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Transcriptional Induction of a Carbon Starvation Gene during Other Starvation and Stress Challenges in *Pseudomonas putida* MK1: A Role of a Carbon Starvation Gene in General Starvation and Stress Responses

Subramanian Chitra†, Ho Sa Lee[1], and Youngjun Kim*

Environmental Biology, Division of Life Science, Catholic University of Korea, Pucheon 420-743, Korea; [1]Department of Biology, Kyunghee University, Seoul 130-701, Korea

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Thirteen transcriptionally-fused carbon starvation mutants, derived from *Pseudomonas putida* ATCC 12633, were analyzed for their survivability and transcriptional induction profiles upon carbon starvation. One of these mutants, MK114, which exhibited the lowest survivability and the highest induction rate, was selected and further examined under different starvation (nitrogen and phosphate) and stress (osmolarity, H_2O_2, salts, alcohol, and heat) conditions. Under all tested conditions MK114 induced β-galactosidase activity, implying that the interrupted gene (*cst114*) is a general starvation and stress response gene. The rate of induction ranged from 2.6-fold for phosphate starvation to 3.7-fold for osmotic shock. The mini-Tn*5* flanking DNA was cloned from the chromosome of MK114. The cloned DNA fragment exhibited carbon starvation activity, indicating that this fragment contains a carbon starvation-related promoter region. This region was partially sequenced. Possible physiological roles of *Cst114* in a carbon sensing mechanism and in other stress responses are also discussed.

Key words: Carbon starvation, bacterial stress response, *P. pudita*

Microorganisms have evolved a variety of mechanisms that allow them to survive in harsh environments they encounter in nature. A particularly prevalent form of stress in nature is starvation. Earlier studies of non-differentiating bacteria, such as *E. coli* or *Pseudomonas* species clearly show that they also undergo an elaborate process of genetic differentiation upon starvation, which leads to the generation of cells that possess enhanced resistance to a variety of stresses, such as heat, osmosis, oxidation, and phosphate as well as nitrogen starvation (10, 11). In *E. coli*, up to 50 proteins were expressed upon carbon starvation, some of which were involved in other stress responses, leading cells to the general resistant state (9, 14, 16).

Pseudomonas spp. are prominent in bioremediation and inhabit soil and groundwater environments which are often very nutrient poor. Earlier studies showed that they expressed similar responses to that of *E. coli*, producing starvation-induced proteins and general resistance to other stresses (7, 8, 12, 23). The degree of resistance for *Pseudomonas* was, however, much greater than that of *E. coli*. Molin and his coworkers have shown that about 72 proteins are induced upon carbon deprivation in *Pseudomonas putida* KT2442 (7). Among 72 proteins some of them are overlapped with other starvation and stress-induced proteins, suggesting that these proteins are involved in increased stress tolerance.

In *E. coli*, a specific sigma factor, RpoS, plays a central role in the induction of genes involved in these starvation and stress-related responses. Sigma factors, which were structurally and functionally similar to RpoS of *E. coli*, were also found in other gram-negative bacteria such as *Pseudomonas* and *Ralstonia* strains (6, 19, 21). However the mechanisms by which the bacteria sense the environmental stimuli and induce genes common to various starvation and stress are largely unknown.

Previously, we reported that a carbon starvation gene is regulated by *rpoN* product, σ^{54}, in *P. putida* ATCC 12633 (13). Even in the RpoN mutant, half of the β-galactosidase activity, compared to that in the wild type, was expressed during the starvation period.

* To whom correspondence should be addressed.
(Tel) 82-32-650-3371; (Fax) 82-32-341-9798
(E-mail) yjunkim@www.cuk.ac.kr
† Present address: Department of Microbiology and Immunology, University of Michigan, Ann Arbor, MI 48109, USA

This means that other factors are operational in carbon starvation sensing mechanism in this bacterium.

In this paper, we further screened the previously isolated carbon starvation mutants for their responses to other environmental stimuli. One of the mutants, MK114, exhibited a greater induction rate upon carbon starvation. MK114 also expressed transcriptional activity under various starvation and stress conditions. We partially cloned and characterized a carbon-starvation gene from this mutant, which is interrupted by a mini*Tn5-lacZ* transcriptional fusion plasmid.

Materials and Methods

Bacterial strains, plasmids, and growth media

All strains and plasmids used in this study are listed in Table 1. *E. coli* was grown at 37°C in LB medium. *P. putida* strains were grown at 30°C in either M9 minimal medium for carbon starvation and survivability (13) or MOPS minimal medium for various stress-induced transcriptional activity. MOPS minimal medium which used derived from medium described previously by Thorne *et al.* (22). This medium was prepared as follows; 0.05 M of MOPS (pH 7.2), 0.1 M of Na_2HPO_4 (pH 7.2, phosphate source), 20 mM of NH_4Cl (nitrogen source), 25 mM of KCl, 10 mM of NaCl, 1 mM of $MgCl_2$, 0.1 mM of $CaCl_2$, 10 uM of $FeCl_3$, 10 mM of Na_2SO_4. Glucose was used for all minimal media as a carbon source. The following antibiotics were used at the indicated concentrations (in micrograms per milliliter) for *P. putida*: kanamycin (100), and rifampicin (150). For *E. coli*: ampicillin (50), kanamycin (50), and chloramphenicol (35).

β-Galactosidase assay for transcriptional induction

Liquid assay for β-galactosidase induction for all mutant strains was performed using M9 media containing 0.05% glucose as a sole carbon source. Mutants cells were first grown overnight, then subcultured into the same fresh medium. Samples from the exponential phase were withdrawn from the culture when optical density at 600 nm reached 0.3 to 0.5. Samples from the stationary phase were obtained from the overnight culture. β-galactosidase activities for the mutant MK114 under various starvation and stress conditions were tested as follows. Cells were first grown overnight in MOPS minimal medium containing 0.2% glucose in 100-ml Erlenmeyer flasks at 30°C,

Table 1. Strains and plasmids used in this study

Bacterial strains	Relevant characteristics	References
E. coli		
MC1061	*hsdR2, hsdM+, hsdS+, araD139, D(ara-leu), Dlac galE15, galK16, rpsL, mcrA, mcrB1*	13
Pseudomonas putida		13
ATCC 12633	prototroph	13
MK1	derivative of ATCC12633; Rif^r	
MK101	*Tn5* mutant of *P. putida* MK1; Km^r, Rif^r	13
MK102	*Tn5* mutant of *P. putida* MK1; Km^r, Rif^r	13
MK103	*Tn5* mutant of *P. putida* MK1; Km^r, Rif^r	13
MK104	*Tn5* mutant of *P. putida* MK1; Km^r, Rif^r	13
MK105	*Tn5* mutant of *P. putida* MK1; Km^r, Rif^r	13
MK106	*Tn5* mutant of *P. putida* MK1; Km^r, Rif^r	13
MK108	*Tn5* mutant of *P. putida* MK1; Km^r, Rif^r	13
MK109	*Tn5* mutant of *P. putida* MK1; Km^r, Rif^r	13
MK110	*Tn5* mutant of *P. putida* MK1; Km^r, Rif^r	13
MK111	*Tn5* mutant of *P. putida* MK1; Km^r, Rif^r	13
MK112	*Tn5* mutant of *P. putida* MK1; Km^r, Rif^r	13
MK113	*Tn5* mutant of *P. putida* MK1; Km^r, Rif^r	13
MK114	*Tn5* mutant of *P. putida* MK1; Km^r, Rif^r	13
MK201	*Tn5* mutant of *P. putida* MK1; Km^r, Rif^r	13
Plasmids		
pUT(mini-*Tn5 lacZ1*)	Delivery plasmid for mini-*Tn5::lacZ1*	13
pRK600	ColE1(*ori*) RK2-Mob$^-$ RK2-Tra$^+$; Cm^r	13
pMMB67EH	Tac expression cloning vector with cloning sites of pUC18; Ap^r	13
pMK201	pMMB67EH containing 12.5 kb *Pst*1 fragment from MK114, Ap^r, Km^r	This study
pMK203	pMMB67EH containing 5.7 kb *Pst*1 fragment from MK114. Ap^r, Km^r	This study
pMK2011	pMK201 with 1.3-kb *EcoR*1 fragment deleted	This study
pMKU301	pUC19 with 1.3-kb *EcoR*1-*Pst*1 inserted	This study

Antibiotics: Ap, Cm, Km, and Rif refer to ampicillin, chloramphenicol, kanamycin, and rifampicin, respectively.

then subcultured into the same fresh medium and grown to an optical density of 0.5 at 600 nm . Cells were harvested by centrifugation using a pre-warmed rotor and resuspended in MOPS minimal medium minus carbon, nitrogen, and phosphate sources. Appropriate aliquots were then distributed into each starvation and stress medium. The initial OD_{600} in each culture ranged from 0.07 to 0.09. Glucose, NH_4Cl, and Na_2HPO_4 were omitted from the MOPS medium in order to study carbon, nitrogen, and phosphate starvation, respectively. For other stress responses the medium was prepared as follows: 3 M of NaCl (osmotic shock), 3 M of sodium acetate (acid), 5 mM of H_2O_2 (oxidative stress), and 20% of ethanol (alcohol shock) respectively were added to MOSP medium containing 0.2% glucose. All cultures were grown at 30°C except for heat stress induction, which was incubated at 55°C. Alternatively, microtubes were used for each starvation and stress experiments. The cells prepared as described above were aliquoted into an Eppendorf tube containing a proper starvation and stress medium. The culture volume was about 1 ml with an initial OD_{600} of 0.07 to 0.09. The tubes were vigorously shaken in a microtube incubator maintained at 30°C. Aerobic condition in the tubes was established by punching a hole with a needle on the cap of each Eppendorf tube. The growth pattern of the cells in this microtube is nearly the same as that of the Erlenmeyer flask culture until 5 or 6 h, but after that growth of the cells gradually decreased. Each sample removed at different time intervals was analyzed for its optical density at 600 nm and for β-galactosidase activity. The assay for β-galactosidase activity was performed as described by Clark and Switzer (5).

Viability experiments

Cells were grown in 50 ml of M9 medium plus 0.2% glucose in 250 ml of Erlenmeyer flasks at 150 rpm maintained at 30°C. Zero time samples were taken when the value of optical density reached the maximum level. The viability of starving cultures was determined by spreading serial dilution of cells on LB plates.

Cloning of mutant DNA

The genomic DNA was partially digested with *Pst*I and 5 to 15 kb DNA fragments were isolated after gel electrophoresis in 0.7% agarose. 0.5 g of DNA was ligated with 0.5 g of *Pst*I-digested and dephosphorylated pMMB67EH overnight at 15°C. The ligated mixture was electroporated into *E. coli* MC1061 competent cells. The recombinant plasmid pMK201 was cut with *Eco*RI and *Bam*HI, and a 1.3 kb fragment was subcloned into a pUC19 cut with the same restriction enzymes.

Other genetic techniques

Transformation protocol and preparation of competent cells, DNA extraction, restriction enzyme analysis, ligation of DNA with T4 DNA ligase, calf intestinal alkaline phosphatase treatment, purification of DNA fragments from agarose gel, and other genetic techniques were performed by standard procedures (2, 15) or as recommended by the suppliers. Transfer of plasmids from *E. coli* to *P. putida* was performed by the triparental mating method (1) in the presence of helper plasmid pRK600.

Results

Survivability and transcriptional induction of carbon starvation genes in the mutants

The carbon starvation induced mutants were previously isolated by using the mini Tn*5*::*lacZ*1 transcriptional fusion plasmid. All carbon-starvation mutants were isolated by differentiating the colonies which revealed dark blue color development (attributable to the induction of *lacZ* gene on X-gal plate) on carbon-limited agar plate upon exhaustion of a carbon source, while no such color change was observed in the case of a carbon-enriched plate. hirteen mutants, which showed higher differences in color change out of 30 colonies screened were selected and further analyzed for their survivability and for β-galactosidase induction upon carbon starvation. The survivability of

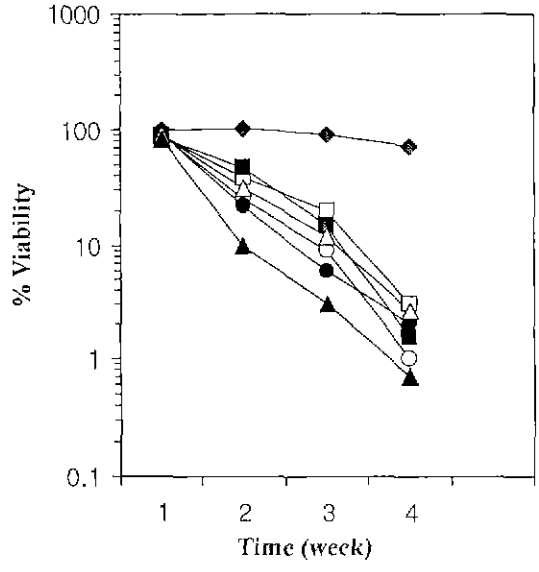

Fig. 1. Viability of *P. putida* MK1 and its mutants. Cells were cultured in 250-ml flasks containing 50 ml of M9 medium with 0.05% glucose as a sole carbon source. One hundred percent viability corresponded to 5×10^8 to 8×10^8 cells per ml. Symbols: ◆, *P. putida* MK1; □, MK101; ■, MK104; ○, MK107; ●, MK109; △, MK112; ▲, MK114.

Table 2. β-galactosidase activities in *P. putida* MK1 mutants during exponential and stationary phases

Mutants	Exponential phase[a]	Stationary phase[b]
MK201	556[c]	525(0.94)[d]
MK101	2134	5862(2.75)
MK102	821	1542(1.88)
MK103	529	897(1.70)
MK104	1650	3781(2.29)
MK105	1206	3560(2.95)
MK106	1612	3489(2.16)
MK107	3578	11253(3.15)
MK108	2985	6339(2.12)
MK109	3125	6244(2.00)
MK110	756	1508(1.99)
MK111	697	1735(2.49)
MK112	1201	2154(1.79)
MK114	3657	12561(3.43)

[a] Samples were withdrawn from the culture when the optical density at 600 nm reached 0.3 to 0.5.
[b] Samples were obtained from the overnight culture.
[c] All values are the average of duplicate experiments.
[d] Induction rates

Table 3. β-galactosidase activities[a] of MK114 under various starvation and stress conditions

Stress conditions	initial or before shock	final or after shock
Control	958[b]	1044(1.08)[c]
Carbon	1124	3749(3.34)
Nitrogen	995	2760(2.77)
Phosphate	1047	2755(2.63)
Osmotic	1001	3690(3.69)
Acid	1104	3540(3.21)
H_2O_2	1018	3576(3.51)
Alcohol	986	2964(3.00)
Temp.	1007	3058(3.04)

[a] The enzyme activity was checked after incubation of 5 h from the onset of nutrient-deprivation for starvation studies. For other stress responses, each culture was grown for 3 h to the exponential phase (OD_{600}= 0.2 to 0.4), then shock was applied and cultures grown for 2 more hours before measuring β-galactosidase activity.
[b] All values are the average of duplicate experiments.
[c] Induction rates

the cells was analyzed by counting CFU (colony forming unit) on LB plates after exhaustion of the carbon source for up to one month.

All the mutants tested appeared to be impaired on their survivability (Fig. 1). During the first week, all mutants showed similar viability to that of the wild type where as the loss of viability in the mutants was remarkable at about 14 days. The number of viable cells in the wild-type remained unchanged during the same period of 14 days. Out of different mutants studied the MK114 mutant, showed a marked decrease in survivability. Similar phenomenon for MK114 can also be seen from a previous result (13). None of the mutants exhibited any difference in growth rate compared with the wild-type (data not shown), indicating that the fused genes in all mutant strains are truely involved in the ability to prolong their life after nutrient starvation.

Liquid assay for β-galactosidase induction was performed with all the mutants. Each mutant showed a different induction profile with the lowest induction rate in case of MK103, and the highest rate in MK114 as they entered the stationary phase (Table 2). The mutant MK201, which showed dark blue color during exponential growth on X-gal plate, was used as a control and did not express any starvation-related activity.

Transcriptional induction of Tn*5*::*cst114* under various stress conditions

Due to its high loss of viability and high -galactosidase induction rate upon carbon starvation, MK114 was further selected and analyzed for the transcriptional induction of the mutated gene under various nutrient starvation and stress conditions as described in Methods and Materials. These included nitrogen and phosphate starvation as well as osmotic, acidic, oxidative, alcoholic and heat stress. All the stress conditions employed induced higher β-galactosidase activity than the control, ranging from 2.6-fold for phosphate starvation to 3.7-fold for osmotic shock (Table 3).

The rate of induction under all the stress conditions employed except for phosphate and nitrogen limitation appeared to be similar to that of carbon starvation, showing more than a 3-fold increase. Under nitrogen and phosphate starvation conditions, similar enzyme activities (2.7- and 2.6-fold increase for nitrogen and phosphate, respectively) were also observed. The above result indicates that a carbon starvation gene (we designated this gene as *cst114*) acts as a general stress response gene. Hence, this gene may play an important role in general stress and starvation resistance in *P. putida*.

Cloning of *cst114* from the MK114 mutant

To clone and further characterize the gene of *cst114* at molecular level, chromosomal DNA was isolated from the MK114 mutant, partially digested with *Pst*I, and ligated into the pMMB67EH plasmid vector to make a plasmid library. The vector is a broad-host-ranged plasmid which can replicate in *P. putida* as well as in *E. coli*. Furthermore, it can easily be transferred from *E. coli* into *P. putida* by conjugation in the presence of a helper plasmid. About fifteen positive clones (Km^r and Ap^r) from the plasmid library were subjected to restriction analysis. The size of insert DNA ranged from 6-kb to more than 15-kb. Further clonal analysis showed that all clones shared a common fragment,

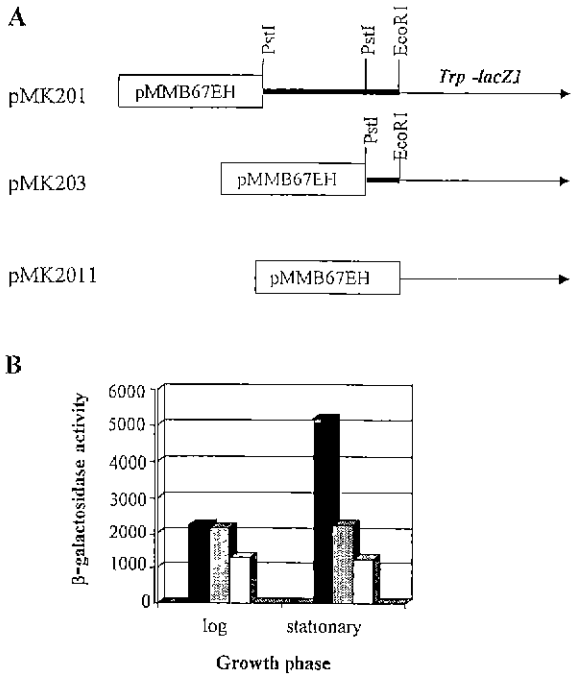

Fig. 2. (A) Restriction maps of mini-*Tn5*::*lacZ1*-containing insert DNA cloned from the MK114 mutant. pMK2011 was constructed from pMK201 by removing 1.25-kb *Pst*I-*Eco*RI chromosomal DNA as described in Materials and Methods. Orientation of transcription is indicated by arrows. (B) β-galactosidase synthesis by three cloned plasmids during log and stationary phase in *P. putida* MK1. All cultures were grown on M9 medium plus 0.05% glucose. Samples from the stationary phase were taken after overnight growth. Symbols: ■, pMK201; ⊠, pMK203; □, pMK2011.

carrying the *lacZ* and kanamycin resistance genes.

Assessment of clones in *P. putida* MK1

All recombinant clones were first transferred from *E. coli* MC1061 into wild-type *P. putida* MK1 by a triparental mating method. *P. putida* MK1 containing each of these clones was analyzed for starvation-induced promoter activity. Two clones carrying the largest and the smallest insert, pMK201 and pMK203, from the mutant were tested for liquid culture assays of -galactosidase. *P. putida* MK1 containing only pMK201 conferred starvation promoter activity, indicating that the region responsible for the starvation activity is deleted in pMK203 (Fig. 2). The pMK2011 was made by removing the 5' flanking DNA of the transposon from pMK201 as a negative control. As expected, starvation-driven promoter activity was not detected in pMK2011.

Discussion

This study was performed as an initial step to elucidate the regulatory circuit commanding the common starvation and stress response at the transcriptional level in *P. putida*. This work can also be applied in the fields of bioprocessing and bioremediation. The starvation promoters can be used to drive the expression of bioremediation genes. This approach is used in order to utilize natural stress, such as starvation itself, as the trigger for the expression of biodegradative activity.

In this study, we investigated thirteen carbon-starvation mutants of *P. putida*, which were isolated previously, for their survivability and transcriptional induction profile upon carbon starvation. Plate-based selection procedure, which was applied for the isolation of starvation gene mutants, was found to be effective, as all the mutants induced β-galactosidase activity upon starvation. The rate of induction of enzyme for all the thirteen mutants tested was 1.7 to 3.4 fold greater than that of the control. Among these mutants, MK114 was selected for other starvation and stress response because it showed the lowest survivability and expressed strong β-galactosidase activity upon starvation. Our results from the MK114 mutant showed that the carbon starvation gene was also induced under other starvation and stress conditions such as nitrogen and phosphate limitation as well as during osmolar, salts, alcohol, and H_2O_2 conditions, indicating that this gene is a general starvation and stress-response gene. To elucidate the gene structure and its promoter activity, the mini-*Tn5* flanking DNA was cloned and partially sequenced (data not shown). The largest clone (pMK201) containing 1.3-kb DNA at the promoter region expressed carbon-starvation induced β-galactosidase activity, whereas the pMK203 or the pMK2011 did not show any enzyme activity. Even though carbon-starvation activity was observed in the case of pMK203, the induction rate was much lower than that of the MK114. It also expressed lower activities upon other starvation (N and P limitation) and stress conditions, implying that the upstream region beyond 1.3-kb DNA is needed for full induction (data not shown). The result of partial DNA sequencing data revealed that the 1.3-kb DNA confers at least two open reading frames. One ORF (ORF1), which might start somewhere beyond the 5' end of the DNA fragment, stops at 169 bp. Partial amino acid sequence homology data showed that this fragment has 76% similarity to OprH, an outer membrane protein of *P. aeruginosa* (3, 4). This protein is known to be associated with resistance to polymyxin B, aminoglycosides, and EDTA. Interestingly McLeod and Spector (17) reported that carbon starvation and stationary phase induced resistance to polymysin B in *Salmonella typhimurium*. It is expected that similar function of ORF1 to that of OprH might operate in *P.*

putida during carbon starvation.

The second ORF starts at about 200 bp from the stop codon of the first ORF. Nucleotide analysis of this region revealed the presence of a long inverted repeat over 150 bp, approximately 20 nucleotides downstream of the first stop codon of the ORF1. It is conceivable that this long inverted repeat sequence of the intercistronic region might be involved in mRNA processing or stability if the genes for ORF1 and ORF2 constitute an operon. On the other hand, if the genes were transcribed separately, this sequence would probably act as a signal for carbon-starvation promoter activity. Considering the fact that the induction rates with the cloned plasmid (pMK201) were negligible under various starvation and stress conditions, the latter would not be the correct assumption. However, we can not exclude the possibility of the sequence being involved in starvation and stress responses.

Another interesting finding from partial DNA sequencing data is that ORF2 shares high similarity with the response regulator of the two component regulatory systems, such as PhoP in PhoP/PhoQ (24) and OmpR in the OmpR/EnvZ (26) system. ORF2 starts 371 bp from the 5' end of the DNA fragment, hence the *Tn5* insertion site is located approximately 900-bp downstream of the start site of ORF2. In light of the fact that the genes for the two component systems are usually co-trancribed and the genes for the response regulators are usually between 600 and 700-bp long, it can be predicted that *Tn5* does not interrupt the gene for ORF2. It also reveals that the gene for the sensor protein which might constitute an operon with the gene for ORF2 exists downstream of ORF2. Whistler *et al.* (25) recently reported that two component regulators GacS and GacA, which are required for antibiotic production, influence accumulation of the stationary-phase sigma factor (RpoS) and the stress response in *P. fluorescens* Pf-5. Morel-Deville *et al.* (18) also reported two-component signal-transducing systems in *Lactobacillus sakei* involved in stress reponses such as temperature, acid, and oxidative conditions.

A carbon starvation gene (*cst114*) we examined in this study might belong to the two component regulatory systems, functioning analogous to the GacS/GacA system. Since the Cst114 gene responds to various starvation and stress conditions, we hope that further molecular analysis of this gene cluster will elucidate the common regulatory circuit through which this bacterium senses various stimuli from the environment. These and other possible physiological roles of these gene products are currently under investigation.

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