Available online at www.sciencedirect.com
ScienceDirect
Research in Microbiology 157 (2006) 811–818
Research in Microbiology
www.elsevier.com/locate/resmic

The alternative sigma factor RpoH2 is required for salt tolerance in *Sinorhizobium* sp. strain BL3

Panlada Tittabutr [a], Waraporn Payakapong [a], Neung Teaumroong [a], Nantakorn Boonkerd [a], Paul W. Singleton [b], Dulal Borthakur [c,*]

[a] *School of Biotechnology, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand*
[b] *Department of Tropical Plant and Soil Sciences, University of Hawaii at Manoa, Honolulu, HI 96822, USA*
[c] *Department of Molecular Biosciences and Bioengineering, University of Hawaii at Manoa, Honolulu, HI 96822, USA*

Received 23 December 2005; accepted 11 July 2006

Available online 2 August 2006

Abstract

The objectives of this investigation were to isolate the *rpoH2* gene encoding an alternative sigma factor from *Sinorhizobium* sp. BL3 and to determine its role in exopolysaccharide (EPS) synthesis, salt tolerance and symbiosis with *Phaseolus lathyroides*. The *rpoH2* gene of *Rhizobium* sp. strain TAL1145 is known to be required for EPS synthesis and effective nodulation of *Leucaena leucocephala*. Three overlapping cosmid clones containing the *rpoH2* gene of BL3 were isolated by complementing an *rpoH2* mutant of TAL1145 for EPS production. From one of these cosmids, *rpoH2* of BL3 was identified within a 3.0-kb fragment by subcloning and sequencing. The cloned *rpoH2* gene of BL3 restored both EPS production and nodulation defects of the TAL1145 *rpoH2* mutants. Three *rpoH2* mutants of BL3 were constructed by transposon-insertion mutagenesis. These mutants of BL3 grew normally in complete or minimal medium and were not defective in EPS synthesis, nodulation and nitrogen fixation, but they failed to grow in salt stress conditions. The mutants complemented with cloned *rpoH2* from either BL3 or TAL1145 showed higher levels of salt tolerance than BL3. The expression of *rpoH2* in BL3 started increasing during the exponential phase and reached the highest level in the mid-stationary phase. These results indicate that RpoH2 is required for salt tolerance in *Sinorhizobium* sp. BL3, and it may have additional roles during the stationary phase.

Keywords: Alternative sigma factor; *rpoH2*; *Sinorhizobium*; Exopolysaccharide; Salt tolerance

1. Introduction

TAL1145 is a strain of *Rhizobium* sp. that forms nitrogen-fixing nodules on the tree legume *Leucaena leucocephala (leucaena)*. It produces large amounts of exopolysaccharide (EPS) in medium containing mannitol. We have shown previously that in this strain, the alternative sigma factor RpoH2 regulates the transcription of several *exo* genes required for EPS synthesis [16]. *rpoH2* mutants of TAL1145 are also defective in nodulation and nitrogen fixation on the tree legume *Leucaena leucocephala*. Conversely, *rpoH2* mutants of *Sinorhizobium meliloti* are not defective in EPS synthesis and symbiosis with the host legume alfalfa [24]. However, cloned *rpoH2* of *S. meliloti* Rm1021 complemented the *rpoH2* mutant of *Rhizobium* TAL1145 for EPS synthesis, suggesting that RpoH2 of TAL1145 and *S. meliloti* may have similar regulatory functions [16]. It is not known if RpoH2 is required for EPS synthesis and symbiosis in other *Rhizobium* and *Sinorhizobium* strains.

Beside EPS synthesis, alternative sigma factors can also be involved in transcriptional regulation of genes for sporulation, flagellum biosynthesis, stationary phase survival, nitrogen fixation and stress response in bacteria [36]. For example, the *rpoS* gene products are responsible for the transcription of many genes expressed during stationary phase and osmotic stress [12]. In *Bacillus subtilis*, an alternative sigma factor, SigB, is expressed during stationary phase in rich medium or after depletion of glucose, phosphate or oxygen in the medium, and also in response to heat and osmotic stresses [11]. In *Mycobac-*

* Corresponding author. Department of Molecular Biosciences and Bioengineering, 1955 East-West Road, Ag. Science 218, Honolulu, HI 96816, USA.
E-mail address: dulal@hawaii.edu (D. Borthakur).

0923-2508/$ – see front matter
doi:10.1016/j.resmic.2006.07.001

terium tuberculosis, SigF is induced during stationary phase, nitrogen depletion, and due to oxidative, osmotic and low temperature stresses [5]. In *E. coli*, RpoH induces the transcription of heat shock proteins including molecular chaperones and proteases [10,37]. The *groESL* operon, which encodes molecular chaperones in *E. coli*, is regulated by RpoH in response to high temperature [18]. In *S. meliloti*, RpoH1 has been shown to induce several genes for heat shock proteins, while RpoH2 has functional but redundant roles in the induction of genes for heat tolerance [24]. Recently, Bittner and Oke [2] found that one of the four *groESL* operons in *S. meliloti*, *groESL5*, was controlled by RpoH1 under free-living conditions, whereas none of the chaperone genes was controlled by RpoH2.

Sinorhizobium sp. strain BL3 is a salt-tolerant root-nodule bacterium that nodulates *Phaseolus lathyroides* [26]. This strain also makes copious amounts of EPS when grown in yeast extract–mannitol medium. Since RpoH2 is required for the transcription of *exo* genes in *Rhizobium* sp. strain TAL1145 but not in *S. meliloti* Rm1021, we wanted to know if RpoH2 is involved in EPS synthesis in *Sinorhizobium* sp. strain BL3. BL3 can be grown in medium containing up to 0.6 M NaCl and it forms nitrogen-fixing nodules on *P. lathyroides* growing in saline soils [26]. Since some alternative sigma factors have been shown to induce genes for stress tolerance in other bacteria, we hypothesized that RpoH2 of *Sinorhizobium* sp. strain BL3 might also be required for tolerance to environmental stress, such as the ability to grow under high salt condition. The aims of this investigation were to isolate the *rpoH2* gene from BL3 and to determine if it is required for EPS synthesis, symbiosis and salt tolerance in this strain.

2. Materials and methods

2.1. Bacterial strains, plasmids and culture conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *Rhizobium* and *Sinorhizobium* strains were grown in yeast extract–mannitol (YEM) medium [29] or YEM medium containing 0.5 mM NaCl for growth under salt stress conditions. In order to determine the colony morphology, a 2% (w/v) stock solution of Calcofluor White M2R (Cellufluor; Polysciences, Warrington, PA) was autoclaved and added to sterilized YEM agar medium at a final concentration of 0.02%. The synthesis of the Calcofluor-binding EPS by the colonies was observed as bright (Cal^+) or dim (Cal^-) fluorescence upon exposure to UV from a laboratory-bench long-wave UV lamp [9]. For EPS analysis, *Sinorhizobium* strains were grown in 10× GMS liquid medium [4].

2.2. Construction of a Sinorhizobium sp. BL3 genomic DNA cosmid library

A *Sinorhizobium* sp. BL3 genomic DNA cosmid clone library was constructed by partial digestion of genomic DNA with the restriction enzyme *Sau*3AI. DNA fragments were size-fractionated using sucrose gradient centrifugation and fragments of 20–30 kb were selected. The fragments were ligated to the cosmid vector pLAFR3, which was previously digested with the restriction enzyme *Bam*HI and dephosphorylated. The ligation mixture was packaged in vitro with Gigapack® II XL packaging extracts and used to transfect *E. coli* strain CVS257 following the protocol specified by manufacturer (STRATAGENE, USA). The transfected *E. coli* cells were plated onto LB containing 5 μg/ml tetracycline, and incubated at 37 °C for 24 h. 7000 colonies were pooled to constitute the cosmid clone library.

Table 1
Bacterial strains and plasmids

Strains and plasmids	Relevant characteristics	Source or reference
Rhizobium		
TAL1145	*Rhizobium* sp., wild-type strain, nodulates *Leucaena leucocephala* and *Phaseolus vulgaris*, EPS^+, Rif^r, Str^r	[8]
RUH102	TAL1145 *rpoH2* mutant, Km^r	[16]
Sinorhizobium		
BL3	*Sinorhizobium* sp., wild-type strain, highly salt tolerant, isolated from Thailand, Rf^r, Sm^r	[26]
RUH178, RUH179, RUH180	*RpoH2*::Tn*3*Ho*gus* insertion mutants of BL3; Rf^r, Sm^r, Km^r	This study
Plasmids		
pLAFR3	Wide-host-range P1 group cloning vector, used for BL3 genomic DNA library construction, Tc^r	[30]
pRK404	Wide-host-range P1 group cloning vector, Tc^r	[6]
pUHR37	The 2.0-kb *Hin*dIII fragment containing *rpoH2* of *Rhizobium* TAL1145, cloned into pRK404, Tc^r	[16]
pUHR323	*rpoH2* of *S. meliloti* Rm1021 cloned in pRK404, Tc^r	[16]
pUHR333, pUHR334, pUHR335	Overlapping cosmid clones from the BL3 genomic library containing *rpoH2*	This study
pUHR336	PLAFR3 containing a 7.0-kb *Eco*RI fragment from pUHR333, contain *rpoH2* of BL3, Tc^r	This study
pUHR338	The 6.0-kb *Hin*dIII–*Eco*RI fragment of pUHR336 containing *rpoH2* of BL3 subcloned in pRK404, Tc^r	This study
pUHR339	The 3.0-kb segment comprising of a 0.7-kb *Bam*HI and a 2.3-kb *Bam*HI–*Eco*RI fragment from pUHR336 containing *rpoH2* of BL3 subcloned in pRK404, Tc^r	This study
pUHR340	The 2.3-kb *Bam*HI–*Eco*RI fragment from pUHR336 in pRK404, Tc^r	This study
pUHR341	The 3.0-kb *Hin*dIII–*Bam*HI fragment from pUHR336 in pRK404, Tc^r	This study
pUHR345	pUHR333*rpoH2*::Tn*3*Ho*gus*-180, which was used to construct the *rpoH2* mutant RUH180 from BL3	This study

2.3. DNA manipulation, Tn3Hogus-insertion mutagenesis, subcloning and sequencing

DNA manipulation, Tn*3*Ho*gus*-insertion mutagenesis, and subcloning of DNA fragments were done as described pre-

viously [16]. The position of Tn*3*Hog*us* insertion in *rpoH2* was determined by sequencing the PCR fragment generated by using *gus* reverse primer (5′-AATTCCACAGTTTTCGCG-ATC-3′) and a forward primer from the subcloned fragment. Deduced amino acid sequences were compared by using online program BLASTP from the National Center for Biotechnology Information (NCBI) with the following parameter settings; database: nr, selected from: all organisms, and filter: low complexity. The GenBank accession number for the 3.0-kb DNA fragment containing *rpoH2* is DQ340403.

2.4. β-Glucuronidase (Gus) activity assay and EPS extraction and analyses

Gus was assayed as described previously [16]. For EPS extraction and analysis, *Sinorhizobium* BL3 and the *rpoH2* mutant RUH180 were grown in 10× GMS liquid medium and EPS was isolated, purified and fractionated as described by Parveen et al. [25]. To determine the amount of EPS (mg/mg of protein) produced by rhizobia, cells were grown in 50 ml of YEM liquid medium for seven days. The supernatant was collected for determination of the hexose content by anthrone analysis [32]. The cell pellets were washed 3 times with 1 M NaCl, and resuspended in 1 ml of lysis buffer containing 140 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4; 1.8 mM KH_2PO_4; 0.1% (v/v) Triton X-100; lysozyme at final concentration 0.2 mg/ml, and incubated at 4 °C for 10 min. Samples were sonicated (amplitude 70, 6 × 60 s) in an ice bath. The amount of soluble protein in supernatant phase was determined using Lowry's method [19].

2.5. Plant experiments

Seeds of *Leucaena leucocephala* (K636), *Phaseolus vulgaris* (Royal) and *Phaseolus lathyroides* (wild) were surface-sterilized and germinated using standard methods [29]. Seedlings of *L. leucocephala* and *P. vulgaris* were grown in modified Leonard jar assemblies containing vermiculite, while the *P. lathyroides* seedlings were grown in sterile sand in pots. All plants were maintained in a controlled growth chamber and watered with nitrogen-free nutrient solution [29]. Three-day-old seedlings were inoculated with 10^8 rhizobia, diluted in sterile plant nutrient solution, from a two-day-old culture. Five replicates were used for each treatment with each Leonard jar containing three seedlings. Uninoculated control plants were grown to check for cross-contamination. *P. vulgaris* were grown for 4 weeks, but *P. lathyroides* and *L. leucocephala* were grown for 8 weeks. The symbiosis functions were assessed by determining the number of nodules, nodule fresh weight, nitrogenase activity, and plant dry weight.

3. Results

3.1. Isolation of cosmids pUHR333, pUHR334 and pUHR335 by complementing an rpoH2 mutant of Rhizobium sp. strain TAL1145

To isolate cosmid clones containing the *rpoH2* gene of *Sinorhizobium* sp. strain BL3, the cosmid clone library of BL3 was transferred to the *Rhizobium* TAL1145 *rpoH2* mutant RUH102, which produces non-mucoid (EPS^-) colonies on YEM agar. Less than 0.5% of the colonies in the transconjugants were mucoid (EPS^+). Fifteen of these EPS^+ colonies was purified and cosmid DNA were isolated from them. Restriction digests of these cosmids showed three distinct types with overlapping fragments. These three cosmids were transferred to the *Rhizobium* TAL1145 *rpoH2* mutant RUH102, and they complemented the *rpoH2* mutation. These cosmids were named pUHR333, pUHR334 and pUHR335.

3.2. Localization of rpoH2 in pUHR333 by subcloning

pUHR333 gave four *Eco*RI fragments of sizes 7.0, 6.6, 6.0, and 2.2 kb. These fragments were subcloned into the *Eco*RI site of pLAFR3 and the resulting plasmids were transferred to RUH102. One of these plasmids, pUHR336 containing the 7.0-kb fragment complemented RUH102. A 6.0-kb *Hin*dIII–*Eco*RI fragment, subcloned from the 7.0-kb *Eco*RI fragment in plasmid pUHR338, also complemented RUH102. A 3.0-kb *Hin*dIII–*Bam*HI fragment or a 2.3-kb *Bam*HI–*Eco*RI fragment, subcloned from pUHR336, did not complement this mutant. However, a 3.0-kb segment comprising a 0.7-kb *Bam*HI and the 2.3-kb *Bam*HI–*Eco*RI fragment, cloned in plasmid pUHR339, complemented the mutant. Therefore, the *rpoH2* gene is located within this 3.0-kb *Bam*HI–*Eco*RI region (Fig. 1).

3.3. The EPS and symbiosis defects of the rpoH2 mutant of Rhizobium TAL1145 are complemented by the cloned rpoH2 gene of Sinorhizobium BL3

To determine whether the function of *rpoH2* in *Rhizobium* can be complemented by *rpoH2* from *Sinorhizobium*, plasmids pUHR339 and pUHR323, containing cloned *rpoH2* from BL3

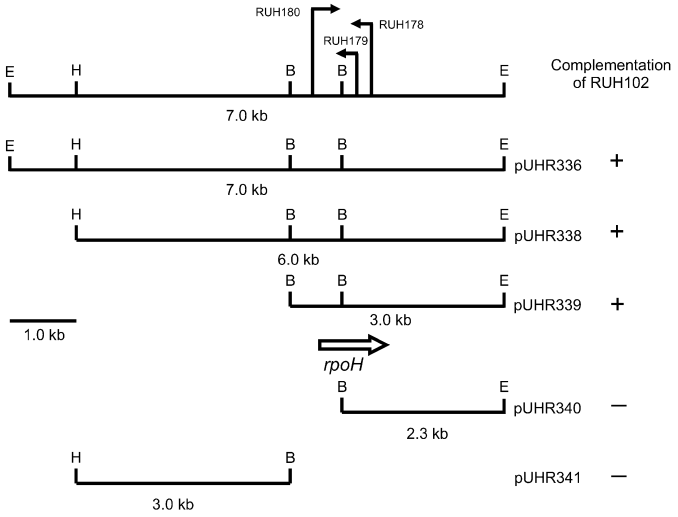

Fig. 1. Restriction map of the 7.0-kb region subcloned from cosmid pUHR336 that contains the *rpoH2* gene from *Sinorhizobium* sp. strain BL3. Smaller subcloned fragments from the 7.0-kb region are also shown. The horizontal open arrow shows the *rpoH2* ORF. The positions of the three *rpoH2*::Tn*3*Hog*us* insertions are shown with solid arrows where the direction of the arrow indicates the orientation of the *gus* reporter gene. B: *Bam*HI, E: *Eco*RI, H: *Hin*dIII.

and *S. meliloti* Rm1021, respectively, were introduced into the *rpoH2* mutant RUH102. The cloned *rpoH2* genes from both *S. meliloti* and *Sinorhizobium* BL3 complemented the EPS defects of RUH102, although the transconjugants of the mutant containing the cloned *rpoH2* gene of *S. meliloti* did not produce as much EPS as TAL1145 (Table 2). The symbiosis defects of RUH102 on *L. leucocephala* were fully complemented by the *rpoH2* genes from both BL3 and *S. meliloti* (Fig. 2). The complemented mutant could develop a cylindrical-shaped nodule that is called 'indeterminate type' with *L. leucocephala*. Mutant RUH102 does not form any nodules on *P. vulgaris*. When the transconjugants of RUH102 containing the cloned *rpoH2* genes of BL3 and *S. meliloti* Rm1021 were inoculated on seedlings of *P. vulgaris*, they formed spherical-shaped nitrogen-fixing nodules called 'determinate type' like TAL1145 (data not shown). Thus the EPS and nodulation defects of the *rpoH2* mutant of TAL1145 were complemented by cloned *rpoH2* from both *Sinorhizobium* BL3 and *S. meliloti*.

Table 2
EPS production by *Rhizobium* sp. strain TAL1145, the *rpoH2* mutant RUH102, and the transconjugants of RUH102 containing the cloned *rpoH2* genes of *Sinorhizobium* sp. strain BL3 and *S. meliloti* Rm1021

Strain	Colony morphologies**	EPS (mg/mg of protein)*
TAL1145	Mucoid, Cal^+	10.96 ± 1.68
RUH102	Non-mucoid, Cal^-	0.68 ± 0.25
RUH102:pUHR339	Mucoid, Cal^+	10.34 ± 2.44
RUH102:pUHR323	Mucoid, Cal^+	8.99 ± 1.28
BL3	Mucoid, Cal^-	4.72 ± 0.32
RUH178	Mucoid, Cal^-	4.14 ± 0.35
RUH179	Mucoid, Cal^-	4.23 ± 0.61
RUH180	Mucoid, Cal^-	3.01 ± 0.77
BL3:pUHR333	Mucoid, Cal^-	4.42 ± 0.78
BL3:pUHR335	Mucoid, Cal^-	4.54 ± 0.19

* The data are mean and standard deviations of three replicates.
** Cal^+: colony produced the Calcofluor-binding EPS and showed a bright UV-fluorescence.
Cal^-: colony did not produced the Calcofluor-binding EPS and showed a dim UV-fluorescence.

3.4. *Isolation of* rpoH2 *mutants of BL3*

pUHR333 was mutagenized with Tn*3*Ho*gus* insertion and the resulting pUHR333::Tn*3*Ho*gus* derivatives were transferred to RUH102. Twenty-two colonies showing the colony morphology similar to RUH102 were selected. In these colonies, the *rpoH2* gene in pUHR333 was expected to be disrupted by the Tn*3*Ho*gus* insertions. Plasmid DNA was isolated from these colonies, and analyzed by restriction digest. Three pUHR333::Tn*3*Ho*gus* derivatives that showed distinguishable insertion positions, were selected (Fig. 1). These insertions were transferred to BL3 and the interrupted genes were marker-exchanged into

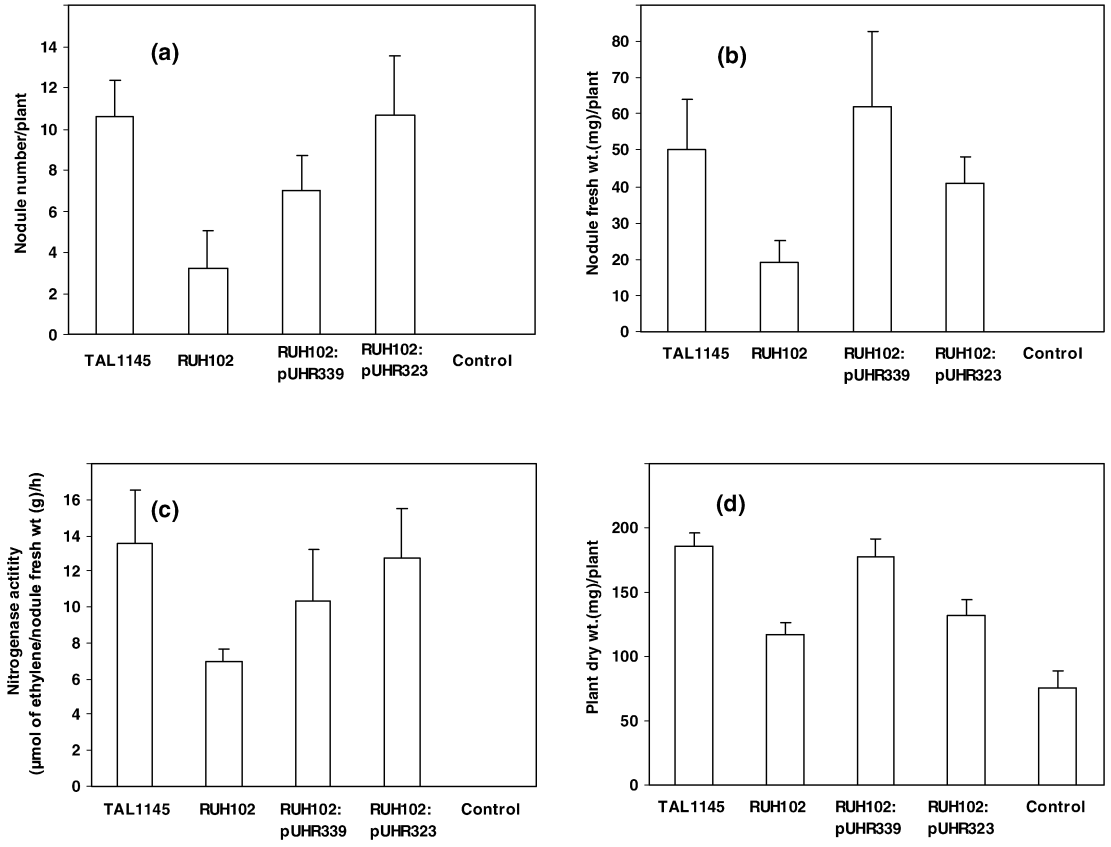

Fig. 2. Complementation of the *Rhizobium* TAL1145 *rpoH2* mutant RUH102 for symbiosis functions with recombinant plasmids pUHR339 and pUHR323, containing the *rpoH2* genes from BL3 and *S. meliloti*, respectively. Bar diagrams show number of nodules (a), nodule fresh weights (b), nitrogenase activities (c), and plant dry weights (d) of *L. leucocephala* plants, inoculated with *Rhizobium* TAL1145, RUH102, RUH102:pUHR339, and RUH102:pUHR323, and uninoculated (control) plants. The error bars indicate standard deviations from three replications.

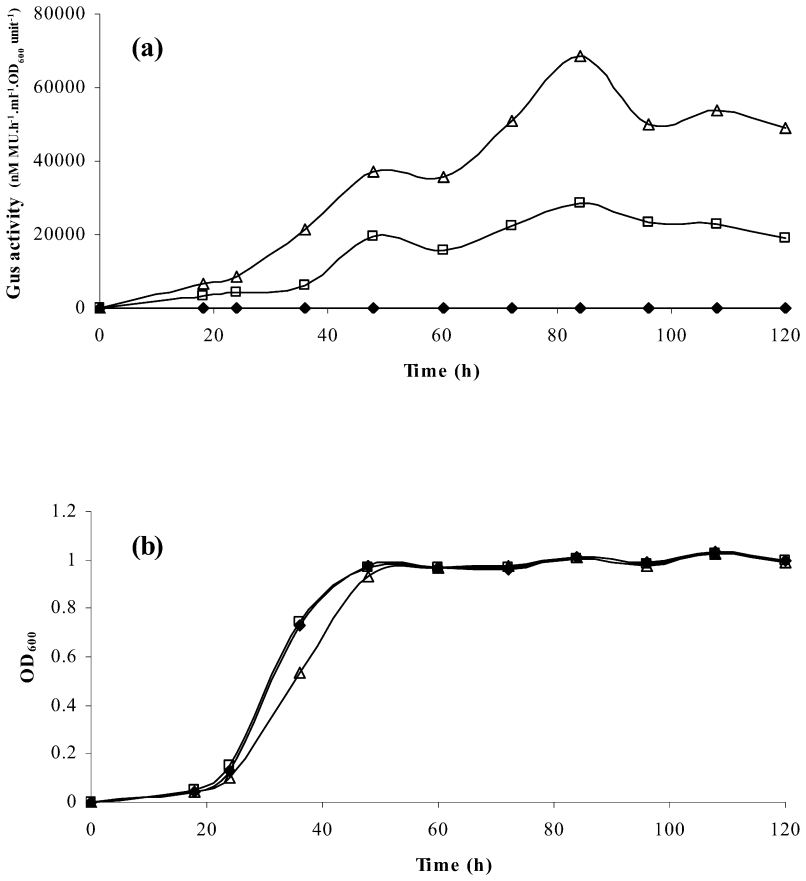

Fig. 3. Expression of *rpoH2* at different phases of growth of wild-type BL3 (close diamond); the *rpoH2* mutant RUH180 (open square); and the transconjugant BL3:pUHR345 (open triangle) in YEM medium at 28 °C. Expression of *rpoH2* is represented by β-glucuronidase activity (a), and the cell densities of the cultures are shown as OD at 600 nm (b).

equivalent positions in the chromosome. In this way, three mutants, RUH178, RUH179, and RUH180 were isolated.

3.5. *The nucleotide sequence of* rpoH2 *isolated from BL3*

To verify the position of insertion of Tn*3*Ho*gus* in the *rpoH2* gene, the 3.0-kb *Bam*HI–*Eco*RI fragment of pUHR339 was sequenced. Analysis of sequence revealed three ORFs between nucleotide positions 394 and 2916. ORF1 encodes a protein of 32.7 kDa that shows 82 and 71% similarities with RpoH2 of *S. meliloti* and *Rhizobium* sp. TAL1145, respectively, based on BLASTP analysis. Because of high similarity to *rpoH2* in *S. meliloti*, the interrupted gene cloned from *Sinorhizobium* sp. BL3 was designated as *rpoH2*. The conserved nine amino acid RpoH motif of RpoH2 protein isolated from *Sinorhizobium* sp. BL3 has one amino acid mismatch [QKALFFNLR] when compared to the common RpoH motif [Q(R/K)(K/R)LFFNLR] [21]. This amino acid mismatch in the RpoH motif was the same as in RpoH2 isolated from *S. meliloti* [23]. The sequence analyses also revealed that the Tn*3*Ho*gus* insertions in mutants RUH180, RUH179, and RUH178 were located 40, 585 and 805 bp downstream from the ATG start site of *rpoH2*, respectively. The ORF2 and ORF3 were located in the reverse direction with ORF1, and showed 72 and 85% similarities with the putative amino acid transport system permease ABC transporter protein and the putative amino acid-binding periplasmic ABC transporter protein of *S. meliloti* Rm1021, respectively.

3.6. *Expression of* rpoH2 *in BL3*

To investigate the pattern of *rpoH2* expression in *Sinorhizobium* sp. BL3 during growth, the Gus activities of the *rpoH2* mutant RUH180, containing a single copy *rpoH2*::*gus* fusion in the chromosome, and the transconjugant BL3:pUHR345, containing multiple copies of *rpoH2*::*gus* fusion, were determined in YEM medium (Fig. 3). As indicated by Gus activities, *rpoH2* was expressed during the early exponential growth at a low level. The Gus activities increased considerably when the cells reached the stationary phase at about 48 h and the highest level of expression was observed at 84 h. These data indicate that *rpoH2* is expressed most strongly during stationary phase.

3.7. *Characteristics of* rpoH2 *mutants*

Mutants RUH178, RUH179, and RUH180 produced almost the same amounts of EPS as BL3 (Table 2). When grown on YEM agar containing the fluorescent dye Calcofluor white, the mutants showed fluorescence like BL3. This suggests that these mutants are not defective in the Calcofluor-binding EPS, known

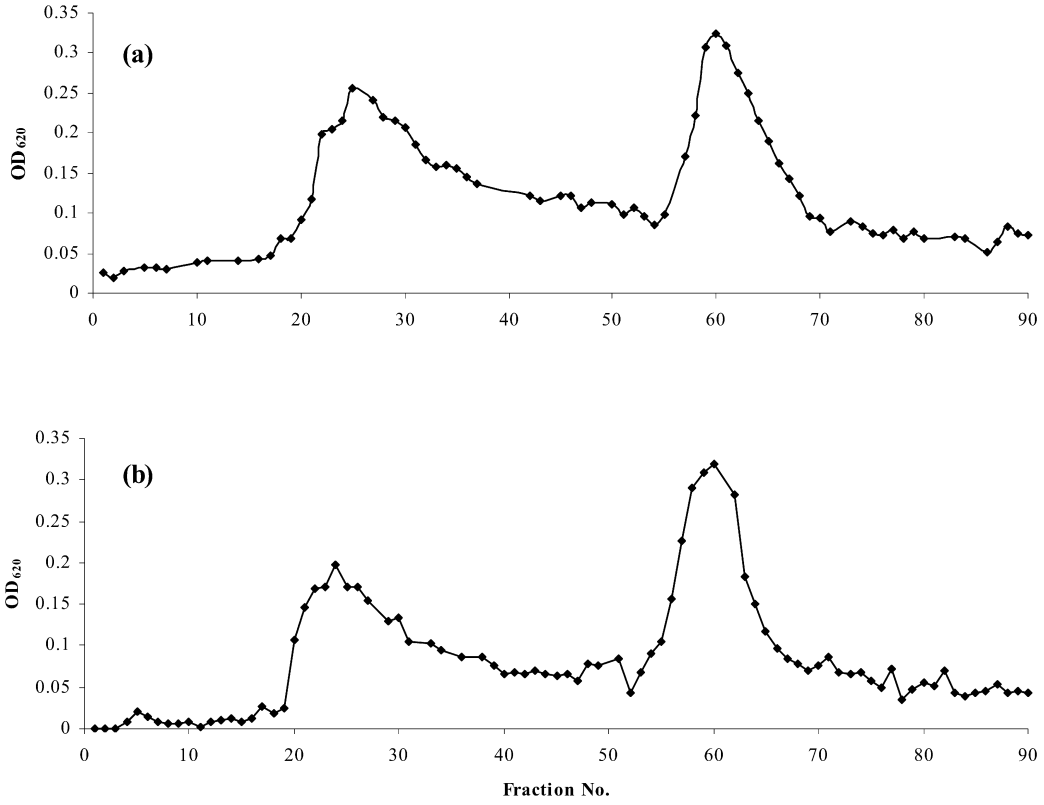

Fig. 4. Gel filtration of the culture supernatant of (a) BL3 and (b) *rpoH2* mutant (RUH180) through a Bio-Gel A-5 m column. The lyophilized culture supernatant was loaded on the column and the hexose content of the samples was determined by the anthrone sulfuric method.

as EPS I. The fractionation of large and small molecular weight fractions of EPS produced by the mutants also showed a similar pattern as BL3 (Fig. 4). However, these mutants failed to grow in YEM broth containing 0.5 mM NaCl (Fig. 5). When these mutants were complemented with the cloned *rpoH2* gene from either BL3 or TAL1145, they grew better than BL3. These results suggest that *rpoH2* is required for growth under salt stress conditions, and multiple copies of *rpoH2* enhanced salt tolerance of the transconjugants.

4. Discussion

RpoH2 regulates the transcription of *exo* genes in *Rhizobium* sp. TAL1145 and is required for effective nodulation of *L. leucocephala* [16]. In this research, we found that the EPS-defective *rpoH2* mutant of *Rhizobium* sp. TAL1145 could be complemented by *rpoH2* from both *Sinorhizobium* sp. BL3 and *S. meliloti* Rm1021 for both EPS production and symbiosis. This was not surprising considering that RpoH2 of *Sinorhizobium* sp. BL3 and *S. meliloti* showed 71 and 75% similarities to RpoH2 of *Rhizobium* sp. TAL1145, respectively. However, the RpoH2 in *Sinorhizobium* sp. BL3 is not involved in EPS biosynthesis and symbiosis with the host legume. The *rpoH2* mutants of BL3 produced the same amount of EPS and showed a similar profile of small- and large-molecular weight EPS fractions as BL3, and these mutants could nodulate and fix nitrogen on the *P. lathyroides* plants as well as BL3.

The expression of *rpoH2* in BL3 was highly increased when the cells entered the stationary phase, suggesting that RpoH2 may have some function when nutrients are depleted in the growth medium. In *E. coli*, the level of the RpoH protein increases during glucose starvation, and the *rpoH* mutant shows decreased survival during starvation [14]. Thorne and Williams [31] reported that entry of bacterial cells to stationary phase leads to multiple stress resistances, including heat, low pH, oxidative and osmotic stresses. In the present study, there were no significant differences in growth between *rpoH2* mutants and wild-type at either 28 or 37 °C. These characteristics of the *rpoH2* mutants of *Sinorhizobium* sp. BL3 were similar to those of the *rpoH2* mutants of *S. meliloti* Rm1021. Oke et al. [23] reported that *S. meliloti rpoH2* mutants could grow at both 30 and 37 °C, and also nodulated alfalfa and fixed nitrogen as well as the wild-type strain. In *S. meliloti*, *rpoH1* is more important than *rpoH2* for growth at high temperature (37 °C) or symbiosis with the host legume. A single *rpoH1* mutant showed low induction of heat shock protein, failed to grow at 37 °C, and formed ineffective nodules with alfalfa. However, the *rpoH1 rpoH2* double mutant of *S. meliloti* showed further reduction of heat-shock proteins from the levels seen in the *rpoH1* single mutant. Moreover, the *rpoH1 rpoH2* double mutant was unable to nodulate alfalfa. These observations suggested that *rpoH1* and *rpoH2* have different but overlapping functions [23,24]. Both RpoH1 and RpoH2 contain an RpoH box, a conserved nine amino acid sequence, which may be involved in binding of the sigma factor to the core RNA polymerase [1,15,21]. The RpoH box of the *Sinorhizobium* sp. BL3 RpoH2 has one mismatch, which is in the same position as in the *S. meliloti* RpoH2.

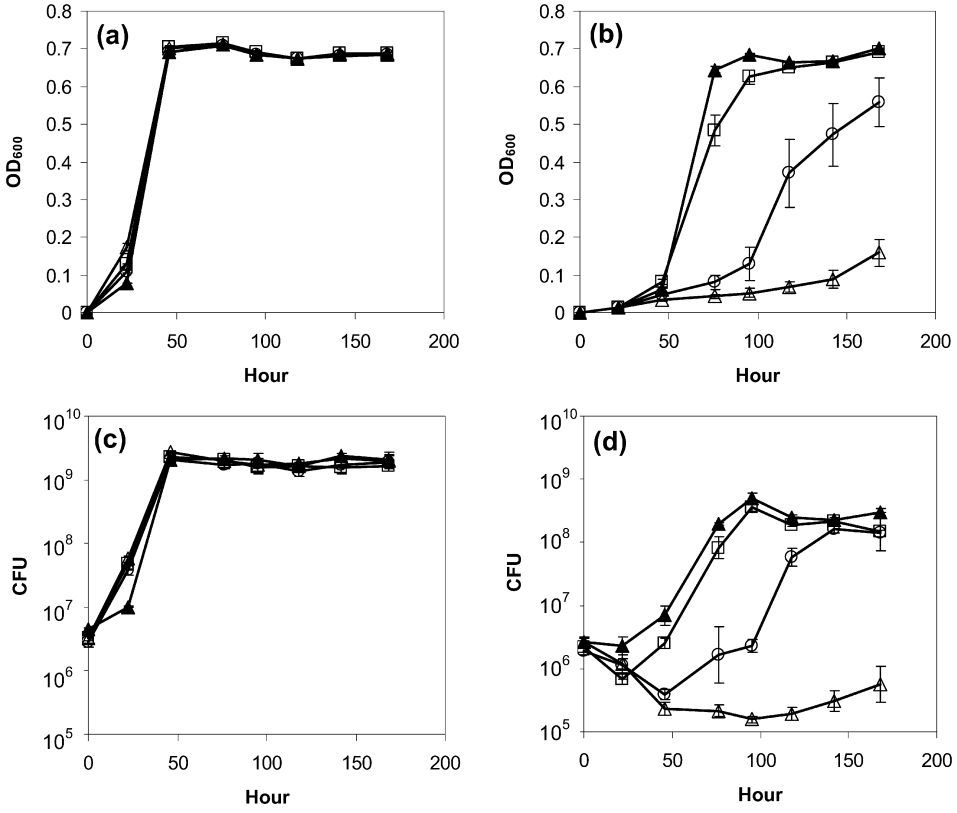

Fig. 5. The growth of BL3 (open circle), the *rpoH2* mutant RUH180 (open triangle), the *rpoH2* mutant RUH180 complemented with pUHR37 (close triangle), and pUHR339 (open square) in YEM broth (a and c), and YEM broth containing 0.5 mM NaCl (b and d) with shaking at 28 °C. The growth data were recorded as OD at 600 nm (a and b), and as viable cell counts (c and d) from the same experiment. Each data point represents the mean of three replications and the vertical bars indicate standard deviations.

Thus, the *Sinorhizobium* sp. BL3 RpoH2 appears to be functionally similar to the *S. meliloti* RpoH2.

In this study, we found that *rpoH2* mutants of *Sinorhizobium* sp. BL3 had a defect in growth under salt-stress conditions. In *E. coli*, RpoH induces transcription of genes for heat-shock proteins (Hsps), such as DnaK, DnaJ and GrpE in response to elevation of temperature [7,22]. Many Hsps are also induced by other environmental changes, such as cold shock, chemical stress, heavy metals, pollutants, starvation, and salt stress [3,11,17,20,28,33–35]. Both heat and salt stresses can induce Hsps in *B. subtilis* [35]. Similarly, in *Lactococcus lactis*, Hsps were induced by salt stress [17]. The Hsps include chaperones and proteases that are presumably essential for overcoming changes that involve protein denaturation during stress conditions [27]. It was found in *E. coli* that the induction of Hsps improved thermotolerance and tolerance to heavy metals [13, 18]. In the present study, the *Sinorhizobium* sp. BL3 *rpoH2* mutants could not grow as well under salt stress conditions as BL3, while the mutants complemented with multiple copies of *rpoH2* grew better than BL3 under high salt condition. In BL3, RpoH2 may serve as a regulatory switch to upregulate genes for salt-tolerance proteins under high salt conditions. BL3 was isolated from the root nodule of *P. lathyroides* that grows on saline soils of Northeastern Thailand. High salt tolerance of BL3 might have evolved through continuous exposure to saline environment for a long time during which some of the genes for salt tolerance might have bridged to the RpoH2 regulatory switch, through a series of mutations and selection. Future research will determine the mechanism of the switch by identifying genes for salt tolerance that are regulated by RpoH2.

Acknowledgements

This work was supported by the Royal Golden Jubilee Ph.D. Program of the Thailand Research Fund, Suranaree University of Technology, Nakhon Ratchasima, Thailand, and the College of Tropical Agriculture and Human Resources, University of Hawaii at Manoa, Honolulu. We would like to thank the referees for their suggestion of examining salt tolerance of the BL3 *rpoH2* mutant.

References

[1] F. Arsène, T. Tomoyasu, A. Mogk, C. Schirra, A. Schulze-Specking, B. Bukau, Role of region C in regulation of the heat shock gene-specific sigma factor of *Escherichia coli*, J. Bacteriol. 181 (1999) 3552–3561.

[2] A.N. Bittner, V. Oke, Multiple *groESL* operons are not key targets of RpoH1 and RpoH2 in *Sinorhizobium meliloti*, J. Bacteriol. 188 (2006) 3507–3515.

[3] A. Blom, W. Harder, A. Matin, Unique and overlapping pollutant stress proteins of *Escherichia coli*, Appl. Environ. Microbiol. 58 (1992) 331–334.

[4] M.W. Breedveld, L.P.T.M. Zevenhuizen, A.J.B. Zehnder, Excessive secretion of cyclic β-(1,2)-glucan by *Rhizobium trifolii* TA-1, Appl. Environ. Microbiol. 56 (1990) 2080–2086.

[5] J. DeMaio, Y. Zhang, C. Ko, D.B. Young, W.R. Bishai, A stationary-phase stress-response sigma factor from *Mycobacterium tuberculosis*, Proc. Natl. Acad. Sci. USA 93 (1996) 2790–2794.
[6] G. Ditta, T. Schmidhauser, E. Yakobson, P. Lu, X.W. Liang, D.R. Finlay, D. Guiney, D.R. Helinski, Plasmids related to the broad host-range vector, pRK 290, useful for gene cloning and monitoring gene expression, Plasmid 13 (1985) 149–153.
[7] J. Gamer, G. Multhaup, T. Tomoyasu, J.S. McCarty, S. Rüdiger, H.J. Schönfeld, C. Schirra, H. Bujard, B. Bukau, A cycle of binding and release of the DnaK, DnaJ and GrpE chaperones regulates the activity of the *Escherichia coli* heat shock transcription factor σ^{32}, EMBO J. 15 (1996) 607–617.
[8] M.L.C. George, J.P.W. Young, D. Borthakur, Genetic characterization of *Rhizobium* sp. strain TAL1145 that nodulates tree legumes, Can. J. Microbiol. 40 (1994) 208–215.
[9] J. Glazebrook, G.C. Walker, A novel exopolysaccharide can function in place of the calcofluor-binding exopolysaccharide in nodulation of alfalfa by *Rhizobium meliloti*, Cell 56 (1989) 661–672.
[10] C.A. Gross, in: F.C. Neidhardt, R. Curtiss III, J.L. Ingraham, E.C.C. Lin, K.B. Low, B. Magasanik, W.S. Reznikoff, M. Riley, M. Schaechter, H.E. Umbarger (Eds.), *Escherichia coli* and *Salmonella* Cellular and Molecular Biology, Function and Regulation of the Heat Shock Proteins, American Society for Microbiology, Washington, 1996, pp. 1382–1399.
[11] M. Hecker, W. Schumann, U. Völker, Heat-shock and general stress response in *Bacillus subtilis*, Mol. Microbiol. 19 (1996) 417–428.
[12] R. Hengge-Aronis, in: F.C. Neidhardt, R. Curtiss III, J.L. Ingraham, E.C.C. Lin, K.B. Low, B. Magasanik, W.S. Reznikoff, M. Riley, M. Schaechter, H.E. Umbarger (Eds.), *Escherichia coli* and *Salmonella* Cellular and Molecular Biology, Regulation of gene expression during entry into stationary phase, American Society for Microbiology, Washington, 1996, pp. 1497–1512.
[13] O. Inbar, E.Z. Ron, Induction of cadmium tolerance in *Escherichia coli* K-12, FEMS Lett. 113 (1993) 197–200.
[14] D.E. Jenkins, E.A. Auger, A. Matin, Role of RpoH, a heat shock regulation protein, in *Escherichia coli* carbon starvation protein synthesis and survival, J. Bacteriol. 173 (1991) 1992–1996.
[15] D.M. Joo, A. Nolte, R. Calendar, Y.N. Zhou, D.J. Jin, Multiple regions on the *Escherichia coli* heat shock transcription factor σ^{32} determine core RNA polymerase binding specificity, J. Bacteriol. 180 (1998) 1095–1102.
[16] P.H. Kaufusi, L.S. Forsberg, P. Tittabutr, D. Borthakur, Regulation of exopolysaccharide synthesis in *Rhizobium* sp. strain TAL1145 involves an alternative sigma factor gene, *rpoH2*, Microbiology 150 (2004) 3473–3482.
[17] M. Kilstrup, S. Jacobsen, K. Hammer, F.K. Vogensen, Induction of heat shock proteins DnaK, GroEL, and GroES by salt stress in *Lactococcus lactis*, Appl. Environ. Microbiol. 63 (1997) 1826–1837.
[18] N. Kusukawa, T. Yura, Heat-shock protein GroE of *Escherichia coli*: Key protective roles against thermal stress, Genes Dev. 2 (1988) 874–882.
[19] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with Folin phenol reagent, J. Biol. Chem. 193 (1951) 256–275.
[20] A. Muffler, M. Barth, C. Marschall, R. Hengge-Aronis, Heat-shock regulation of sigmaS turnover: A role for DnaK and relationship between stress responses mediated by sigmaS and sigma32 in *Escherichia coli*, J. Bacteriol. 179 (1997) 445–452.
[21] K. Nakahigashi, H. Yanagi, T. Yura, Isolation and sequence analysis of *rpoH* genes encoding σ^{32} homologs from gram negative bacteria: Conserved mRNA and protein segments for heat shock regulation, Nucleic Acids Res. 23 (1995) 4383–4390.
[22] F. Neidhardt, R.A. VanBogelen, Heat shock response, in: F.C. Neidhardt, J.L. Ingraham, K.B. Low, B. Magasanik, M. Schaechter, H.E. Umbarger (Eds.), *Escherichia coli* and *Salmonella typhimurium*, vol. 2, American Society of Microbiology, Washington, 1987, pp. 1334–1345.
[23] V. Oke, B.G. Rushing, E.J. Fisher, M. Moghadam-Tabrizi, S.R. Long, Identification of the heat-shock sigma factor RpoH and a second RpoH-like protein in *Sinorhizobium meliloti*, Microbiology 147 (2001) 2399–2408.
[24] Y. Ono, H. Mitsui, T. Sato, K. Minamisawa, Two RpoH homologs responsible for the expression of heat shock protein genes in *Sinorhizobium meliloti*, Mol. Gen. Genet. 264 (2001) 902–912.
[25] N. Parveen, D.T. Webb, D. Borthakur, *Leuceana leucocephala* formed by a surface polysaccharide defective mutant of *Rhizobium* sp. strain TAL1145 are delayed in bacteroid development and nitrogen fixation, Mol. Plant–Microbe Interact. 9 (1996) 364–372.
[26] W. Payakapong, P. Tittabutr, N. Teaumroong, N. Boonkerd, P.W. Singleton, D. Borthakur, Identification of two clusters of genes involved in salt tolerance in *Sinorhizobium* sp. strain BL3, Symbiosis 41 (2006) 47–51.
[27] E.Z. Ron, G. Segal, R. Sirkis, M. Robinson, D. Graur, in: C.R. Bell, M. Brylinsky, P. Johnson-Green (Eds.), Proceedings of the 8th International Symposium on Microbial Ecology, Regulation of Heat-Shock Response in Bacteria, Atlantic Canada Society for Microbial Ecology, Halifax, Canada, 1999.
[28] P. Salotra, D.K. Singh, K.P. Seal, N. Krishna, H. Jaffe, R. Bhatnagar, Expression of DnaK and GroEL homologs in *Leuconostoc esenteroides* in response to heat shock, cold shock or chemical stress, FEMS Microbiol. Lett. 131 (1995) 57–62.
[29] P. Somasegaran, H.J. Hoben (Eds.), Handbook for Rhizobia, Methods in Legume-*Rhizobium* Technology, Springer-Verlag, New York, 1994.
[30] B. Staskawicz, D. Dahlbeck, N.T. Keen, C. Napoli, Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. glycinea, J. Bacteriol. 169 (1987) 5789–5794.
[31] S.H. Thorne, H.D. Williams, Adaptation to nutrient starvation in *Rhizobium leguminosarum* bv. phaseoli: Analysis of survival, stress resistance, and changes in macromolecular synthesis during entry to and exit from stationary phase, J. Bacteriol. 179 (1997) 6894–6901.
[32] W.E. Trevelyan, J.A. Harrison, Studies on yeast metabolism, fractionation and microdetermination of cell carbohydrates, Nature 50 (1952) 298–303.
[33] R.A. VanBogelen, P.M. Kelley, F. Neidhardt, Differential induction of heat-shock, SOS and oxidation stress regulons and accumulation of nucleotides in *Escherichia coli*, J. Bacteriol. 169 (1987) 26–32.
[34] T.K. VanDyk, T.R. Reed, A.C. Vollmer, R.A. LaRossa, Synergistic induction of the heat-shock response in *Escherichia coli* by simultaneous treatment with chemical inducers, J. Bacteriol. 177 (1995) 6001–6004.
[35] U. Völker, H. Mach, R. Schmid, M. Hecker, Stress proteins and cross-protection by heat-shock and salt stress in *Bacillus subtilis*, J. Gen. Microbiol. 138 (1992) 2125–2135.
[36] M.M.S.M. Wösten, Eubacterial sigma-factors, FEMS Microbiol. Rev. 22 (1998) 127–150.
[37] T. Yura, Regulation and conservation of the heat-shock transcription factor sigma32, Genes Cells 1 (1996) 277–284.