# Characterization of lysine decarboxylase-negative strains of *Salmonella enterica* serovar Enteritidis disseminated in Japan

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## Abstract

*Salmonella enterica* serovar Enteritidis is one of the leading causes of food-borne diseases in Japan. Typically, *Salmonella* spp. test positive for lysine-decarboxylase. However, the number of isolates of serovar Enteritidis without lysine-decarboxylase activity increased in Japan in 2003. Among 109 strains from distinct outbreaks, 10 lacked lysine-decarboxylase activity. Nine of the ten lysine-decarboxylase-negative strains showed quite similar pulsed-field gel electrophoresis profiles. Their lysine-decarboxylase phenotype was recovered by introduction of the *cadBA* locus from an lysine-decarboxylase-positive strain. Although the *cad* loci of the lysine-decarboxylase-negative strains seemed to be intact without any insertion sequences, *cadC*, a positive regulator of *cadBA*, had a single-base deletion at the same position, the 973rd base (cytosine), in all the nine lysine-decarboxylase-negative strains, whereas the wild-type *cadC* gene has a 1542 bp coding region (514 amino acids). This deletion was expected to produce a truncated (338 amino acids) form of CadC due to a frameshift. Because CadC senses environmental cues such as external pH and lysine through its putative C-terminal periplasmic domain, it is likely that the truncated CadC is not sensitive enough to external signaling to activate the *cadBA* operon, resulting in loss of the lysine-decarboxylase activity. Our results suggest that dissemination of these genetically closely related strains of serovar Enteritidis accounts for the unusual increase in the isolation of lysine-decarboxylase-negative strains.

## Introduction

Nontyphoidal salmonellae are the predominant causative agents of food-borne diseases in Japan. *Salmonella enterica* serovar Enteritidis has been predominant since 1989 (National Institute of Infectious Diseases, 2003). Biochemical testing of amino acid decarboxylase activities is the most popular means of identifying *Enterobacteriaceae*. A test for lysine decarboxylase (LDC) is used for the identification of *Salmonella* spp. as typical nontyphoidal salmonellae are positive for LDC (Farmer, 2003). The mechanism of LDC activity has been studied in *Escherichia coli* and is considered to depend on the *cad* locus. The *cad* locus consists of three genes, *cadC*, *cadB* and *cadA*. *cadA* and *cadB* encode lysine decarboxylase and lysine-cadaverine antitransporter, respectively, and constitute the *cadBA* operon. The *cadBA* operon is expressed under conditions of low external pH in the presence of lysine. *cadC* is located

upstream of *cadBA* and encodes a positive regulator for the expression of *cadBA* through sensing signals induced by external pH and lysine (Tabor *et al.*, 1980; Meng & Bennett, 1992a, b; Watson *et al.*, 1992; Neely & Olson, 1996; Soksawatmaekhin *et al.*, 2004). The deduced amino acid sequence of CadC has three domains: a cytoplasmic domain at the amino terminus, a transmembrane domain, and a periplasmic domain at the carboxyl terminus. The N-terminal cytoplasmic domain is similar to DNA-binding domains of bacterial response regulators belonging to the $RO_{II}$ subclass (Parkinson & Kofoid, 1992; Dell *et al.*, 1994).

In 2003, there was an unusual increase in the frequency of identification of LDC-negative isolates of *S. enterica* serovar Enteritidis in Japan. The present study examined LDC-negative isolates by bacteriophage typing and genotyping, and also analyzed the *cad* loci to elucidate the mechanism underlying this phenotype in *Salmonella* spp.

## Materials and methods

### Bacterial strains

A total of 802 isolates related to 109 apparently independent outbreaks that occurred in 2003 were collected from local public health institutes. Of these, 109 strains of *Salmonella enterica* serovar Enteritidis representative of these outbreaks were used in this study.

### Phenotypic and genotypic characterization

The LDC test was performed using Moller Lysine medium (Eiken Chemical Co. Ltd., Tokyo, Japan) (Moller, 1955).

Bacteriophage typing was performed according to the method of the Health Protection Agency (HPA) (London, UK) (Ward *et al.*, 1987).

Pulsed-field gel electrophoresis (PFGE) was performed as previously described using the *S. enterica* serovar Braender-up H9812 as a standard strain (Ribot *et al.*, 2002; Hunter *et al.*, 2005), with the following modifications. The DNA in agarose plugs was digested with *Bln*I (Takara Bio Inc., Shiga, Japan). Digested DNA was separated through a 1% SeaKem Gold agarose gel (Cambrex Bio Science Rockland Inc., Rockland, ME) in $0.5 \times$ TBE buffer at $14\,^{\circ}\text{C}$ in a CHEF DR-III (Bio-Rad Laboratories Inc., Hercules, CA) with the following electrophoresis conditions: initial switch time of 2.2 s, final switch time of 63.8 s, $6\,\text{V cm}^{-1}$, at an angle of $120^{\circ}$ for 19 h. The resulting profiles were scanned and saved in the TIFF format to be analyzed using Fingerprinting II software (Bio-Rad Laboratories Inc.). Similarity was determined using the Dice coefficient, and clustering was based on the unweighted pair group method with arithmetic averages (UPGMA) with a band position tolerance of 1.2%.

### Analyses of the *cad* locus

PCR of the *cad* locus was performed with Takara Ex Taq DNA polymerase (Takara Bio Inc.) using chromosomal DNA of LDC-negative strains as a template. *cadC* and *cadBA* were amplified using primer pairs of 5′-GCCGGATCCCCT GCTTATTGCGCTGC-3′ and 5′-GCCAAGCTTAATCTTCT GCCAGAAAAC-3′, and 5′-GCCGGATCCGTTTTCTGGCA GAAGATTAA-3′ and 5′-GCCAAGCTTATTTCGTATTTTC TTTCAGC-3′, respectively.

The amplified fragment of *cadC* was purified using Sigma Spin Post-Reaction Purification Columns (Sigma-Aldrich Inc., St Louis, MO). The nucleotide sequence was determined with an ABI PRISM 3100-Avant Sequencer (Applied Biosystems, Foster City, CA).

### Complementation of the *cad* genes

To construct *cad* expression plasmids, wild-type *cadC* and *cadBA* were cloned into pUC18 and pUC19 vectors digested with *Bam*HI and *Hin*dIII (Takara Bio Inc.) after performing PCR as described above using chromosomal DNA of an LDC-positive strain, E-1219, as a template. The resulting plasmids (pUC18-cadC, pUC19-cadC, pUC18-cadBA, and pUC19-cadBA) were introduced into LDC-negative strains by electroporation for the assay of complementation.

## Results and discussion

### Typing of outbreak-related strains of *Salmonella enterica* serovar Enteritidis

A total of 109 strains of serovar Enteritidis were subjected to bacteriophage typing and LDC testing. The results are summarized in Table 1. Ten (9.2%) strains were LDC-negative, with five identified as phage type (PT) 4, four as PT14b, and one as PT1. As shown in Table 1, PT4, PT47 and PT1 strains were predominant in 2003 as well as in previous years (Izumiya *et al.*, 2003). PT14b strains were also not uncommon. However, strains testing negative for LDC are considered unusual among *Salmonella* spp. PFGE genotyping showed unique PFGE profiles of LDC-negative PT4 and PT14b strains of serovar Enteritidis (Fig. 1), but these profiles were quite similar to each other. The profile of the LDC-negative PT1 strain differed, however, with less than 50% similarity to the other strains (Fig. 1). Thus, these PT4 and PT14b strains were probably responsible for the unusual increase in the frequency of the LDC-negative strains. We therefore analyzed their *cad* locus further.

**Table 1.** Distribution of phage type and lysine-decarboxylase phenotype of the serovar Enteritidis strains used in this study

| PT | LDC | | Total |
|---|---|---|---|
| | + | − | |
| 4 | 18 | 5 | 23 |
| 47 | 23 | | 23 |
| 1 | 20 | 1 | 21 |
| 6a | 10 | | 10 |
| 14b | 5 | 4 | 9 |
| RDNC | 7 | | 7 |
| 36 | 5 | | 5 |
| 5c | 4 | | 4 |
| 6 | 2 | | 2 |
| 1b | 1 | | 1 |
| 3 | 1 | | 1 |
| 21 | 1 | | 1 |
| 29 | 1 | | 1 |
| UT | 1 | | 1 |
| Total | 99 | 10 | 109 |

LDC, lysine-decarboxylase; PT, phage type; RDNC, reacted but did not conform; UT, untypeable.
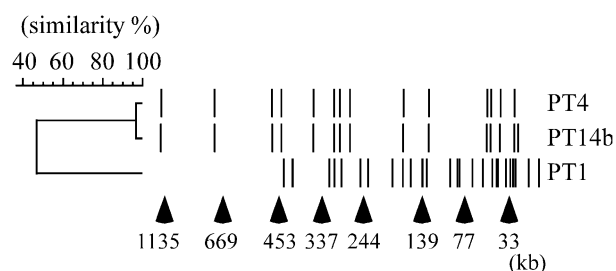
**Fig. 1.** Dendrogram of representative *BlnI*-digested PFGE profiles of the lysine-decarboxylase-negative strains. Corresponding phage types are shown on the right. Positions and sizes of the bands of the *Salmonella enterica* serovar Braenderup H9812 are indicated at the bottom.

## LDC negativity of the PT4 and PT14b strains of serovar Enteritidis due to a mutation in *cadC*

The LDC phenotype has been shown to depend on the *cad* locus in *Escherichia coli*. Inactivation of *cadC* by an insertion sequence has been reported to also cause LDC negativity in *E. coli* (Casalino *et al.*, 2003). PCR was used to detect the *cadBA* operon and the *cadC* gene of the LDC-negative strains. The results showed that bands with the expected sizes were amplified in each one (data not shown). This indicates that there were no apparent genetic changes due to insertion sequences.

We then cloned the *cadBA* operon and the *cadC* gene of an LDC-positive strain of *S. enterica* serovar Enteritidis in the pUC18 and pUC19 vectors for use in complementation assays of LDC negativity. The resulting plasmids were named pUC18-cadBA, pUC18-cadC, pUC19-cadBA and pUC19-cadC, respectively. The cloned *cadBA* operon and the *cadC* gene contained the putative authentic promoters. It was expected that expression of the genes cloned in pUC18 would be constitutive and dependent upon the *lac* promoter in the vector as well as on the genes' own promoters, whereas the genes cloned in pUC19 were ex-

**Table 2.** Complementation of the *cad* genes in lysine-decarboxylase-negative strains

| LDC-negative strains | LDC activities after introduction of | | | |
|---|---|---|---|---|
| | pUC18-cadC | pUC19-cadC | pUC18-cadBA | pUC19-cadBA |
| E–1211 | + | + | + | − |
| E–1217 | + | + | + | − |
| E–0548 | − | − | + | − |

+, positive; − , negative; LDC, lysine-decarboxylase.

pected to be expressed under the control of only their own promoters.

Two LDC-negative strains were used for complementation experiments: one representative of PT4 strains (E-1211) and the other representative of PT14b strains (E-1217). The LDC phenotype of both strains was restored following transformation with pUC18-cadBA, pUC18-cadC and pUC19-cadC. However, the introduction of pUC19-cadBA did not restore the LDC phenotype (Table 2). These results suggest that (1) the LDC phenotype depended on expression of the *cadBA* operon in *S. enterica* as well as in *E. coli*, and (2) the activity of the positive regulator CadC was insufficient for the activation of the *cadBA* promoter in the LDC-negative strains, as pUC19-cadBA could not restore the phenotype, whereas pUC19-cadC could.

The nucleotide sequences of the coding regions for the CadC proteins and their 0.1 kb upstream regions were determined. Both the PT4 and PT14b LDC-negative strains exhibited a single-base deletion at position 973 (cytosine) of the 1542 bp coding region (514 aa), which was predicted to result in a truncated CadC (338 aa) on the basis of the predicted amino acid sequence (Fig. 2). The sequences of *cadC* of all the other LDC-negative strains of PT4 and PT14b were examined, and the same deletion mutation found, which is consistent with the similarity in their PFGE profiles.
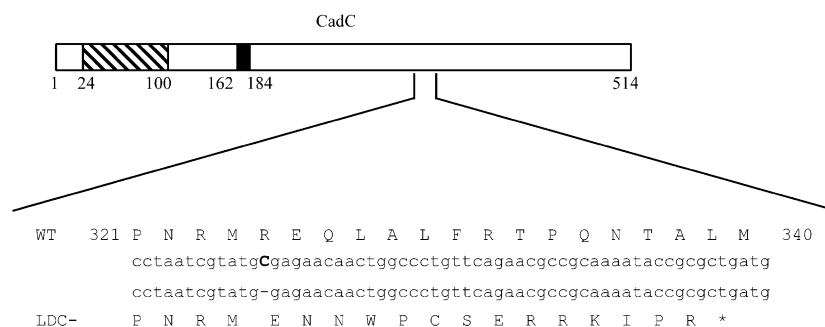


**Fig. 2.** Schematic diagram of the *cadC* mutational site in the lysine-decarboxylase-negative strains. Numbering starts at the putative initiation codon. A frameshift mutation due to the deletion of the cytosine at position 973 (shown in bold uppercase) was expected to lead to truncation of the deduced CadC protein (514 aa) to 338 aa. The hatched box indicates the position of the putative DNA-binding domain. The solid box indicates the position of the transmembrane domain.

This mutation causes a frame shift in the *cadC* gene to produce a truncated form of CadC. The truncated CadC protein retained its putative DNA-binding and transmembrane domains, but lacked its C-terminal domain. Single amino acid substitutions in the carboxyl terminal periplasmic domain cause derepression of pH- and lysine signaling, suggesting that this domain is important for the function of CadC as a sensor (Dell *et al.*, 1994). The truncation identified in this study seemed to be a novel type of mutation in that it was not due to any insertion sequences and would cause loss of the ability of CadC to sense such external signals. However, the truncation might not cause complete loss of function because multicopy expression of the truncated CadC by a pUC18-based plasmid restored the phenotype of the LDC-negative PT4 and PT14b strains (data not shown). Thus, the mutation identified in this study is likely to reduce the ability of CadC to activate *cadBA* transcription due to a weak response to signals induced by external pH or lysine.

### Analysis of the LDC-negative PT1 strain of serovar Enteritidis

PCR, complementation assay and sequence analysis of *cadC* were carried out to examine the *cad* locus in the LDC-negative PT1 strain E-0548 as well as the LDC-negative PT4 and PT14b strains. The PCR results revealed no apparent genetic changes (data not shown). The LDC negativity of the PT1 strain was suppressed by overexpression of *cadBA* from pUC18-cadBA. However, neither pUC18-cadC nor pUC19-cadC could restore the phenotype (Table 2). In addition, no mutations were found in *cadC* of the LDC-negative PT1 strain. These results indicate that the PT1 strain has mutation(s) in gene(s) which differ from those found in the LDC-negative PT4 and PT14b strains. Further investigations are needed to elucidate the mutation(s) responsible for the phenotypes.

### Concluding remarks

An LDC-positive phenotype is a major characteristic of *Salmonella* spp., and is widely used in simplified screening tests (Wilson, 2004). However, there was an unusual increase in the frequency of LDC-negative strains isolated in local public health institutes in Japan in 2003, accounting for no less than 9% of outbreak-related strains. Almost all of them showed similar PFGE profiles and had the same point mutation in *cadC*, though they included two phage types, PT4 and PT14b. It would appear that the dissemination of these genotypically related strains was responsible for the unusual increase in the LDC-negative isolates.

We identified a novel point mutation in *cadC* which was acquired naturally and might affect the function of the CadC protein as a sensor. Such a mutation would cause unusual biochemical characteristics such as LDC negativity in *Salmonella* spp. Conversely, unusual characteristics may reflect genetic changes, as described in this paper. Such unusual features would be useful as epidemiological markers along with other typing methods such as PFGE and bacteriophage typing.

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