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Cloning and Sequencing Analysis of *cadC* Encoding Transcriptional Activator CadC from *Salmonella typhimurium*

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***Salmonella typhimurium* possesses a *cad* operon, which contributes to an adaptive response against an acidifying environment. In *Escherichia coli*, the activation of the *cad* operon is dependent on *cadC*, which is located upstream of the operon. However, the activator of *cad* operon in *S. typhimurium* has not been known until now. In this study, we selected a putative *cadC* mutant by trasposon mutagenesis and cloned the *cadC* of *S. typhimurium*. Moreover, the *cadC* mutant was complemented by *cadC* clone. The *cadC* gene from *S. typhimurium* LT-2 consists of 1539 bp encoding a polypeptide ob 512 amino acids, and shows sequence similarity to *cadC* of *E. coli* with 53% identity and 67% similarity. The hydrophobicity profile of the *S. typhimurim* CadC sequence is very similar to *E. coli* CadC.**

Key words: *Samonella typhimurium, cad* operon, adaptive response, acidifying, *acd*

Sudden as well as gradual exposure to acid stress occurs in a variety of ecological niches occupied by *Salmonella typhimurium*. During its entry into a host, the microbe must endure severe acid conditions in the stomach and volatile fatty acid in the intestine, mild to moderate acid in the phagosomes and phagolysosomes (5). Consequences of acid stress include an acidified internal pH followed by damage to various macromolecules (1). Evolutionary processes have engineered a variety of acid survival systems designed to weather or neutralize acid stress. These systems include the acid tolerance responses (ATRs) and a series of amino acid decarboxylases (6, 8, 11). From *S. typhimurium*, we previously cloned the *cadBA* operon encoding lysine decarboxylase and a lysine/cadaverine antiporter and reported that the low pH-inducible lysine decarboxylase contributed significantly to pH homeostasis in environments as low as pH 3.0. The *cadBA* operon from *S. typhimurium* was found to be 79% homologous to the *cadBA* operon from *Escherichia coli* (17).

Degradative lysine and lysine decarboxylase are expressed in *E. coli* and *S. typhimurium* under conditions of low external pH in the presence of their respective amino acid substrate (13, 16, 17). The products of these reactions include the decarboxylated amino acids (cadaverine for lysine and agmatine for arginine) and CO_2. Concomitant with the production and excretion of these molecules is an increase in the pHs of the media (due to the consumption

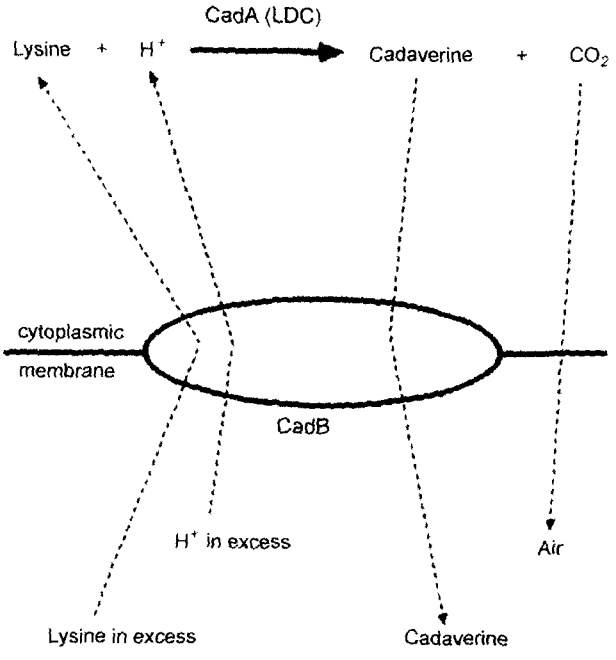

Fig. 1. Model for detoxification of extracellular high H^+ concentration by CadB and CadA. CadB and CadA function as a lysine/cadeverine antiporter and a lysine decarboxylase, respectively.

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of a proton during the decarboxylation reaction) (see Fig. 1) (13, 20).

In *E. coli*, located immediately upstream of *cadBA*, but transcribed separately, is *cadC*, a gene encoding a protein requried for *cadBA* transcription. A lysine-responsive regulatory gene, *cadR* encodes lysine-specific permease and also negatively regulates *cadBA* operon (19). Positive and negative regulators of *cadBA* operon in *S. typhimurium* have not been investigated yet. In this study, we selected a putative *cadC* mutant by transposon mutagenesis. Recently, *S. typhimurium cadC* was partially sequenced in the research of the *hmp* gene (3). Using this result, we acquired *cadC*-containing PCR product (by left primer on *hmp* and right primer on *cadB*) and sequenced that product. Moreover, the putative *cadC* mutants were complemented by this PCR clone.

Materials and Methods

Strains and plasmids

The bacterial strains used throughout this study are all derivatives of *S. typhimurium* wild types, LT-2 and UK-1. All strains and plasmids used in this work are listed in Table 1.

Growth conditions, media and chemicals.

In this study, cells were cultured at 37°C. Luria-Bertani (LB) and Salt Glucose (SG) medium (2% 50 × E buffer and 0.4% glucose) were used for bacterial growth. Moeller lysine decarboxylase (LDC) medium (0.5% bacto-peptone, 0.5% bacto-beef extract, 0.05% bacto-dextrose, 0.001% bacto-brom cresol purple, 0.0005% cresol red, and 10 mM L-lysine) was used for the LDC test. LB-5.8 was buffered to pH 5.8 with a final concentration of 100 mM MES and LB-8.0 was buffered to pH8.0 with a final concentration of 100 mM MOPS. Antibiotics were used at the concentration of 20 μg/ml for kanamycine, 60 μg/ml for ampicillin, and 10 μg/ml for tetracycline. Cadaverine (free-base form, $C_5H_{14}N_2$), lysine (L-lysine) and X-gal were added to a final concentration of 3 mM, 10 mM, and 40 μg/ml, respectively.

Genetic manipulations

General transduction was performed with P22 HT105/int and nonlysogenic segregants were identified by sensitivity to P22 H5 (2, 12). The Mu*dJ* and Tn10*d*Tet insertion library in UK1 (SF530) were generated by the technique of transitory *cis* complementation outlined by Hughes and Roth (9).

β-Galactosidase assays

β-Galactosidase assays were performed as previously described and are reported in units as defined by Miller (14). The assay was done at least twice at each time point.

Table 1. Bacterial strains and plasmids used in this study

	Genotype	Sources
S. typhimurium		
LT-2	Non-virulent wild type	SGSC[a]
JF2238	LT-2, *cadA*::Mu*dJ*	16
JF2640	LT-2, *cadC*1::Tn10*d*Tc *cadA*::Mu*dJ*	This study
JF2643	LT-2, *cadC*3::Tn10*d*Tc *cadA*::Mu*dJ*	This study
JF2644	LT-2, *cadC*::Tn10*d*Tc	This study
SCDC1	JF2643/pGSCadC	This study
SCDC2	JF2644/pGSCadC	This study
SCDC3	JF2643/pSSCadC	This study
SCDC4	JF2644/pSSCadC	This study
SCDC5	JF2643/pUSCadC	This study
SCDC6	JF2644/pUSCadC	This study
E. coli		
DH5		
ECDC7	DH5/pUSCadC	This study
ECDC8	DH5/pUC119	This study
Plasmids		
pGEM-T-Easy	TA cloning vector	Promega co.
pBSSK(+)	Cloning vector	Stratagene
pUC119	Cloning vector	Stratagene
PGSCadC	2.1 kb insert including *cadC* in pGEM-T-Easy	This study
PSSCadC	2.1 kb *Eco*RI frag. From pGSCadC in pBSSK(+)	This study
PUSCadC	2.1 kb *Eco*RI frag. From pGSCadC in pUC119	This study
pPF86	*cadBA* clone in pBR322	This study

[a]SGSC: *Salmonella* Genetic Stock Center, University of Calgary, Canada.

Cloning of the cadC gene from S. typhimurium

The entire *cadC* sequence was amplified by using CAD1 (5'-TTCGGCCCGCATAAAGTG-3') and CAD2 (5'-TGCAATCCCGCTCCCCATCA-3'). Chromosomal DNA was used as the template. Polymerase Chain Reaction (PCR) was performed by *Ex-Taq* polymerase (Takara, Japan) using a DNA thermal cycler (model TP3000, Takara PCR Thermal Cycler MP). The following cycle was used: 30 cycles consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and 2 min extension at 72°C for 2 min. The resulting 2.1kb product was ligated with a pGEM-T-Easy vector system by using T4 DNA ligase (Takara, Japan). Resulting plasmid pGSCadC was transformed to *cadC* mutants for complementation tests as previously described by Sambrook *et al.* (18). The inserted PCR product was digested with *Eco*RI. This insert was ligated with pBlueScriptSK(+) (pSSCadC) and pUC119 (pUSCadC).

Sequence analysis

DNA sequence was performed with a DNA Sequencing Kit (ABI prism, Part No. 4303152) and analyzed by ABI model 310 system (version 3.0). This DNA sequence was converted with an amino acid sequence by DNASIS program (Hitachi Software Company, Ltd.), and the homology search was performed by BLASTN and BLASTP (NCBI). The DNA and amino acid sequence alignments were performed using the DNASIS and DNASTAR MegAlign program (DNASTAR, Inc.). Hydrophobicity is displayed as previously described by Kyte and Doolittle (10).

Nucleotide sequence accession number

The nucleotide sequence of the cadC gene has been deposited in the EMBL, GenBank, and DDBJ databases under accession number AF360364.

Results

Selection of a putative cadC mutant

Previously, we constructed the *cadA-lacZ* mutant (*cadA::*Mu*dJ*) and LacZ-expression of the strain was confirmed at pH 5.5 with lysine (17). Into this mutant, we transduced the Tn10*d*Tet pool and selected two mutant strains (JF2640 and JF2643) which repressed the LacZ-expression of the *cadA*::Mu*dJ*. As shown in Table 2, the putative *cadC* mutants decreased the *cadA-lacZ* expression in the acidic medium contaning L-lysine, which is the inducing condition of *cadA* (17).

Polymerase chain reaction for cloning of a cadC gene

The gene for lysine decarboxylase (*cadA*) was first identified in *S. typhimurium* from a series as low pH-inducible genes and characterized by Lee *et al.* (11). The sequence of *cadBA in S. typhimurium* is 79% homologous (90% at the protein level) to that of *E. coli* (13, 17). The only known significant difference between the *E. coli* and *S. typhimurium cad* loci is the map position (93.7 for *E. coli* and 56.2 for *S. typhimurium*). Lee *et al.* have demonstrated the presence for *cadC* and *lysP* (*cadR*) in *S. typhimurium* and suggested that regulation of *cadBA* in both organisms will be similar (11). Recently, Crawford and Goldberg (3) revealed a partial sequence of the *cadC* gene in virulent *S. typhimurium* strain 14028. The *cadC* gene was located directly downstream of the *hmp* gene and the *hmp* gene did not appear to be part of an operon with *cadC*. However, the *cadC* gene from *S. typhimurium* has not been completely cloned. Based on this, we designed two primers, CAD1

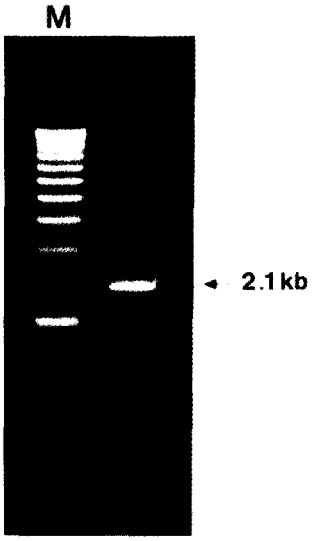

Fig. 2. PCR product containing *cadC* gene. DNA size marker (lane 1) and 2.1 kb PCR product (lane 2). CAD1 and CAD2 were used as primers (see Materials and Methods).

Table 2. Effect of a putative *cadC* mutant on *cadA-lacZ* expression

Strain	Genotype	β-Galactosidase activity			
		pH 5.8		pH 7.8	
		Lys (−)	Lys (+)	Lys (−)	Lys (+)
JF2238	*cadA10*::Mu*dJ*	0.6	858.8	9.1	8.3
JF2640	*cadA10*::Mu*dJ cadC*1::Tn10	13.4	13.3	12.2	6.7
JF2643	*cadA10*::Mu*dJ cadC*3::Tn10	10.2	9.1	11.3	10.1

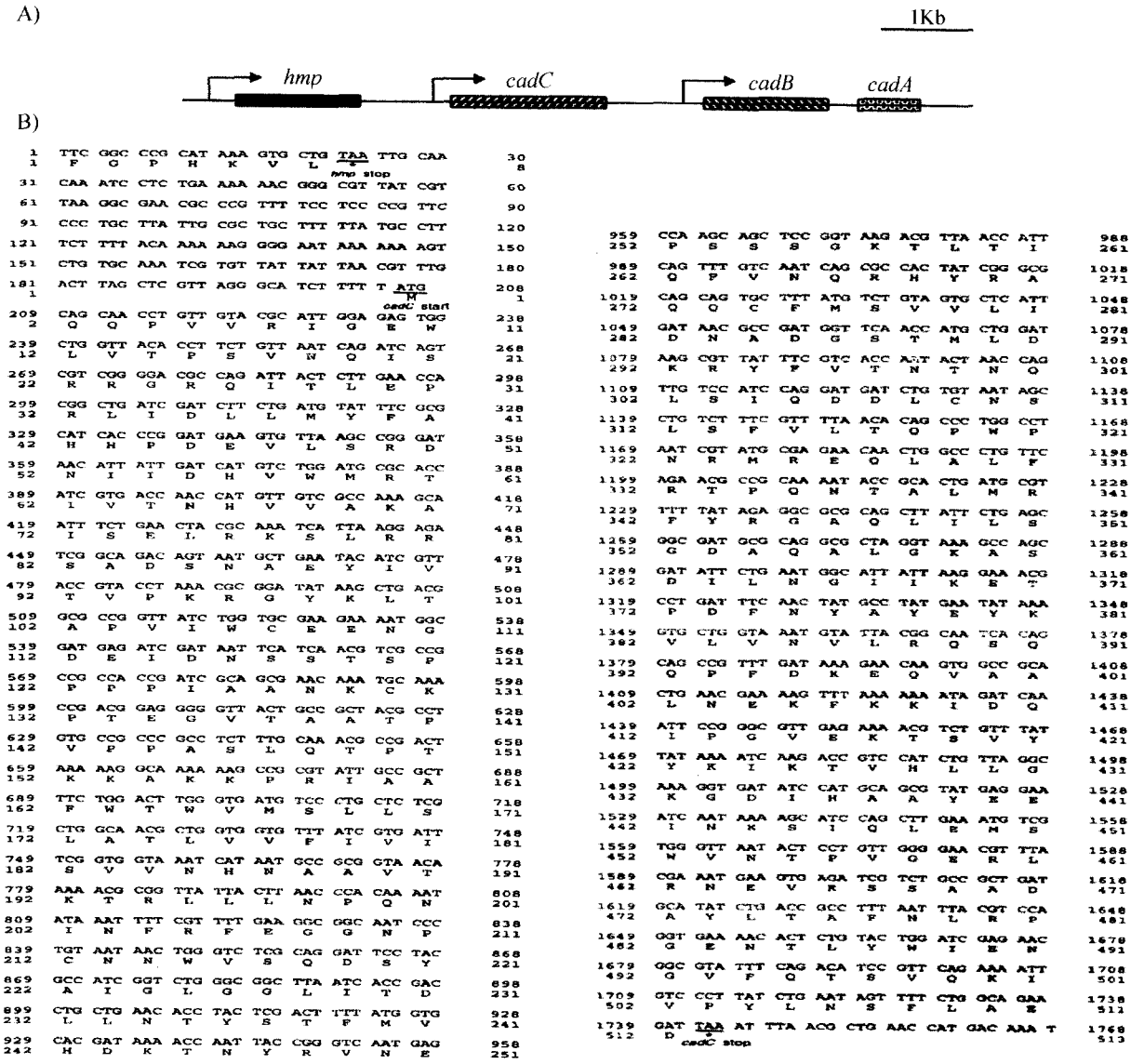

Fig. 3. A) Gene composition near *cadC* on chromosome of *S. typhimurium*. Arrows indicate the directions of transcription. B) Nucleotide and deduced amino acid sequence of *cadC* from *S. typhimurium*. One letter notation indicates the amino acid sequence.

and CAD2: The upper primer (CAD1) in the 3-end region of the *hmp* gene, and the lower primer (CAD2) in the 5-region of the *cadB* gene. As shown in Fig. 2, the PCR product is 2.1 kb (Fig. 2).

In this study, the PCR product was cloned and sequenced. The sequence analysis revealed that the *cadC* gene from *S. typhimurium* is located between *hmp* and the *cadBA* operon (Fig. 3A). The DNA sequence and deduced amino acid sequence is represented in Fig. 3B. As is the *cadC* from *E. coli* (21), the *cadC* gene from *S. typhimurium* is 1539 bp. The *cadC* from *S. typhimurium* shows 67% DNA sequence homology with that of *E. coli* (data not shown). Deduced amino acid sequence shows 53% identity and 67% similarity with *E. coli* CadC sequence (Fig. 4).

Complementation of putative cadC mutants

We transformed the *cadC* clone to putative *cadC* mutants. By LDC test, we confirmed that the clone complemented the mutants (Fig. 5). If Lysine decarboxylase is present in LDC medium (yellow), it turns purple. We also detected that the β-galactosidase activity of JF2640 and 2643 was restored on LB-X-gal and SG-X-gal plates containing lysine (pH 5.8) by the *cadC* clone (data not shown). From these results, we suggest that the strains are *cadC* mutants.

Discussion

Work with the *E. coli cad* locus showed that it is composed of two genes: *cadA* encodes lysine decarboxylase,

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1   MQQPVVRIGEWLVTPSVHQISRRGRQITLEPRLIDLLMYFAHHPDEVLSRDNIIDHVWMR 60
    MQQPVVR+GEWLVTPS+HQISR GRQ+TLEPRLIDLL++FA H  EVLSRD +ID+VW R
1   MQQPVVRVGEWLVTPSINQISRNGRQLTLEPRLIDLLVFFAQHSGEVLSRDELIDNVWKR 60

61  TIVTNHVVAKAISELRKSLRRSADSNAEYIVTVPKRGYKLTAPVIW-CEENGDEIDNSST 119
    +IVTNHVV ++ISELRKSL+ + + +  YI TVPKRGYKL  PVIW  EE G+EI  SS
61  SIVTNHVVTQSISELRKSLKQNDEDSPVYIATVPKRGYKLNVPVIWYSEEEGEEIMLSSP 120

120 SPPPPIAANKCKPTEGVTAATPVPPASLQTPTKKAKKPRIAAFWTWVMSLLSLATLVVFI 179
      P P       P+  +       P   Q+P K     R   FW W   LLSL   V  +
121 PPIPEAVPATDSPSRSLNIQNTATPPE-QSPVKSK---RFTTFWVWFFFLLSLGICVALV 176

180 VISVVNRNAAVTKTRLLLNPQNINFRTEGGNPCNMWVS--QDSYAIGLGGLITDLLMTYS 237
      S ++     ++K+R+LLNP++I+      + CN+W S  Q SYAIG+G L+   LMT+S
177 AFSSLDTRLPMSKSRILLNPRDIQINHVNKS-CNSWSSPYQLSYAIGVGDLVATSLNTFS 235

238 TFMVHDKTNYRVNEPSSSGKTLTIQFVNQRHYRAQQCFMSVVLIDNADGSTMLDKRYFVT 297
    TFMVHDK NY ++EPSSSGKTL+I FVNQR YRAQQCFMS+ L+DNADGSTMLDKRY +T
236 TFMVHDKINYNIDEPSSSGKTLSIAFVNQRQYRAQQCFMSIKLVDNADGSTMLDKRYVIT 295

298 NTNQLSIQDDLCNSLSFVLTQPWPNRMREQLALFRTPQNTALMRFYRGAQLILSGDAQAL 357
    N NQL+IQ+DL  SLS  L QPWP RM+E L       +   L  FY+    +L GD ++L
296 MGNQLAIQNDLLESLSKALNQPWPQRMQSTLQKILPHRGALITNFYQAHDYLLHGDDKSL 355

358 GKASDILNGIIKETPDFNYAYEYKVLVNVLRQSQQPFDKEQVAALNEKPKEIDQIPGVEK 417
     +AS++L  I++ +P+P YA   K LV+++R SQ P D++Q+AALN +   I  +P +
356 NRASELLGEIVQSSPEFTYARAEKALVDIVRHSQHPLDEKQLAALNTEIDNIVTLPELNN 415

418 TSVYYKIKTVRLLGKGDIHAAYEEINKSIQLEMSNVNTPVGERL--RNRVRSSAADAYLT 475
     S+ Y+IK V  L KG    +Y+ IN  I LEMSW+N  +  ++     +   AADAYLT
416 LSIIYQIKAVSALVKGKTDESYQAINTGIDLEMSWLNYVLLGKVYEMKGMNREAADAYLT 475

476 AFNLRPGENTLYWIENGVFQTSVQKIVPYLNSFLAED 512
    AFNLRPG NTLYWIENG+FQTSV  +VPYL+ FLA +
476 AFNLRPGANTLYWIENGIFQTSVPYVVPYLDKFLASE 512
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Fig. 4. Homology of amino acid sequence between *E. coli* and *S. typhimurium cadC*. The upper sequence is *cadC* from *S. typhimurium* and lower sequence from *E. coli* (Acession no. M67452). The identity and positivity between *E. coli* and *S. typhimurium* CadC are presented by capital letters and +, respectively.

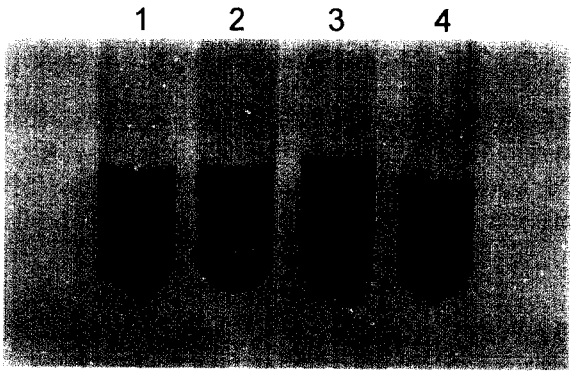

Fig. 5. Complementation test. Using Moeller decarboxylase medium, test of lysine decarboxylase activity was performed (1.LT-2, 2.JF2644, 3.SCDC2, and 4.SCDC4). Yellow and purple colors indicate the absence and presence of lysine decarboxylase, respectively.

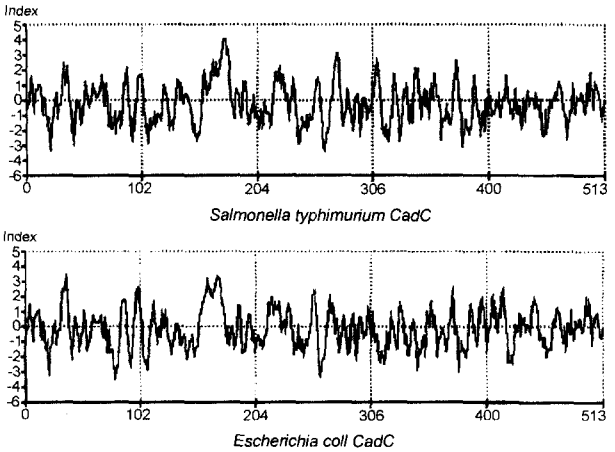

Fig. 6. Comparison of hydrophobicity profiles of *S. typhimurium* and *E. coli* CadC. More positive values indicate the more hydrophobic regions.

and *cadB* encodes a lysine/cadaverine antiporter (13, 21). *cadC*, as a positive regulator, is located upstream of the *cadBA* locus. Also, *S. typhimurium* possesses the *cadBA* operon homologous to that of *E. coli* (17). However, a positive regulator of the *cadBA* operon has not been investigated.

We isolated a *cadC* gene and its mutant from *S. typhimurium*. Sequencing analysis showed that *S. typhimurium cadC* has a relatively low nucleotide sequence (67% identity) and amino acid sequence homology (53% identity and 67% similarity) with *cadC* from *E. coli* (Fig. 4). The amino acid and nucleotide homologies between *E. coli* and *S. typhimurium cadC* are less similar than those of *E. coli* and *S. typhimurium cadBA*. However, the hydrophobicity plots showed that the CadC from *S. typhimurium* has a similar structure with *E. coli* CadC protein (Fig. 6). Previously, analysis of the amino acid sequence of CadC from *E. coli* revealed that it has three domains: an amino terminal DNA-binding domain, a transmembrane domain, and a periplasmic domain at the carboxy terminus (21). The domain from 153 to 187 residues, which

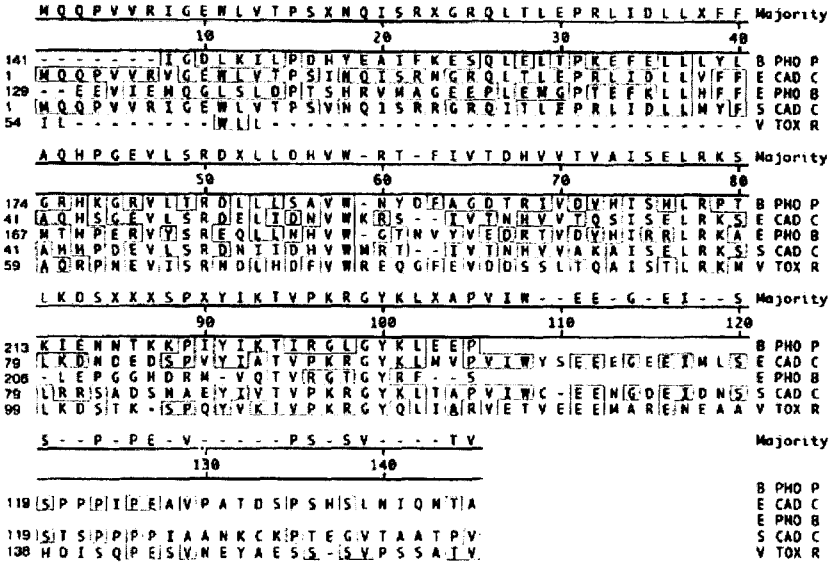

Fig. 7. Sequence alignment of transcriptional activators: PhoP, ToxR, PhoB, and CadC. Boxes indicate complete identity. The sequences are from various organisms as follows: B, *B. subtilis* (accession No. M16775 for PhoP); E, *E. coli* (M67452 for CadC and X04026 for PhoB); S, *S. typhimurium*; V, *V. cholerae* (M21249 for ToxR).

shows a high positive value, is the transmembrane domain in *E. coli* CadC (4). The high positive value indicates the more hydrophobic region. From hydrophobicity profile of the *S. typhimurium* CadC, we predicted that it has a transmembrane domain similar to the transmembrane domain of *E. coli* CadC. As shown in Fig. 7, CadC from *S. typhimurium* also has a hydrophobic region at 153 to 190 residues of amino acid. Overall sequences in both the 5' and 3' ends of CadC from *S. typhimurium* are very similar to *E. coli* (Fig. 4). Here, we suggest that the structure of *S. typhimurium* CadC may be similar with *E. coli* CadC, although the sequence of the predicted transmembrane domain shows a low homology with the *E. coli* CadC sequence.

The putative DNA-binding domain of *E. coli* CadC shows a sequence similarity to the DNA-binding domain of a group of bacterial response regulators referred to as the RO_{II} sub-group (15). In this study, we compared several transcriptional activators with *S. typhimurium* CadC: PhoP (alkaline phosphatase regulatory protein) from *Bacillus subtilis*, CadC from *E. coli*, PhoB (phosphate regulon transcriptional regulatory protein) from *E. coli*, and ToxR (cholera toxin transcriptional activator) from *Vibrio cholera* (Fig. 7). This sequence alignment shows that the CadC from *S. typhimurium* is highly homologous with other transcriptional activators, especially ToxR, with CadC from *E. coli*. In *E. coli* CadC, the codon 265 (Arg) plays an important role in the response to the lysine signal and the pH signal (13). In this study we found that *S. typhimurium* CadC also has the codon at 267 amino acid (Arg) (Fig. 4). This result indicates that the carboxyl end of the *S. typhimurium* CadC is important in responding to the pH.

From these results we propose that CadC from *S. typhimurium* is located in the membrane because of a hydrophobic region and activates the *cadBA* operon by binding its promoter. The precise function and expression of *cadC* in acidic environments is worth further investigation.

Acknowledgments

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