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Characterization of the *ves* Gene, Which is Expressed at a Low Temperature in *Escherichia coli*

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Abstract

A gene, designated *ves,* that is expressionally responsive to temperature was found in *Escherichia coli*. Experiments with a single-copy *lacZ* operon fusion and primer extension analysis revealed that *ves* was expressed at a low temperature with a peak around 25°C but was hardly expressed at 42°C. After a temperature downshift, the mRNA level increased until 6 to 12 h and then decreased. Consistently, an A + T-rich sequence similar to UP elements seen in cold-shock inducible cold-shock protein (Csp) genes was found upstream of the *ves* promoter, and its 5′-untranslated region was found to share similarity with those of the cold-shock inducible and cold-adaptive *cspA* and *cspB* genes. Additionally, a putative downstream box, which also exists in cold-inducible proteins, was found. The *ves* product was identified by overproduction and determination of its N-terminal sequence. Similarity of the C-terminal portion of Ves to the CspA family suggests that Ves belongs to this family. The results of gene-disruption experiments suggest that *ves* is not essential for *E. coli*.

Introduction

Genome projects have revealed that there are about 4300 ORFs in *Escherichia coli* (Blattner *et al.*, 1997), but the organization of most promoters of these ORFs and their expressional control remain to be characterized. We have analyzed about 10% of the promoter-proximal genes to identify many genes responsive to various stresses (Talukder *et al.*, 1994 and unpublished data). Of those, five genes were found to be under RpoS-dependent negative control in the stationary phase (Talukder *et al.*, 1996) and one of them was found to be related to a decrease in the number of viable cells in the early stationary phase (Yamada *et al.*, 1999).

A new gene, *ves*, was also identified as an unknown protein-coding gene (Talukder *et al.*, 1994). This gene is located next to the *spy* gene at 39.2 min on the *E. coli* genome (Hagenmaier *et al.*, 1997) and appears to be regulated under *v*arious *e*nvironmental *s*tresses, including osmotic shock (unpublished) and oxygen (Talukder *et al.,* 1994). Here, we present evidence of the specific expressional regulation of the *ves* gene at a low temperature.

E. coli cells induce a number of proteins when the culture temperature is reduced from 37°C to 20°C or lower as a cold-shock response (Jones and Inouye, 1994; Graumann and Marahiel, 1996; Panoff *et al.*, 1998). Such cold-induced proteins may be classified into two classes (Mitta *et al.,* 1997). One class includes proteins of CspA, CspB, CspG, CsdA and RbfA, which are induced by more than 10 fold upon cold shock, and the other class consists of RecA, Hns, Pnp, NusA, InfB and GyrA, which are induced by less than 10 fold. Interestingly, although nine Csps have been identified as members of the CspA family, a well-characterized cold-shock protein family of small (7.4 kDa), mostly acidic proteins in *E. coli* (Yamanaka *et al.*, 1998; Graumann and Marahiel, 1998), only CspA, CspB and CspG are cold-inducible. Most of the Csps are thought to function as RNA chaperones with a positively charged RNA-binding epitope (Yamanaka *et al.*, 1998; Graumann and Marahiel, 1998). The family members seem to share common functional properties because a growth defect at low temperature was not observed until four *csp* genes (*cspA*, *cspB*, *cspE* and *cspG*) were deleted, and the defect was suppressed by most of the family members (Xia *et al.*, 2001).

As has been demonstrated in *cspA*, *cspB* and *cspG* genes, specific control of such gene induction at a low temperature may be achieved by at least three elements: the UP element, cold box and downstream box. The UP element is an A + T-rich sequence located upstream of the −35 hexamer in many bacterial promoters, and it is responsible for enhancing promoter activity and may be a specific interaction site for α subunit of RNA polymease (Ross *et al.*, 1993; Ross *et al.*, 1998). This element was demonstrated to be required for *cspA* transcription (Mitta *et al.*, 1997). The cold box that was found in the 5′-untranslated regions (UTR) of the *cspA* or *cspB* mRNAs is thought to be a specific element for a putative repressor and may be required for cold-shock adaptation (Jiang *et al.*, 1996). The

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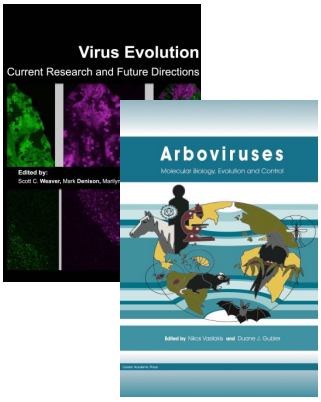

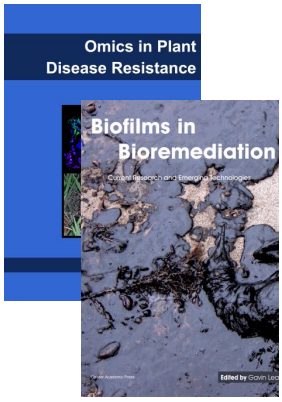

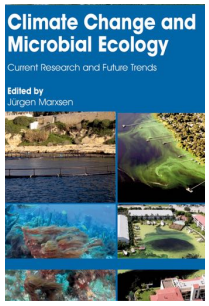

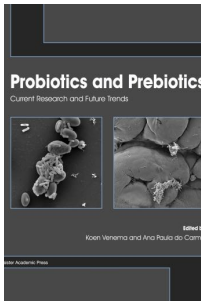

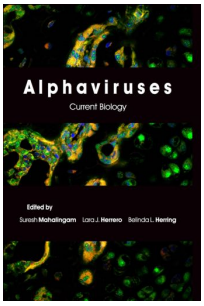

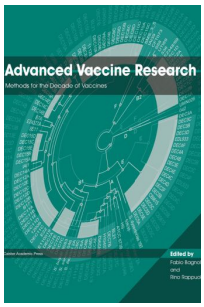

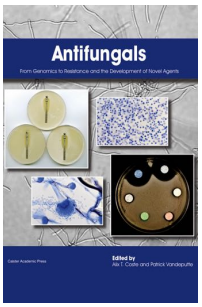

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downstream box is known to enhance translation initiation by interacting with 16S rRNA (Sprengart and Porter, 1997; Goldenberg *et al*., 1997) and is located near the initiation codons in most cold-shock proteins (Mitta *et al*., 1997).

We analyzed the expression of the *ves* gene at various temperatures by using *lacZ* operon fusion, primer extension, and reverse transcriptase-polymerase chain reaction (RT-PCR). The expression was induced more than 10 fold by reducing the culture temperature from 37°C to 25°C, and the mRNA level appeared to increase and then decrease after the downshift. These features of its temperature-responsive expression seem to be similar to those of some CspA family genes. We found that there are putative sequences of a UP element, cold box and downstream box in the *ves* gene and that Ves possesses a C-terminal domain similar in primary sequence to CspH.

Results and Discussion

Organization of the *ves* Gene

The *ves* gene is located next to the *syp* gene encoding spheroplast protein y, which is synthesized in spheroplasts but not in intact cells (Hagenmaier *et al*., 1997), on the *E. coli* genome. The *spy* gene has a relatively large inverted repeat structure (positions 154 to 212 in Figure 1A) that was suggested to be a palindromic transcription terminator (Hagenmaier *et al*., 1997), and a sequence (GCCGGTCTTGTCCAC—TGCAGTAATGCGGTGGAC at positions 160 to 197) in this structure is similar to the canonical sequence of repetitive extragenic palindromic sequences (Stem *et al.,* 1984). We performed primer extension analysis to determine whether the *ves* gene has its own promoter or not and to determine the transcription start site, if there is one (Figure 1B). We detected a single band corresponding to position 290, suggesting that *ves* has at least one promoter in this region. Its putative promoter sequence is thus GATAAC (−10 sequence) and CTAACG (−35 sequence), somewhat similar to the cannonical sequences for σ^{70} (Miller, 1992). Thus, for the transcript synthesized from the site determined, the translation of *ves* should start from a point downstream of that indicated in databases. We determined the N-terminal amino acid sequence of Ves after its overproduction as described below. Ves is thus predicted to be a 192-amino-acid peptide, which

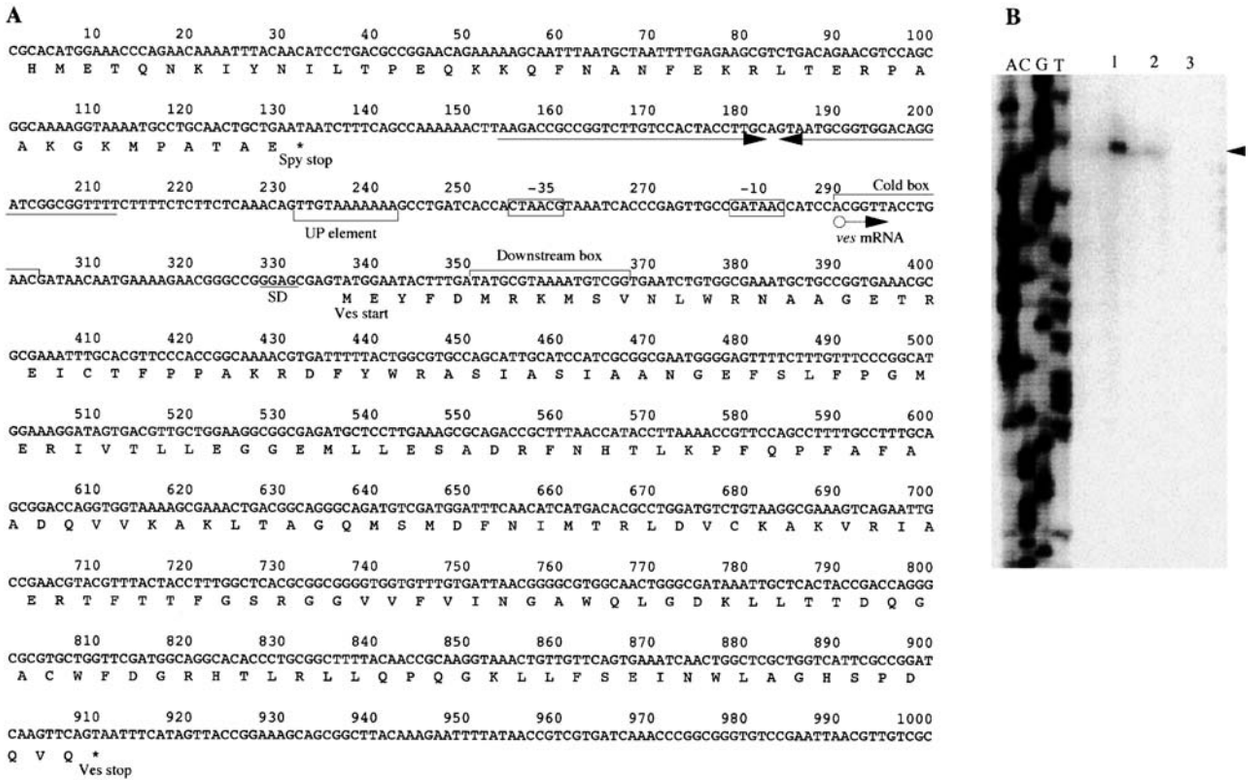

Figure 1. Organization of the *ves* gene and determination of its transcription start site. A. The nucleotide sequence of the 3′-portion of *spy* (Accession no. Y07714) and the whole region of *ves* and their amino acid sequences are shown. The -10 and -35 promoter sequences, ribosome recognition sequence (SD) and transcription initiation site of *ves* are indicated on the nucleotide sequence. A putative UP element, cold box, and downstream box are also indicated by brackets. B. Primer extension was performed using an FITC-labeled primer as described in Experimental Procedures. Total RNA (1 μg) from NK7049 cells harboring pRSVESLAC, grown in LB medium at 25°C (lane 1), 37°C (lane 2) and 42°C(lane 3) was used. The sequencing ladder (lanes A, C, G and T) was obtained by using pRSVESLAC DNA as a template and the same primer. An arrowhead indicates the extended bands.

is 21 amino acid residues shorter than that published in databases, with a mass of 21,577 Da. No structure forming a strong terminator was found just downstream of the *ves* coding sequence, but, interestingly, a 40-b segment coding for the C-terminal portion of Ves overlaps with that for the C-terminal portion of the proximal gene (b1741) product, a putative exinuclease subunit (databases). Therefore, *ves* may constitute a monocistronic operon.

Expression of the *ves* Gene at a Low Temperature

The expression of the *ves* gene was examined using strain YU522, which bears a single-copy *ves-lacZ* operon fusion on the genome. After preculture at 37°C, the cells were grown in LB medium at various temperatures, and β-galactosidase activity from the fusion gene was measured using cell cultures withdrawn at the times indicated (Figures 2A and B). The activities at 25°C and 30°C gradually increased along with cell growth and were significantly higher than those at 16°C and 37°C at each sampling time, but almost no increased activity was observed at 8°C or 42°C. Consistently, strong and weak extended bands were detected at 25°C and 37°C, respectively, but not at 42°C in primer extension analysis (Figure 1B). Essentially similar results were obtained by using NK7049, which harbors a multi-copy *ves-lacZ* operon fusion (data not shown). These results suggest that the *ves* gene is regulated to be transcribed at a low temperature.

We also performed RT-PCR analysis to examine the change in the *ves* mRNA level after a temperature shift from 37°C to 25°C (Figure 2C). Total RNA was isolated from YU522 cells grown for 3, 6, 12 and 16 h after the temperature downshift. Amplified bands were observed at 20, 25 and 30 cycles in 6-h and 12-h samples, and the intensities at these cycles were nearly the same in the two samples. However, in the case of 3-h and 16-h samples, bands were observed at 30 cycles and at 25 and 30 cycles, respectively. Therefore, it is likely that the peak of accumulation of the *ves* mRNA is around 6 to 12 h and then the mRNA level gradually decreases. The discrepancy of these results to those obtained in experiments with *lacZ*-operon fusions may be due to the stability of the LacZ fusion protein inside cells.

Identification of the *ves* Gene Product by Using a T7 RNA Polymerase System

To identify the *ves* gene product, a 606-bp DNA fragment encompassing the region from the ribosome-recognition site to the stop codon of *ves* was amplified by PCR and cloned under the control of the T7 promoter, generating pVEXVES. After the induction of transcription from the promoter, proteins of BL21 cells harboring pVEXVES were compared with those of cells harboring the vector plasmid. A band of 21-kDa protein, being equivalent to the size (21,577 Da) calculated from the deduced amino acid sequence, was detected (lane 2 in Figure 3). The N-terminal 9-amino-acid sequence of the protein was then determined, and it completely agreed with the sequence deduced from the nucleotide sequence. The protein was found in the precipitate fraction but not in the supernatant fraction separated by low-speed centrifugation following cell disruption by sonication, and this finding is contrary to the hydropathy prediction that Ves is a typical soluble protein (data not shown). Therefore, Ves protein in this sample may form an inclusion body or a relatively large complex.

Sequence Characteristics of the *ves* Operator

The *ves* gene expression in response to temperature may be caused by elements demonstrated in several cold-shock protein genes, which include a UP element (Ross *et al.*, 1993), cold box (Jiang *et al.*, 1996), and downstream box (Sprengart and Porter, 1997). Thus, we attempted to search such sequences around the *ves* promoter-operator region. There is a long A + T-rich sequence at positions 209 to 242, and in this sequence, TTGTAAAAAAA is located 40 to 60 b upstream of the mRNA start site like the UP elements extensively examined (Ross *et al.*, 1998). A sequence, UAUGCGUAAAAUGUCGG, similar to those of downstream boxes in cold-shock proteins is located at positions 351 to 367, and the matching number in this sequence to the 16S rRNA sequence (GC + AU + GU) is 10 (Mitta *et al.*, 1997). In the case of a cold box, no sequence that is very similar to the consensus sequence of UGACGUACAGA (Jiang *et al.*, 1996) is located at the 5′ UTR. Instead, there is a sequence of ACGGUUACCUGAAC (positions +1 to +14 from the mRNA start site) similar to ACGGUUUGACGUAC (positions +1 to +14) of *cspA* and UCGGUUUGAAGAAC (positions +2 to +15) of *cspB*. The sequence of the *ves* gene might be a cold box because these sequences of *cspA* and *cspB* overlap with the originally proposed cold boxes (Jiang *et al.*, 1996), and it might be the interaction site of the putative repressor for cold shock adaptation as postulated by Jiang *et al.* (1996).

Possible Function of Ves

To determine the physiological function of *ves*, we constructed a *ves*-disruptant strain, YU615, and compared its growth with that of the wild-type strain W3110 under various conditions. However, no significant difference was observed in rich and minimal media at a low or high temperature (data not shown), indicating that the *ves* gene is not essential for the organism.

A homology search was then carried out, and two homologues of Ves were found in databases as ORF6 (AL031866) in *Yersinia pestis* and as a hypothetical 21.2-kDa protein (YHUT-PSEPU) encoded by a gene at the 3′-region of the *hutC* gene in *Pseudomonas putida*. The N-terminal portions of both proteins share 44% and 32% identities, respectively, with the N-terminal portion (1 to 115 in amino acid number) of Ves but not with the

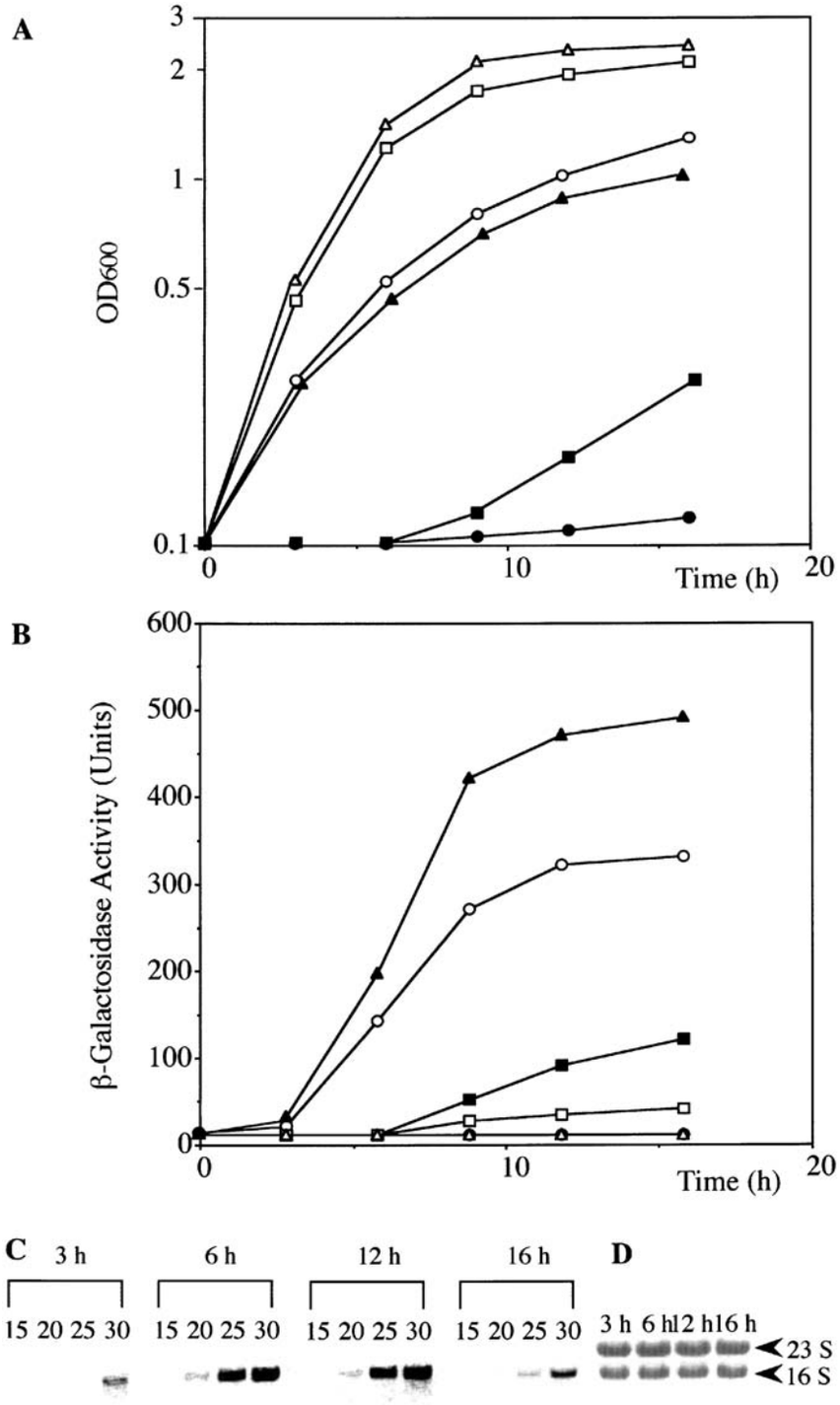

Figure 2. Expression of the *ves* gene at a low temperature. YU522 {NK7049 $\phi\lambda$(*ves-lacZ*)} cells were grown in LB at various temperatures and OD600 (A) and β-galactosidase activity (B) were measured using the cultures at the times indicated. Closed circles, closed squares, closed triangles, open circles, open squares, and open triangles represent OD600 and the activities at 8°C, 16°C, 25°C, 30°C, 37°C and 42°C, respectively. C. RT-PCR was carried out by using total RNA isolated from the cells grown at 25°C for 3, 6, 12 and 16 h. The number of the PCR cycles is indicated as 15, 20, 25, and 30.

remaining C-terminal portion. However, the physiological function of Ves could not be predicted from the similarity because the two proteins have not been functionally defined. Interestingly, the C-terminal portion of Ves (116 to 191 in amino acid number) was found to be similar (24% identity and 42% similarity) to the

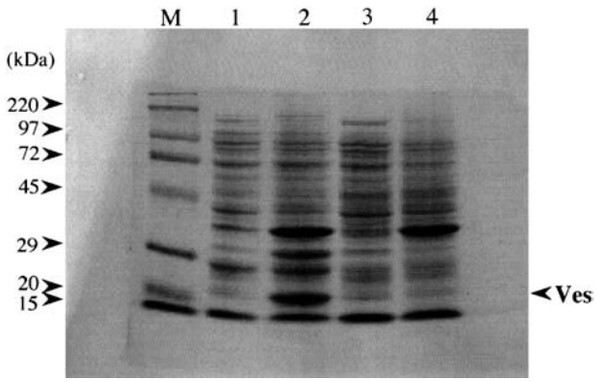

Figure 3. Identification of the *ves* product. After BL21 (DE3) cells harboring pVEXVES (lanes 1 and 2) and the vector (lanes 3 and 4) were grown at 25°C until mid-log phase, 1 mM IPTG was added to the culture and cultivation was further continued for 4 h. The cells were then harvested, disrupted by sonication and centrifuged. The supernatant as crude extracts (lanes 1 and 3) and the remaining precipitate (lanes 2 and 4) were subjected to SDS-12% polyacrylamide gel electrophoresis. Lane M, molecular markers.

whole region of cold-shock protein CspH, although the putative RNP1 and RNP2 sequences of CspH (Yamanaka *et al*., 1998) are not similar to the corresponding sequences of Ves. Weak similarity (from 20 to 30%) with other *E. coli* Csps was found. Therefore, the C-terminal portion of Ves might function as a cold-shock domain (Graumann and Marahiel, 1998) and share a certain function with CspH.

Experimental Procedures

Bacterial Strains, DNA Manipulations, and Nucleotide Sequencing

The *E. coli* K-12 strains used in this study are shown in Table 1. Conventional recombinant DNA techniques (Sambrook *et al.,* 1989) were used. Restriction enzymes, T4 DNA ligase, Taq DNA polymerase, and a DNA sequencing kit (Takara Shuzo, Kyoto, Japan) were used according to the specifications of the manufacturers. Recombinations were confirmed by restriction mapping and/or nucleotide sequencing by the dideoxy-chain termination method (Sanger *et al*., 1977). Homology searches were carried out by using the EMBL, GenBank, and SWISS-PROT databases.

Construction of Multi- and Single-Copy *ves-lacZ* Operon Fusions

To construct a multi-copy *ves-lacZ* operon fusion, a 420-bp DNA fragment encompassing the region from 210 b upstream to 210 b downstream of the initiation codon of the gene was prepared by PCR (Yamada *et al*., 1993) using two primers, 5′-TTGGGATCCCTGAA-TAATCTTTCAGC-3′ (corresponding to positions 127 to 143) and 5′-TAAGAATTCCTGCGCTTTCAAG-GAGC-3′ (corresponding to positions 553 to 537), which have *Bam*HI and *Eco*RI sites, respectively, at their 5′-ends, and W3110 genomic DNA as a template. The DNA fragment was digested with *Bam*HI and *Eco*RI and inserted into the *Bam*HI-*Eco*RI site of pRS550 (Simons *et al*., 1987), generating pRSVESLAC. The inserted DNA fragment was confirmed by nucleotide

Table 1. Bacterial strains and plasmids used in this study

Strain / Plasmid	Genotype or description	Reference / Source
E. coli strains		
NK7049	Δ*lacX74 galOP308 rpsL*	Simones *et al*., 1987
P90C	*ara* Δ*(lac-pro) thi*	Simones *et al*., 1987
TG1	*supE hsd*Δ*5 thi* Δ*(lac-proAB)*	
	F' *traD36 proAB lacI*^{q} *lacZ*Δ*M15*	Sambrook *et al*., 1989
YU522	NK7049 $\phi\lambda$(*ves-lacZ*)	This study
CT690	*recB21 recC22 thi-1 thr-1 leu-6 lacY1*	
	mtl-1 xyl-1 ara14 galK2 his-4 proA2	
	argF3 rpsL31 tsx-33 sup-37 sbcB15	M. Tsuda
W3110	IN(*rrnD-rrnE) rph-1*	K. Mizobuchi
YU615	W3110 *ves::cml*	This study
BL21 (DE3)	F' *dcm ompT hsdS(rB*$^-$ *mB*$^-$*) gal* lDE3	
	(*lacI lacUV5-T7* gene 1 *ind1 Sam7 nin5)*	RIKEN DNA Bank
Plasmids		
pRS550	*lacZ* Amp^r Kan^r	Simones *et al*., 1987
pRSVESLAC	pRS550 with the 420-bp PCR	
	fragment bearing the *ves* promoter region	This study
pBR322	Amp^r Tet^r	Bolivar *et al*., 1977
pBRVES	pBR322 with the 2.5-kb DNA fragment	
	bearing the deleted *ves* gene	This study
pCM4	Amp^r *cml* cassette	Pharmacia Biotech
pBRVESCML	pBRVES with the *cml* cassette	This study
pVEX11	pBR322 with T7 promoter of	
	of ϕX 10 gene and its terminator	Studier *et al*., 1990
pVEXVES	pVEX11 with the 606-bp PCR	
	fragment bearing the *ves* gene	This study

sequencing. A single-copy *ves-lacZ* operon fusion was then constructed as described by Simons *et al.* (1987) by the transfer of the *ves-lacZ* fusion portion of pRSVESLAC onto the NK7049 genome, generating YU522.

Construction of a *ves*-Disruptant Mutant

A *ves*-disruptant mutant from W3110 was constructed as follows. A 1.3-kb DNA fragment extending from the upstream region of the *ves* gene to its 5′-coding region and a 1.2-kb DNA fragment from its 3′-coding region to the donwstream region were amplified by PCR with primer sets of VES-DIS5′:5′-AGGAAGCTTTTGCGCTGCATACT-3′ (corresponding to positions −954 to −939) and VES-in3′:5′-TAAGGATCCGCAGCATTTCGCCACAG-3′ (corresponding to positions 389 to 373), and VES-in5′:5′-ATAGGATCCAATCAACTGGCTCGCTG-3′ (corresponding to positions 870 to 886) and VES-DIS3′:5′-AAAGTCGACACATAAACGCCGTCCGC-3′ (corresponding to positions 2041 to 2025), respectively, and W3110 genomic DNA as a template. These primers have *Hin*dIII, *Bam*HI, *Bam*HI and *Sal*I sites, respectively, at their 5′-ends. Both PCR products were digested with either *Hin*dIII and *Bam*HI or *Bam*HI and *Sal*I, and they were inserted together into the *Hin*dIII-*Sal*I site in pBR322. Subsequently, the *Bam*HI *cml* cassette from pCM4 (Pharmacia Biotech) was inserted between the two PCR fragments, generating pBRVESCML. The DNA fragment was amplified by PCR using primers of VES-DIS5′ and VES-DIS3′ and pBRVESCML DNA as a template, and it was introduced into CT690 to allow homologous recombination between the *cml*-inserted *ves* gene on the fragment and the *ves* gene on the genome. Recombinants were screened on LB plates containing chloramphenicol (15 μg/ml). Gene disruption was confirmed by PCR using the same set of primers and the genomic DNA from isolated strains. From the resultant CT690 *ves*::*cml*, W3110 *ves*::*cml* (YU615) was made by P1 transduction (Miller, 1992).

Primer Extension and RT-PCR Analyses

Total RNA was prepared by the hot phenol method as described by Aiba *et al.* (1981). Primer extension was performed as described previously (Yamada *et al.*, 1998). Total RNA (1 μg) isolated from NK7049 cells harboring pRSVESLAC (bearing *ves-lacZ* operon fusion), which were grown at 25°C, 37°C or 42°C for 8 h, was subjected to a reaction using a fluorescein isothiocyanate (FITC)-labeled primer (5′-CGCCAGGGTTTTCCCAGTCACGAC-3′). The labeled primer corresponds to the amino acid sequence from positions 20 to 12 in LacZ. A nucleotide sequencing reaction was carried out in parallel using the same FITC-labeled primer and pRSVESLAC DNA as a template. Both samples were analyzed using an SQ3000 nucleotide sequencer (Hitachi Electronics Engineering, Tokyo, Japan) with 6.1-M urea-6% polyacrylamide gel. To analyze the expression along with the cell growth, RT-PCR was carried out using an mRNA Selective PT-PCR kit (Takara Shuzo). RT reaction was carried out at 60°C for 60 min with 0.1 μg of total RNA from YU522 cells and a 3′-primer (5′-GTTTTCCCAGTCACGAC-3′ corresponding to the amino acid sequence from 17 to 12 in LacZ), and then PCR consisting of denaturing at 94°C for 1 min, annealing at 60°C for 2 min and extension at 72°C for 5 min was carried out using the same 3′-primer and a 5′-primer (5′-CGGGAGCGAGTATGGAAT-3′ corresponding to positions 326 to 343). The PCR products were analyzed by 0.9% agarose gel electrophoresis and staining with ethidium bromide. Their relative amounts were compared by measuring band density after the color of the image taken was reversed by using a Model GS-700 Imaging Densitometer (BIO-RAD). Linearity of the amplification was observed at least up to the 25th cycle. In our conditions, the mRNA Selective RT-PCR was able to specifically detect mRNA because no band was observed when reverse transcriptase was omitted.

Bacterial Growth and Enzyme Assay

Cells were precultured until the late exponential phase at 37°C in LB medium (1% Bacto tryptone, 0.5% yeast extract, 0.5% NaCl) containing appropriate antibiotics, ampicillin (100 μg/ml), or kanamycin (50 μg/ml) for cells harboring a multicopy plasmid, or tetracycline (15 μg/ml) or kanamycin (15 μg/ml) for cells having a *ves-lacZ* fusion on the genome. The cells were then diluted 1000-fold with fresh LB medium, grown at different temperatures, and then subjected to a β-galactosidase assay by the Miller method (1992). Reported values are averages of at least three independent experiments.

Overproduction of Ves

A 0.8-kb DNA fragment bearing the *ves* coding region was amplified by PCR using primers 5′-AAATCTAGACCGGGAGCGAGTATGGA-3′ (corresponding to positions 325 to 341) and 5′-CCCGGATCCCGGTAACTATGAAATTA-3′ (corresponding to positions 926 to 910), which have the *Xba*I and *Bam*HI sites, respectively, at their 5′-ends, and W3110 genomic DNA as a template. The amplified fragment was digested with *Xba*I and *Bam*HI, and it was inserted into the *Xba*I-*Bam*HI site downstream of the T7 promoter in pVEX11. The region encompassing *ves* in the recombinant, pVEXVES, was sequenced to ensure that no mutations had been introduced during amplification of the DNA fragment. BL21 (DE3) cells harboring pVEXVES were grown at 25°C in LB medium containing ampicillin, and after 1 mM IPTG was added at the mid-exponential phase, cultivation was further continued for 4 h. The cells were then harvested, washed with saline, and disrupted by sonication. The disrupted cells were centrifuged at 14,000 rpm for 5 min to separate the supernatant and precipitate fractions. The latter was resuspended in an equivalent volume of 20 mM Tris-

HCl (pH 7.0) to that of the supernatant fraction. Both fractions were then subjected to SDS-12% polyacrylamide gel electrophoresis. The proteins in the gel were visualized by staining with Coomassie brilliant blue.

Proteins from the precipitate fraction were similarly separated by electrophoresis and transferred to a PVDF membrane. A protein band enhanced in density by the T7 promoter system on the membrane was cut out and subjected to amino acid sequencing as described previously (Yamada *et al*., 1993).

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