA/RNA* – technology (Fig. 7)
- Optimised surface coating to allow the handling of single molecules in large volumes
- Assess the efficacy of *DNA* and *RNA* handling and quantification
- Automated pathogen monitoring system with computer control

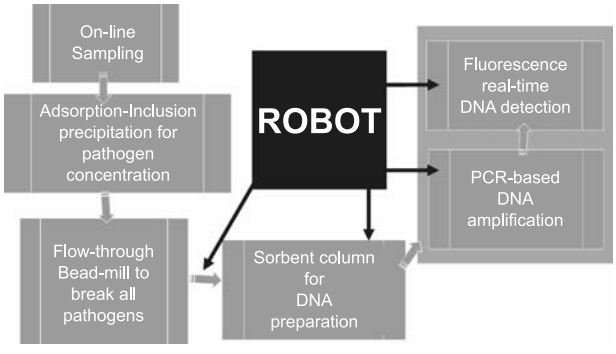

Fig. 6. Schematic diagram of the automated pathogen analyzer

Fig. 7. Photo of the central PCR-handling unit

Microorganisms

Various natural strains and K12 of *E.coli* had been used in this work.

Fermentation and Media

Organisms were either grown in Luria Broth (LB) media in 100 ml *Erlenmeyer* flasks at 37°C or in MMA minimal media [6, 7]: $(NH_4)_2SO_4$(1 g); K_2HPO_4(10.5 g); KH_2PO_4(4.5 g); $MgSO_4 \cdot 7H_2O$(0.1 g); H_2O and 1 l at $pH = 7$.

Shake flask experiments were performed in air shaker at 230 rpm or water bath for heat shock experiments.

Ca^{++} Precipitation Process

1. Increase temperature of sample to 50°C; 2. Add 20 ml of *Tris* 8.5 (0.5 *M*) → 5 m*M* final concentration; 3. Add 40 ml of calcium acetate solution (20%), mix 30 sec; 4. Add 30 ml of Na_2CO_3 solution (1 *M*); 5. Precipitate 5–10 min; 6. Elute supernatant; 7. Add 14 ml of formic acid; 8. CO_2 escape.

Induction of Stress Response and Sample Preparation

Heat shock was induced in the mid exponential phase of growths to 50°C. Culture size was 20 ml throughout all experiments. Culture was first grown on at 30°C, then diluted into fresh media, and again grown until OD ~ 0.2–0.3. All samples (1 ml) were immediately taken before the stress, 5, 15, and 40 min after the stress. Negative control was always kept on starting temperature.

Extra heat shock was performed from 4 to 50°C within 1 min. Cultures were grown identically but put for at least 2 hours on 4°C and then shocked immediately to 50°C.

Nutrition stress experiment was performed by altering the media from LB to MMA [6, 7] without any C-source.

Stabilizing the starving culture with 0.6% glucose and following heat shock experiment was performed by inoculating a culture from LB media in MMA + 0.6% glucose [7], growing cells for ~7 hours until OD reached 0.2–0.3. The culture was placed on 4°C for 2 hours and shocked immediately to 50°C within 1 min.

Chemical stress experiments were performed with the common stress inducer 4% *Et*OH. UV stress experiments were performed with UV light under the flow bench for 10 min.

RNA Purification

RNA was immediately isolated with Qiagen *RN*easy Isolation Kit. Isolation was performed according to *DNA* free protocol. Total *RNA* content was measured at 260 nm in an Amersham photometer after every isolation. *RNA* samples were stored at −20°C.

RT-PCR Primers

The specific combination of primers [1–3] utilized for RT-PCR were either obtained from literature or custom designed by "Oligo" software.

The optimised primer sequences of the *degP* gene are degPEC1F 5′-CCA CAT TAG CAC TGA G′-3 and degPEC1R 5′-TTT AAT GAC CGT CGC G′-3.

RT-PCR

The RT PCR was performed with a Biorad Real-time I-Cycler in a one step RT-PCR reaction with a Qiagen One Step RT PCR Kit. Each reaction mixture (50 μl) contained 1 μl of respective *mRNA*

template, 5 μl of 5 pmol/μl forward and reverse primer, 10×10 μl buffer, 2 μl *dNTP*, 2 μl of one step enzyme mix, 1 μl of a 1:2000 SyBR® Green dilution provided by Roche, and 24 μl of *RNase* free water.

Cycle Program

1 × 30 min 60°C; 1 × 15 min 95°C; 30 × 1 min 94°C; 1 min respective annealing temperature (see Table 2), 1 min 72°C; 1 × 10 min 72°C; 79 × 15 sec 60°C + 0.5°C for melting curve determination.

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