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Identification of a Temperature-Sensitive Mutation in the *ribE* Gene of an *Escherichia coli* Keio Collection Strain

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All *Escherichia coli* genes are classified into three groups, specifically, genes essential for cell growth (essential), dispensable for cell growth (non-essential), and unknown (essential or non-essential). Basically, essential genes are critical for survival of an organism and more highly conserved in evolution. On the other hand, a gene in a growing strain with a null-type mutation is classified as non-essential.[1-5] Gene essentiality is highly dependent on the living environment of an organism. Essential genes encode critical cellular functions that are not buffered by redundant functions or pathways. Profiling of the *E. coli* chromosome database based on MG1655 led to the identification of 4746 genes in total, among which 302 were classified as essential, 4439 as non-essential and 5 as unknown.[6,7] Despite the importance of essential genes, functional studies on essential *E. coli* genes based on direct experiments are currently lacking.[6,8,9]

Temperature-sensitive mutations – typically missense mutations that retain the function of a specific essential gene at standard (permissive) low temperature, but lose activity at a defined high (nonpermissive) temperature – can be exploited as a fundamental and powerful approach to identify gene sets essential for various aspects of biology and elucidate their functions.[4,5,8] Such mutants facilitate the analysis of physiologic changes resulting from inactivation of a gene or gene products by shifting cells to a nonpermissive temperature. Temperature-sensitive mutants can be generated *via* random mutagenesis, typically with a chemical mutagen, often followed by laborious screening of large numbers of progeny.[1,2,10,11] However, this protocol does not allow prediction of the accurate position of the mutation, which may occur at an undesired position. Temperature-sensitive mutations are usually found in noncore residues or surface positions that play important roles in folding, proteolytic sensitivity or interactions with other proteins.

The *E. coli* Keio collection provides researchers with single-gene deletion mutations for all non-essential *E. coli* K-12 genes.[3] During a study on the roles of RNA helicases in RNA metabolism, we observed that a Keio strain lacking *dbpA* grew well at 37 °C, but showed no growth at 43 °C (Table 1). *E. coli dbpA* encoding a DEAD box RNA helicase is a non-essential gene.[3,12] The DbpA protein functions in the late stages of biogenesis of the 50S subunits of the ribosome by acting on 23S rRNA.[13-15] To determine whether the growth defect of the Keio *dbpA* deletion strain at 43 °C results from lack of DbpA protein, we examined the growth ability of the strain at the nonpermissive temperature under conditions of ectopic DbpA expression (Table 1). Notably, cells did not grow at 43 °C, clearly suggesting that thermosensitivity of the Keio *dbpA* deletion strain is not associated with *dbpA*. Accordingly, we assumed that a mutation in another region is responsible for thermosensitive growth. To confirm this hypothesis, we transferred the *dbpA::kan* cassette from the original Keio deletion strain into the Keio wild-type strain to generate a new *dbpA* deletion strain. The new strain grew well at 43 °C (Table 1), supporting our finding that DbpA is not related to temperature sensitivity for growth of the Keio *dbpA* deletion strain and a separate mutation causes growth defects at the nonpermissive temperature.

As a preliminary step to identifying the mutation triggering growth defects of the Keio *dbpA* deletion strain at the nonpermissive temperature, we isolated genes that complemented the defect using a pBR322-based *E. coli* genomic library. Five independent clones that allowed growth of the Keio *dbpA* strain at 43 °C were isolated. The identities of the clones were established by determining the sequences of both ends of insert DNAs. Sequence analyses revealed that all the clones share a 1,808 bp fragment from positions 433515 to 435322 of the *E. coli* K-12 MG1655 genome (Figure 1) comprising the *ribD*, *ribE*, *nusB*, and *thiL* genes. The corresponding 1,808 bp fragment of the Keio *dbpA* deletion strain was amplified using PCR, and subjected to sequence analysis. We detected a single T to G mutation at position 161, leading to conversion of valine 54 of RibE to

Table 1. Growth defect of the Keio *dbpA* deletion strain at 43 °C

Strain	Growth[a]	
	37 °C	43 °C
ΔdbpA	+	no colonies
ΔdbpA/*pDbpA*	+	no colonies
ΔdbpA-P1	+	+

[a]Growth of the Keio *dbpA* deletion strain (*ΔdbpA*) on LB plates at indicated temperatures. + designates that the colony formation. Growth of the Keio *dbpA* deletion strain containing the ASKA DbpA expression plasmid (*ΔdbpA*/pDbpA) in the presence of 0.1 mM IPTG. Growth of a new *dbpA* deletion strain (*ΔdbpA*-P1) constructed by transferring the *dbpA::kan* cassette of the Keio *dbpA* deletion strain into wild-type BW25113.

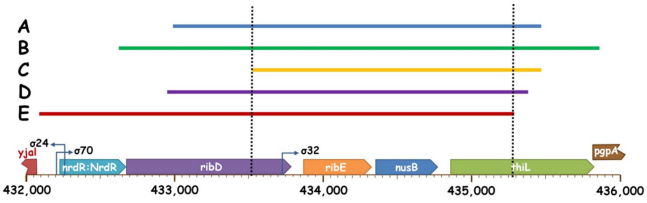

Figure 1. Sequence analysis of the clones complementing growth of the Keio *dbpA* deletion strain at 43 °C. Plasmid DNAs were purified from cells able to grow at 43 °C. Both ends of insert DNA were sequenced using two opposite pBR322 primers flanking the insert DNA within each plasmid. Sequence analysis revealed five independent clones capable of complementing cell growth at 43 °C. By aligning DNA sequence data with the *E. coli* K-12 MG1655 genome sequence, whole insert DNA sequences were predicted. Their positions in the *E. coli* genome sequence are shown. The 1,808 bp region shared by all five clones includes partial *ribD*, *ribE*, *nusB*, and partial *thiL* sequences.

Figure 2. Mutation in the Keio *dbpA* deletion strain. The 1,808 bp chromosomal region encompassing *ribD*, *ribE*, *nusB*, and *thiL* was amplified using PCR, sequenced and compared with the corresponding region of the wild-type counterpart, BW25113. A point mutation within the *ribE* coding region was identified (specifically presented for comparison). The T to G mutation at nucleotide 161 starting from the initiation codon (residue 54) is indicated by an arrow.

glycine (Figure 2). The *ribE* gene encodes 6-7-dimethyl-8-ribityllumazine synthase, an enzyme that catalyzes the penultimate step in the riboflavin biosynthesis pathway.[16-18] Our data suggest that the valine 54 to glycine mutation of RibE is responsible for the temperature sensitivity of the Keio *dbpA* deletion strain. To further confirm the involvement of this mutation in the growth defect of our strain, RibE was ectopically expressed and growth examined at the nonpermissive temperature. Notably, ectopic expression of RibE restored growth of the Keio *dbpA* deletion strain at 43 °C (Table 2). In view of this finding, we propose that the T to G mutation (altering valine 54 to glycine in the RibE protein) is responsible for the temperature sensitivity of the Keio *dbpA* deletion strain.

Since enterobacteria such as *E. coli* and *Salmonella* for riboflavin have been thought to lack an uptake system of riboflavin, mutant strains can grow only when extremely high concentrations of exogenous riboflavin are given.[19] However, it is unclear what concentrations of exogenous riboflavin are required for growth of the mutant strains.

Table 2. Growth recovery of the Keio *dbpA* deletion strain at 43 °C *via* ectopic expression of RibE

Strain	Plasmid	Growth at 43 °C[a]		
		0.01 mM IPTG	0.1 mM IPTG	1 mM IPTG
BW25113	No plasmid	+	+	+
	pCA24N	+	+	+
	pRibE	+	+	+
ΔdbpA	No plasmid	no colonies	no colonies	no colonies
	pCA24N	no colonies	no colonies	no colonies
	pRibE	no colonies	+	+

[a]The Keio *dbpA* deletion strain or wild-type counterpart strain BW25113 containing the RibE expression plasmid (pRibE) was tested for growth on LB plates at 43 °C in the presence of specified concentrations of IPTG. + designates that the colony formation. Plasmid pCA24N was used as a control plasmid.

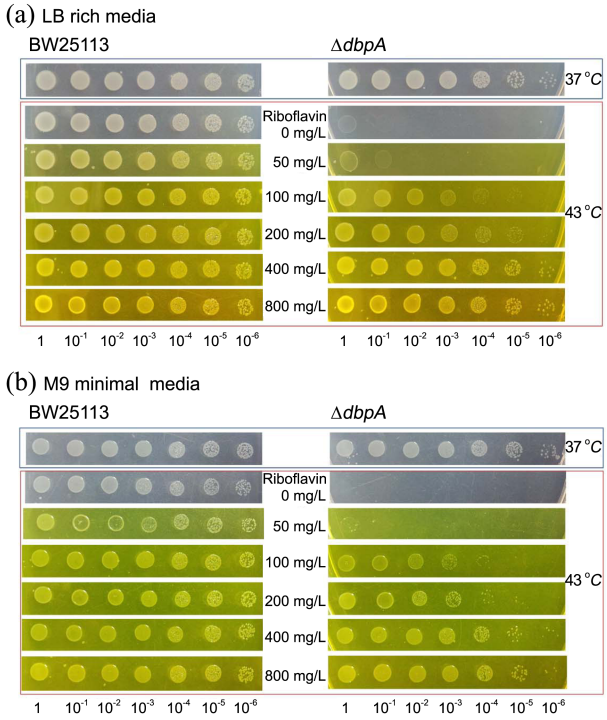

Figure 3. Growth of the Keio *dbpA* deletion strain at 43 °C on riboflavin-complemented media. The Keio *dbpA* deletion or the wild type counterpart strain BW25113 were tested for the growth at 43 °C on rich (a) or minimal (b) media supplemented with increasing concentrations of riboflavin. The growth was evaluated by analysis of colony forming abilities of serial 10-fold dilutions of the cultures containing 1.5×10^5 cells.

Using our temperature mutant strain we tried to determine a minimal concentration of the riboflavin supplement for the growth of the *ribE* temperature sensitive mutant strain in both rich and minimal media (Fig. 3). The cells required more than 800 mg/L of riboflavin for optimal growth in both media and grew slowly at lower concentrations. The growth rate of the mutant cells was proportional to riboflavin concentrations, suggesting that their growth rate is determined by the concentration-dependent influx of exogenous ribo-

flavin. However, it remains to be established whether or not an uptake system of riboflavin exists in *E. coli*. In any cases, the data could be useful if the temperature-sensitive mutant strain is used for screening inhibitors of the riboflavin biosynthetic enzymes in enterobacterial species. Furthermore, the RibE protein forms an icosahedral capsid of 60 subunits through the pentamer substructure.[17] Since valine 54 plays a role in the pentamer contact,[20] the temperature sensitivity through the valine 54 to glycine mutation may result from lack of formation of the icosahedral capsid. Therefore, the *ribE* temperature-sensitive mutation may be used for elucidating the roles of the icosahedral capsid formation in the RibE function.

Experimental Section

Media, Chemicals, and Other Reagents. Cells were grown in LB containing 1% Bacto Tryptone (LPS solution, Daejeon, Korea), 0.5% yeast extract (BD, Pont de Claix, France), and 1% NaCl (Junsei, Tokyo, Japan) with or without antibiotics at 50 μg/mL kanamycin (EMD Chemicals, San Diego, USA), 100 μg/mL ampicillin (Duchefa biochemie, Haarlem, Netherlands), and 34 μg/mL chloramphenicol (Sigma, St. Louis, USA). Cells were also grown in M9 medium (42.2 mM Na_2HPO_4, 22 mM KH_2PO_4, 18.7 mM NH_4Cl, and 8.6 mM NaCl) containing 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 0.4% glucose. When necessary, the media were supplemented with riboflavin (Sigma, St. Louis, USA). Oligonucleotides were obtained from Genotech (Daejeon, Korea). Enzymes were acquired from Enzynomics (Daejeon, Korea), unless indicated otherwise. *Taq* polymerase (Solgent, Daejeon, Korea) was used for PCR. A DNA-spin plasmid (Intron, Hiden, Germany) kit was used to isolate plasmid DNAs. DNA ladder markers were from Enzynomics.

Bacterial Strains and Plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. The Keio *dbpA* deletion strain was from the Keio collection constructed from *E. coli* K-12 strain BW25113 ($lacZ^q$ $rrnB_{T14}$ $\Delta lacZ_{WJ16}$ *hsdR514* $\Delta araBA$-$DAH33$ $\Delta rhaBAD_{CD78}$).[2] A *dbpA::kan* cassette was transferred from the Keio *dbpA* deletion strain to BW25113 to generate the new deletion strain *via* P1 transduction.[2,21] The *dbpA* deletion was confirmed *via* polymerase chain reaction (PCR) using primers k1 (5′-CAGTCATAGCCGAATAGCCT-3′) and d1 (5′-CAGCGTCTACCCTTTAAGAG-3′). Corresponding plasmids from the ASKA library were used for ectopic expression of DbpA or RibE.[22] pCA24N was used as a control plasmid in experiments employing ASKA plasmids.

Identification of Genes Complementing Growth Defects of the Keio *dbpA* Deletion Strain. We constructed a pBR322-based genomic library with chromosomal DNA from the *E. coli* strain, MG1655. Partial *Sau3*AI-digested DNA fragments of 1.5-5 kb were inserted into the *Bam*HI site of pBR322 to generate the genomic library. The Keio *dbpA* deletion strain was transformed with the *E. coli* genomic library. In total 23,000 transformants were screened for cell growth at 43 °C. Plasmid DNAs were purified from the positive clones and subjected to sequence analysis using two pBR322-sequencing primers (5′-CTTGGAGCCACTATCGAC-3′) and reverse primer (5′-GGTGATGTCGGCGATATAGG-3′) to obtain sequence information from both ends of DNA inserted into each plasmid.

Complementation with Ectopic Expression of DbpA and RibE. Cells of the Keio *dbpA* deletion strain were transformed with a DbpA or RibE expression plasmid from the ASKA library and subjected to complementation assays. Complementation tests were performed by examining the growth of cells containing the DbpA or RibE expression plasmid on LB plates supplemented with 34 μg/mL chloramphenicol in the absence or presence of IPTG at 43 °C.

Growth Analysis on Riboflavin-Complemented Media. The plate spotting assay was performed to verify the growth of the Keio *dpbA* deletion strain. Tenfold serial dilutions of overnight cultures were prepared and each 5 μL of the dilutions was plated onto agar plates containing a series of concentrations of riboflavin ranging from 50 to 800 mg/L. Cells were grown for 16 h and 30 h at 43 °C on LB rich and M9 minimal media, respectively.

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References

1. Mori, H.; Isono, K.; Horiuchi, T.; Miki, T. *Res. Microbiol.* **2000**, *151*, 121.
2. Baba, T.; Ara, T.; Hasegawa, M.; Takai, Y.; Okumura, Y.; Baba, M.; Datsenko, K. A.; Tomita, M.; Wanner, B. L.; Mori, H. *Mol. Syst. Biol.* **2006**, doi:10.1038/msb4100050
3. Kato, J.; Hashimoto, M. *Mol. Syst. Biol.* **2007**, *3*, 132.
4. Ben-Aroya, S.; Pan, X.; Boeke, J. D.; Hieter, P. *Methods Enzymol.* **2010**, *470*, 181.
5. Deng, J.; Su, S.; Lin, X.; Hassett, D. J.; Lu, L. J. *PLoS One* **2013**, *8*, e58178.
6. Gerdes, S. Y.; Scholle, M. D.; Campbell, J. W.; Bala, G.; Ravasz, E.; Daugherty, M. D.; Somera, A. L.; Kyrpides, N. C.; Anderson, I.; Gelfand, M. S.; Bhattacharya, A.; Kapatral, V.; D'Souza, M.; Baev, M. V.; Grechkin, Y.; Mseeh, F.; Finstein, M. Y.; Overbeek, R.; Barabasi, A. L.; Oltvai, Z. N.; Osterman, A. L. *J. Bacteriol.* **2003**, *185*, 5673.
7. Hashimoto, M.; Ichimura, T.; Mizoguchi, H.; Tanaka, K.; Fujimitsu, K.; Keyamura, K.; Ote, T.; Yamakawa, T.; Yamazaki, Y.; Mori, H.; Katayama, T.; Kato, J. *Mol. Microbiol.* **2005**, *55*, 137.
8. Chakshusmathi, G.; Mondal, K.; Lakshmi, G. S.; Singh, G.; Roy, A.; Ch, R. B.; Madhusudhanan, S.; Varadarajan, R. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 7925.
9. Hupert-Kocurek, K.; Sage, J. M.; Makowska-Grzyska, M.; Kaguni, J. M. *Appl. Environ. Microbiol.* **2007**, *73*, 7075.
10. Fukagawa, T.; Regnier, V.; Ikemura, T. *Nucleic. Acids Res.* **2001**, *29*, 3796.
11. Tan, G.; Chen, M.; Foote, C.; Tan, C. *Genetics* **2009**, *183*, 13.
12. Jagessar, K. L.; Jain, C. *RNA* **2010**, *16*, 1386.
13. Diges, C. M.; Uhlenbeck, O. C. *EMBO J.* **2001**, *20*, 5503.
14. Karginov, F. V.; Uhlenbeck, O. C. *Nucleic. Acids Res.* **2004**, *32*, 3028.

15. Henn, A.; Cao, W.; Licciardello, N.; Heitkamp, S. E.; Hackney, D. D.; De La Cruz, E. M. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 4046.
16. Kis, K.; Volk, R.; Bacher, A. *Biochemistry* **1995**, *34*, 2883.
17. Mörtl, S.; Fischer, M.; Richter, G.; Tack, J.; Weinkauf, S.; Bacher, A. *J. Biol. Chem.* **1996**, *271*, 33201.
18. Illarionov, B.; Kemter, K.; Eberhardt, S.; Richter, G.; Cushman, M.; Bacher, A. *J. Biol. Chem.* **2001**, *276*, 11524.
19. Eberhardt, S.; Richter, G.; Gimbel, W. *Eur. J. Biochem.* **1996**, *242*, 712.
20. (a) Persson, K.; Schneider, G.; Jordan, D. B.; Viitanen, P. V.; Sandalova, T. *Protein Science* **1999**, *8*, 2355. (b) Arifuzzaman, M.; Ioka-Nakamichi, T.; Inamoto, E.; Toyonaga, H.; Mori, H. *DNA Res.* **2005**, *12*, 291.
21. Datsenko, K. A.; Wanner, B. L. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 6640.
22. Kitagawa, M.; Ara, T.; Arifuzzaman, M.; Ioka-Nakamichi, T.; Inamoto, E.; Toyonaga, H.; Mori, H. *DNA Res.* **2005**, *12*, 291.