cAMP-CRP acts as a key regulator for the viable but non-culturable state in *Escherichia coli*

Kazuki Nosho,† Hiroko Fukushima,† Takehiro Asai, Masahiro Nishio, Reiko Takamaru, Koseki Joseph Kobayashi-Kirschvink, Tetsuhiro Ogawa, Makoto Hidaka and Haruhiko Masaki†*

Abstract

A variety of bacteria, including *Escherichia coli*, are known to enter the viable but non-culturable (VBNC) state under various stress conditions. During this state, cells lose colony-forming activities on conventional agar plates while retaining signs of viability. Diverse environmental stresses including starvation induce the VBNC state. However, little is known about the genetic mechanism inducing this state. Here, we aimed to reveal the genetic determinants of the VBNC state of *E. coli*. We hypothesized that the VBNC state is a process wherein specific gene products important for colony formation are depleted during the extended period of stress conditions. If so, higher expression of these genes would maintain colony-forming activities, thereby restraining cells from entering the VBNC state. From an *E. coli* plasmid-encoded ORF library, we identified genes that were responsible for maintaining high colony-forming activities after exposure to starvation condition. Among these, *cpdA* encoding cAMP phosphodiesterase exhibited higher performance in the maintenance of colony-forming activities. As *cpdA* overexpression decreases intracellular cAMP, cAMP or its complex with cAMP-receptor protein (CRP) may negatively regulate colony-forming activities under stress conditions. We confirmed this using deletion mutants lacking adenylate cyclase or CRP. These mutants fully maintained colony-forming activities even after a long period of starvation, while wild-type cells lost most of this activity. Thus, we concluded that the lack of cAMP-CRP effectively retains high colony-forming activities, indicating that cAMP-CRP acts as a positive regulator necessary for the induction of the VBNC state in *E. coli*.

INTRODUCTION

Microbiologists have conventionally used ‘colony-forming activity’ as an indicator of cell viability, especially for readily culturable bacteria such as *Escherichia coli* or *Vibrio cholerae*. It had been believed that these bacteria would always multiply to form visible colonies in the presence of sufficient nutrients. However, in 1982, a research group led by R.R. Colwell demonstrated that even *E. coli* and *V. cholerae* lose their colony-forming activity but retain their viability upon exposure to prolonged starvation [1]. In their study, they applied ‘direct count of viable bacterial cells (DVC),’ proposed by Kogure *et al.*, as a reliable indicator of the number of viable cells [2]; for these strains, the colony-forming unit (c.f.u.) was significantly smaller than DVC. In addition, Colwell’s group proposed the distinct status ‘viable but non-culturable (VBNC) state,’ referring to the great gap between c.f.u. and the number of viable cells estimated by DVC [3, 4]. Since then, other indicators evaluating the viability of bacterial cells were proposed based on specific metabolic activities such as respiration [5, 6], transcription [6] or protein synthesis [7, 8] to define cells existing in the VBNC state. Now, the concept of VBNC has been widely accepted and over 80 species of bacteria are confirmed to enter the VBNC state [9].

Aside from starvation, various unfavourable environmental conditions such as low temperatures [10, 11], changes in the pH [12], low salinity [13] and oxygen stress [14, 15] were reported to induce the VBNC state. The natural environmental conditions surrounding micro-organisms are unstable and constantly changing. Therefore, a considerable number of bacterial cells in the natural environment may fall into VBNC-like state, impeding the isolation and detection of novel bacteria. Furthermore, other than *E. coli* and *V. cholerae*, numerous pathogens such as *Aeromonas hydrophila* [16, 17], *Listeria monocytogenes* [18, 19] and *Vibrio vulnificus* [20] are known to enter the VBNC state [9]. These pathogens existing in the VBNC state may easily evade surveillance by...
conventional plating methods while retaining or regaining virulence after suitable conditions are achieved [5, 21–23]. Therefore, the VBNC state in these pathogens provokes serious issues related to pathogenesis, food hygiene and public health. To address these issues, other workers have attempted to elucidate the genetic determinants of the VBNC state. For instance, significant changes were reported in transcription and protein synthesis during the entry of bacteria into the VBNC state [24–26]. In addition, a sigma factor RpoS, which acts as the key regulator of general stress response and stationary phase gene expression [27–29], was reported to be involved in VBNC state induction in E. coli [30] and Salmonella enterica [31]. In both cases, inactivation of RpoS resulted in an immediate decrease in bacterial culturability and subsequent entry into the VBNC state. Aside from RpoS, polyphosphate kinase I and glutathione S-transferase were also reported to be involved in the regulation of the VBNC state in Campylobacter jejuni [32] and V. vulnificus [33], respectively. Although these studies have expanded our knowledge about the regulation of the VBNC state, the molecular mechanism determining VBNC state largely remains to be elucidated. To our current knowledge, no large-scale screening has ever been employed to elucidate genetic mechanisms of the VBNC state.

We hypothesised that the VBNC state is induced by the depletion of specific gene products important for colony formation in response to exposure to certain stress conditions. When such genes are artificially overexpressed, cells may maintain colony-forming activities and hence do not enter the VBNC state even after exposure to such stress conditions. In this study, we attempted to obtain genes that contribute to maintaining colony-forming activities after prolonged exposure to starvation in E. coli. Among several genes obtained, we focused on cpdA, which led to a finding that cAMP is a positive factor in E. coli for the entry of the cell into the VBNC state. cAMP is an important second messenger found among diverse organisms, and functions in E. coli as a complex with a specific cAMP receptor protein (CRP) [34–36], which are known to regulate catabolite repression. Besides catabolite repression, bacterial transcription factors of the CRP family are also involved in a variety of physiological changes or environmental adaptation such as virulence factor production, biofilm formation, quorum sensing and secondary metabolism [37–40]. Here, we report a new role of cAMP-CRP in the regulation of VBNC state induction in E. coli.

In this article, we also discuss the possible involvement of RpoS, which is regulated by cAMP-CRP and previously reported to be involved in the formation of VBNC cells [30, 31, 41, 42].

**METHODS**

**Bacterial strains and culture conditions**

E. coli strains K-12 AG1, BW25113 and MG1655 were used in this study. The E. coli ASKA library, a complete set of E. coli AG1 clones harbouring each E. coli open reading frame (ORF) on a pCA24N vector plasmid [43], was provided by the National BioResource Project (NBRP) at the National Institute of Genetics (NIG, Japan). Each clone produces proteins with an N-terminal His tag. Keio collection, a set of single-gene knockout mutants for non-essential genes of E. coli BW25113 [44], was also provided by NBRP at NIG. E. coli cells were cultivated in L-broth containing 1.0 % Bacto tryptone (Becton Dickinson and Co., Franklin Lakes, NJ), 0.5 % Bacto yeast extract (Becton Dickinson and Co.) and 0.5 % sodium chloride (NaCl). For preparation of solid media, 1.5 % Bacto tryptone (Becton Dickinson and Co.) was used.

**Preparation of chemical reagents**

Adenosine 3’, 5’-cyclic monophosphoric acid (cAMP) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was dissolved in L-broth at 10 mM concentration and then added to media in appropriate amounts. Isopropyl-β-d-thiogalactoside (IPTG) (Wako Pure Chemical Industries, Ltd.) was dissolved in sterile water at 0.1 M concentration and added to media at the final concentration of 0.1 mM. Kanamycin sulfate (Wako Pure Chemical Industries, Ltd.) was added to L-broth at 20 µg ml⁻¹ for the selection of MG1655 deletion mutants. Chloramphenicol (Wako Pure Chemical Industries, Ltd.) was added to L-broth at 30 µg ml⁻¹ concentration for the cultivation of ASKA library clones and MG1655 cells carrying the pCA24N vector or pCA24N-cpdA.

**Construction of deletion mutants in MG1655 strain background**

For the construction of deletion mutants, cpdA, cyaA, crp and rpoS ORFs were each replaced with a kanamycin-resistance gene cassette, derived from Keio collection strains, by P1 transduction [45] or homologous recombination using the Quick and Easy E. coli Gene Deletion Kit (Gene Bridges GmbH, Heidelberg, Germany). These constructed mutants, ΔcyaA and Δcrp, were further used for the construction of double-deletion mutants, ΔcyaAΔrpoS and ΔcrpΔrpoS, as follows: the kanamycin-resistance gene of ΔcyaA or Δcrp was eliminated by homologous recombination using plasmid 707-FLPe (Gene Bridges GmbH), followed by replacement of the ORF of rpoS of these strains with the kanamycin-resistance gene cassette by P1 transduction.

**Induction and evaluation of the VBNC state in E. coli**

E. coli cells were grown in L-broth at 37 °C to an optical density of 0.8 at 660 nm wavelength. The cells were washed twice with saline and suspended in sterile water at a density of approximately 10¹⁰ cells ml⁻¹, and then incubated at 3 °C to induce the VBNC state. After the number of days of incubation indicated in each figure, cells were collected and subjected to total cell count, viable cell count and c.f.u. count. The total cell count was determined with a Multisizer 3 Coulter Counter (aperture diameter: 30 µm; Beckman Coulter, Inc., Brea, CA). The viable cell count was determined with a LIVE/DEAD BacLight bacterial viability kit (Molecular Probes, Inc., Eugene, OR) or CFDA fluorescent staining with -Bacstain- CFDA solution (Dojindo Laboratories Co. Ltd., Kumamoto, Japan) according to the manufacturer’s
recommendations. The number of c.f.u. was determined by counting colony numbers formed on L-agar after overnight cultivation at 37 °C. The growth curve of deletion mutants in L-broth was determined by turbidity, which was recorded with a SPECTRONIC 20D+ (Thermo Fisher Scientific Inc., Waltham, MA).

Screening of genes maintaining high colony-forming activity

For screening, whole clones of the ASKA library were arbitrarily divided into eight groups. Each of these clone mixtures was cultured at 37 °C in L-broth containing 0.1 mM IPTG until late exponential phase. These growing cells were subjected to induction of the VBNC state, followed by total cell count, viable cell count and c.f.u. count as described above. When a significant decrease in c.f.u. (roughly below $10^2–10^3$ ml$^{-1}$), i.e. entry to the VBNC state, was observed, 16 colonies were randomly selected and subjected to colony polymerase chain reaction (PCR) analysis to evaluate the enrichment of specific genes using the following primers: forward, ATGAGAGATCTCACC; reverse, AAGCTCAGCTAAATGAA. The enriched genes were identified by sequence determination.

Western blotting

The expression of CpdA was confirmed by Western blotting using anti-His antibody (GE Healthcare, Chicago, IL), antimouse IgG and horseradish peroxidase (HRP)-linked whole Ab sheep (GE Healthcare). ImageQuant LAS 500 (GE Healthcare) was used for detection. MagicMark XP western protein standard (Life Technologies Japan Ltd., Tokyo, Japan) was used as a protein-mass ladder.

RESULTS

Screening of genes contributing to the maintenance of colony-forming activity under cold and starvation conditions

Initially we assumed that *E. coli* possesses specific genes required for colony formation, and that the depletion of such gene products under unfavourable conditions causes a decrease in colony-forming activity, leading to the VBNC state. Under such circumstances, the high expression of these genes in *E. coli* cells would retain higher colony-forming activities even under starvation conditions. To identify these genes, we used the ASKA library – a complete *E. coli* clone set that carries plasmids encoding every *E. coli* ORF [43]. The expression of each gene in the ASKA library can be induced with IPTG, which would result in the enrichment of such clones that carry genes important for maintaining colony-forming activities, as shown in Fig. 1(a). We aimed to isolate such clones in the subsequent screening.

In the screening step, all clones of the ASKA library were divided into eight groups (approximately 500 clones per group). Each group was inoculated in L-broth with IPTG and cultivated to the late exponential phase for the expression of all genes carried on the plasmids. Cells were washed, suspended in sterile water and placed at 3 °C to induce the VBNC state (hereafter, such an incubation under cold and starvation condition is termed ‘VBNC induction’). At fixed intervals, aliquots of cell suspensions were withdrawn to determine c.f.u., viable cell and total cell counts. LIVE/DEAD staining, a method based on the integrity of the cell membrane, is often used for the detection of viable cells in the VBNC state [11–13, 17]. However, it was unclear whether this method would be applicable to our study. Like other indicators of viability, CFDA staining is also widely used and is based on esterase activity [46]. To validate the use of LIVE/DEAD staining, we stained cells with CFDA and compared the results to that of LIVE/DEAD staining (Fig. S1, available in the online version of this article). The scores of these two methods were almost identical, showing that LIVE/DEAD staining is reliable in this study. We chose the LIVE/DEAD staining method for its convenience.

Colony PCR results revealed the enrichment of several genes, particularly in groups 4, 6 and 8 (Fig. 1b–d). Genes enriched in these three groups were identified by sequencing as *cpdA* (group 4), *thiL* (group 6) and *yeiW* (group 8). However, the library *yeiW* gene seemed to be defective, as the plasmid encoded only its 3′ half and thus it was excluded from the present analysis. *thiL* encodes thiamine-phosphate phosphotransferase, which is involved in the final step of thiamine biosynthesis [47]. However, the same phenotype was not reproduced upon introduction of the *thiL*-encoding expression plasmid into MG1655 cells (Reiko Takamaru, unpublished data). The thiamine auxotrophy of the host strain AG1 of ASKA library was probably responsible for this false isolation. The remaining candidate gene, *cpdA*, encodes cAMP phosphodiesterase, which decreases the intracellular cAMP level [48]. cAMP in *E. coli* functions as a complex with CRP [34–36], and cAMP-CRP can act as either a positive or negative transcriptional regulator of diverse genes in response to various stress conditions [49, 50]. Our present finding suggests the involvement of cAMP-CRP in regulation of the VBNC state.

Next, the effect of *cpdA* on the formation of VBNC cells was investigated in *E. coli* MG1655 rather than AG1 as the host strain. Cells carrying *cpdA*-encoding plasmid (pCA24N-*cpdA*) were compared to those carrying empty plasmids (pCA24N) for their colony-forming activity after VBNC induction. Cells carrying the empty plasmid immediately lost colony-forming activity regardless of the addition of IPTG (Fig. 2a). Compared to this, cells carrying pCA24N-*cpdA* showed a significant increase in colony-forming activity, especially in the presence of IPTG (Fig. 2b). Even without IPTG, cells carrying pCA24N-*cpdA* showed relatively higher colony-forming activity compared to those carrying empty plasmids, probably due to the leaky expression of *cpdA* from the plasmid promoter. Fig. 2(c) confirmed high-level and leaky production of CpdA from the plasmid with and without IPTG induction, respectively. On the other hand, the *E. coli* mutant lacking *cpdA* showed no significant difference in colony-forming activity compared to the wild-type strain, suggesting that the efficiency of
Screening of genes responsible for maintaining high colony-forming activity after VBNC induction. (a) Screening strategy applied here is schematically represented. ASKA library clones were arbitrarily divided into eight groups, and each clone mixture was cultured in L-broth with IPTG at 37°C till late exponential phase. These exponentially growing cells were suspended in sterile water at 3°C to induce the VBNC state. Total cell count, viable cell count and c.f.u. count were determined over time. Clones carrying genes responsible for maintaining high colony-forming activity would be enriched among colony formers (left panel, orange-coloured clone). The enrichment of such clones was detected by colony PCR and electrophoresis (right panel). (b) Total cell count, viable cell count and c.f.u. were determined over time. Clones carrying genes responsible for maintaining high colony-forming activity would be enriched among colony formers (group 6) or 27 (group 8) days of VBNC induction, colonies formed on the plates were randomly selected and subjected to colony PCR analysis to confirm the enrichment of specific gene(s) (right panels). Each electrophoretic gel contains the cloned ORF fragments that were PCR-amplified from the 16 randomly selected colonies formed on a plate, with the DNA marker at both end lanes.

From the above observations, we assumed that the cellular cAMP level would negatively correlate with colony-forming activity under cold and starvation conditions. We confirmed this by cultivating ΔcyaA cells in L-broth, with a controlled concentration of cAMP ranging from 0.01 to 1 mM (Fig. 4a). These cells were subjected to VBNC induction, and their colony-forming activities were compared (Fig. 4b). The addition of 0.01 or 0.1 mM cAMP exerted no effect on the colony-forming activity of ΔcyaA cells. The addition of 0.5 mM cAMP to the ΔcyaA strain resulted in only a slight decrease, and with 1 mM cAMP, a significant decrease in colony-forming activity, close to that of the wild-type strain. These results suggest that the cellular cAMP level critically determines the colony-forming activity of cells exposed to cold and starvation conditions.

We also noted the growth rate of ΔcyaA strain in L-broth. Fig. 4(a) shows the growth rates of the wild-type and ΔcyaA strains cultured in L-broth before VBNC induction. The growth rate of ΔcyaA was relatively slower than that of the wild-type strain, which is consistent with previous studies [52, 53]. The addition of cAMP at 0.5 mM or more corrected the growth rate of ΔcyaA strain. We observed an apparent negative correlation between the growth rate in L-broth and colony-forming activity after VBNC induction, both of which depend on the cAMP level.

Participation of RpoS in the regulation of VBNC state induction via cAMP-CRP

RpoS, a sigma factor that works as a key regulator of global stress response, has been reported to regulate the formation of VBNC cells [30, 31]. The inactivation of RpoS accelerates the entry of E. coli cells into the VBNC state, suggesting that RpoS acts as a negative regulator in the formation of VBNC
cells [30]. In addition, studies have shown that cAMP-CRP negatively regulates the transcription of rpoS [41, 42]. Therefore, the overexpression of rpoS in strains ΔcyaA and Δcrp may be responsible for the absence of the VBNC state in these mutants. To test this possibility, we compared the colony-forming activity of mutants ΔcyaA, Δcrp and ΔrpoS and double mutants ΔcyaAΔrpoS and ΔcrpΔrpoS to those of the wild-type strain after VBNC induction (Fig. 5). As expected from previous reports [30, 31], deletion of rpoS caused an immediate decrease in the colony-forming activity as compared to the wild-type strain. If the effect of cAMP-CRP on the regulation of VBNC state induction had been exerted exclusively through the rpoS expression, the decrease in c.f.u. of double mutants ΔcyaAΔrpoS and ΔcrpΔrpoS would have been similar to that of mutant ΔrpoS. However, the colony-forming activity of strains ΔcyaAΔrpoS and ΔcrpΔrpoS was much higher than that of ΔrpoS or the wild-type strain, and slightly lower than that of ΔcyaA or Δcrp. These results suggest that rpoS is partly involved in the effect of cAMP-CRP, but there exists a cAMP-CRP-specific pathway independent of rpoS expression that contributes critically to repression of the formation of VBNC cells in strains ΔcyaA and Δcrp.

To further investigate the RpoS-independent pathway, we conducted the same experiment as that shown in Fig. 4 with strain ΔcyaAΔrpoS (Fig. 6). Cells were cultivated in L-broth containing varying amounts of cAMP (Fig. 6a), followed by VBNC induction and measurement of colony-forming activity. The extent of colony-forming activity of strain

![Fig. 2.](image)

**Fig. 2.** Overexpression of cpdA contributes to the maintenance of colony-forming activity after VBNC induction. (a and b) Exponentially growing MG1655 cells carrying pCA24N (a) or pCA24N-cpdA (b) in L-broth with or without IPTG at 37°C were suspended in sterile water and maintained at 3°C to induce the VBNC state. Total cells, viable cells and c.f.u.s are represented as in Fig. 1. In these panels, total cells, viable cells and c.f.u.s of cells with or without IPTG are indicated by solid and dashed lines, respectively. Each experiment was conducted in triplicate, and error bars indicate standard deviation. (c) Expression of cpdA from pCA24N-cpdA was confirmed by Western blot analysis.

![Fig. 3.](image)

**Fig. 3.** Effects of cyaA or crp deletion on colony-forming activity after VBNC induction. (a–c) Exponentially growing MG1655 wild-type cells (a) and those lacking cyaA (b) or crp (c) in L-broth at 37°C were suspended in sterile water and maintained at 3°C to induce the VBNC state. Total cell, viable cell and c.f.u. counts are represented as shown in Fig. 1. Each experiment was conducted in triplicate, and error bars indicate standard deviation.
ΔcyaAΔrpoS varied widely according to the concentration of the added cAMP (Fig. 6b). Again, these results suggest the existence of a RpoS-independent pathway in the regulation mediated by cAMP-CRP.

On the other hand, the growth rate of strain ΔcyaAΔrpoS in L-broth before VBNC induction was almost identical to that of the wild-type strain even without cAMP (Fig. 6a). Therefore, slow growth accompanied by decreased abundance of cAMP-CRP, as observed in Fig. 4(a), could be corrected by deletion of rpoS. These data imply that RpoS regulates the growth rate in L-broth depending on the concentration of cAMP (Fig. 4a), but contributes less to colony-forming activity under cold and starvation conditions.

From these results, we maintain that an unknown RpoS-independent pathway mediated by cAMP-CRP regulates the formation of VBNC cells more markedly than RpoS itself.

To gain a comprehensive insight into this unknown pathway regulated by cAMP-CRP, we analysed previously obtained RNA-Seq data [54]. The transcriptomes of E. coli
MG1655 wild-type and ΔcyaA cells grown under several cAMP concentrations were compared (original sequence reads were deposited at the DRA/SRA database with accession number DRA006091). Based on the RNA-Seq data, we listed 20 candidate genes that predominantly showed a positive or negative correlation with the concentration of cAMP. However, single-deletion or high-expression constructs of the listed genes had no effect on the formation of VBNC cells, suggesting that the critical effect on VBNC state induction may be exerted by other candidate gene(s) hitherto not examined, or exerted only when relevant genes interact with each other, wherein any alteration in those single genes has no effect. In this study, we failed to detect the downstream target genes contributing to the phenotype of ΔcyaA or Δcrp.

DISCUSSION

We aimed to reveal the genetic mechanism underlying the VBNC state in E. coli. We performed a screening to identify genes that contribute to the maintenance of high colony-forming activity after exposure to cold and starvation conditions, thereby preventing cells from entering the VBNC state. The gene cpdA exhibited a considerably higher performance in maintaining colony-forming activity; cAMP or cAMP-CRP was found to act as a strong 'negative' factor in the maintenance of colony-forming activity, i.e. it acted as a strong 'positive' factor for the entry of the cell into the VBNC state. This result supports the concept that the VBNC state is a type of survival strategy in the face of unfavourable environmental stress; however, the actual reason for the abolition of colony-forming activity is unknown.

The molecule cAMP is a universal second messenger and acts in a complex with CRP to positively or negatively regulate the transcription of a vast variety of genes in response to environmental changes [34–40, 49, 50]. To address the downstream pathway of cAMP-CRP in the regulation of VBNC state induction, we investigated its relation with rpoS. Transcription of rpoS is reported to be negatively regulated by cAMP-CRP [41, 42], which we confirmed by the RNA-Seq analysis mentioned above (data not shown), and RpoS is known to suppress the formation of VBNC cells in E. coli [30]. Therefore, we examined whether the marked effect of cAMP-CRP on induction of the VBNC state was exclusively mediated through its regulation of rpoS. Although RpoS partially contributes to the phenotype of strains ΔcyaA and Δcrp, our results strongly imply the existence of an unknown RpoS-independent pathway that regulates the formation of VBNC cells downstream of cAMP-CRP (Figs 5, 6). Although we conducted RNA-Seq analysis to gain insight into this unknown pathway, we have not yet identified the key genes contributing to this effect by cAMP-CRP.

Slow growth of strain ΔcyaA was almost corrected by the addition of 0.5 mM CAMP to the medium (Fig. 4a). In contrast, the same amount of cAMP did not seem to be sufficient to fully restore CAMP-stimulated entry into the VBNC state (Fig. 4b). These data suggest that the cAMP level required for VBNC state induction is higher than that required to correct the defect in growth rate. In other words, there could be two different modes of action of cAMP-CRP: one on growth rate at lower concentrations of cAMP and the other on VBNC state induction at higher concentrations. CRP is known to have two cAMP-binding sites: the first site, of higher affinity, at the N-terminal domain related to the allosteric transition of CRP, and the second site, of lower affinity, at the C-terminal domain close to the helix-turn-helix DNA-binding motif [36]. CAMP at a higher concentration binds to the second site and possibly modulates the specificity of the target DNA [36, 55]. It is intriguing that (cAMP)_2-CRP supposedly controls induction of the VBNC state through an unknown regulator protein that is distinct from that involved in general catabolite regulation.

The role of cAMP-CRP, or possibly (cAMP)_2-CRP, in induction of the VBNC state observed in our study may represent a new aspect of the diverse adaptations by CRP family transcription factors. In our experiment, the behaviour of the mutants lacking cAMP-CRP was examined only during the late exponential phase and under a limited condition to induce the VBNC state. Further investigation is needed to confirm the general significance of cAMP-CRP in induction of the VBNC state against a variety of stress factors. Also, in the present screening, we isolated and characterized one candidate, cpdA. Other genes involved in regulation of the VBNC state may be masked, owing to the marked effect of cpdA overexpression. We are now conducting another screening trial with other combinations of ASKA clones and different experimental designs to enable the isolation of other candidates. These data may elucidate the unknown genes...
pathway involved in the regulation of the VBNC state by cAMP-CRP.

We believe that there is a common mechanism between the VBNC state of *E. coli* and the extremely low culturable state of bacteria in the natural environment. Therefore, the significance of cAMP-CRP, or (cAMP)$_2$-CRP, observed in our study may provide a key to understanding the difficulty involved in the culturing of bacteria from the natural environment under laboratory conditions.

**Funding information**
This work was funded by the Institute for Fermentation, Osaka (to H.M.) and JSPS KAKENHI (grant nos. 24380044, 15K14688 and 17H03789 to H.M.).

**Acknowledgements**
We wish to thank Dr Yu Kanesaki, Dr Taichiro Ishige and Dr Shunsuke Yajima, Nodai Genome Research Center, Tokyo University of Agriculture for cooperation with the RNA-seq analysis. We are grateful for the granting of the ASKA library and Keio collections to NBRP at NIG.

**Conflicts of interest**
The authors declare that there are no conflicts of interest.

**References**

**Fig. 6.** Effects of cAMP addition on the growth rate of $\Delta$cyA$\Delta$rhoS cells in L-broth and their colony-forming activity after VBNC induction. (a) Growth curves of $\Delta$cyA$\Delta$rhoS cells cultured at 37°C in L-broth containing varying amounts of cAMP are shown, along with those of MG1655 wild-type and $\Delta$rhoS cells. (b) Exponentially growing MG1655 wild-type, $\Delta$rhoS and $\Delta$cyA$\Delta$rhoS cells cultured in L-broth were suspended in sterile water and maintained at 3°C to induce the VBNC state, and c.f.u.s were determined over time. Each experiment was conducted in triplicate, and error bars indicate standard deviation.


41. Lange R, Hengge-Aronis R. The cellular concentration of the sigma S subunit of RNA polymerase in *Escherichia coli* is...


45. Thomason LC, Costantino N, Court DL. *E. coli* genome manipulation by P1 transduction. *Curr Protoc Mol Biol* 2007;Chapter 1:1.17.11–11.17.18.


Edited by: D. Lee and D. Grainger