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Identification and Characterization of Genes Involved in Cysteine Auxotrophy in *Salmonella typhi*

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In spite of long research period for *Salmonella typhi*, little information is known about the pathogenesis mechanism of human typhoid fever caused by *S. typhi* due to lack of infection model in animals. A wild-type of *S. typhi* Ty2 strain requires cysteine to grow on minimal media. We hypothesized that this cysteine requirement may restrict colonization of *S. typhi* in animals during infection process. Among the *S. typhi* strains carrying *Salmonella typhimurium* genomic library, we have isolated three *S. typhi* transformants growing on minimal media without cysteine. Although there were three ORFs in DNA of pBP71, the STM1490 ORF complemented cysteine auxotrophy of *S. typhi*. Analysis of the deduced amino acid sequence of the *STM1490* homolog in *S. typhi* revealed that there are differences in two amino acids. Plasmids containing amino acid substitutions in STM1490 supported *S. typhi* growth on minimal media without cysteine, indicating irrelevance of these two amino acids to STM1490 function. These results tells us that there are other factors or systems involved in cysteine requirement of *S. typhi*.

Key words : *Salmonella typhi*, cysteine auxotrophy, STM1490

Introduction

Salmonella enterica is composed of more than 2,400 serovars that can infect human and a great diversity of animals resulting in diseases ranging from gastroenteritis to life-threatening systemic infection [1,2]. *Salmonella enterica* serovar Typhi (*S. typhi*) is strictly adapted to human hosts, in whom it causes a systemic disease known as typhoid fever which results in some 600,000 deaths annually [3]. One limitation to studying the pathogenesis of typhoid fever is the absence of a good animal model, because *S. typhi* is strictly human adapted, causing disease only in higher primates [4]. Mice infected with *S. typhimurium* develop a systemic typhoid-like disease, which is commonly used to model *S. typhi* infections in humans [5]. However, an obvious shortcoming of this mouse model is the fact that *S. typhimurium* dose not cause typhoid fever in humans, suggesting that genetic differences between *S. typhi* and *S. typhimurium* are critically important for the disease outcome.

It is not known that the reason why *S. typhi* can't survive in the animals. Since *S. typhi* Ty2 is a cysteine auxotroph, we assumed that this phenotype may contribute little or much to accomplish *S. typhi* colonization in the animals. To convert *S. typhi* to the virulent strain in animal at least in mouse, we have to modify *S. typhi* to generate *S. typhimurium*-like *S. typhi*. As a beginning stage for the development of *S. typhi* animal model, we screened and characterized genes which allow *S. typhi* growing in the minimal media without cysteine.

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Materials and Methods

Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Salmonella typhimurium*, *Salmonella typhi* and *Escherichia coli* were grown at 37°C in Luria-Bertani (LB) broth or LB agar [6]. When required, *Salmonella* strains were cultured in Curtiss minimal medium (CMM) and M9 minimal medium. Media were supplemented with appropriate antibiotics at the following concentration (μg/ml) : ampicillin, 100; tetracycline, 15; kanamycin 50; chloroamphenicol, 30; streptomycin, 50. Diaminopimelic acid was added (50 μg /ml) for the Asd^- strains [7]. LB agar containing 5% sucrose was used for *sacB* gene-based counterselection in allelic exchange experiment [8]. Amino acids, when required, were

Table 1. Bacterial strains used in this study

Strains or plasmids	Relevant characteristics*	Reference or source
Escherichia coli		
E. coli DH5α	Transformation host for cloning vector, F⁻,ϕ80d*lac*ZΔM15 Δ(*lac*ZYA⁻*arg*F) U169 *rec*A1 *end*A1 *hsd*R17(r_k^-,m_k^+) *pho*A *sup*E44 Γ⁻ *thi*-1 *gyr*A96 *rel*A1	Promega
E. coli χ7213	*E. coli* DH5α delivative (Δ*asd*), Km^R, DAP required	Roland, K.
Salmonella		
S. typhimurium χ3339	SL1344, *his*G, Sm^R, wild type	Gulig, P.
S. typhi χ3769	Ty-2, wild type	Lab collection
S. typhi CK44	χ3769 derivative, ΔSTM1490, Km^R	This study
Plssmid		
pBSL86	Vector carrying a Km^R gene, ColE1*ori*, Ap^R, Km^R	Alexeyev M. F.
pBR322	Cloning Vector, ColE1*ori*, Ap^R, Tet^R	Balbas P.
pGEM-T vector	Cloning vector for PCR product, ColE1*ori*, Ap^R	Promega
pMEG375	Suicide vector, R6K*ori*, Ap^R, Cm^R	Megan Health Inc.
pWSK29	Cloning vector, pSC101*ori*, Ap^R	Wang, R. F.
pBP71 - pBP81	pBR322 derivative containig a part of DNA segment of *S. typhimurium* LT2	This study
pBP104	1.1kb ynfK *Bam*HI-*Hin*dIII product in pBR322, Ap^R	This study
pBP105	1.8kb STM1490(χ3339) *Bam*HI-*Hin*dIII product from pBP100 in pBR322, Ap^R	This study
pBP115	1.8kb STM1490(χ3339) *Bam*HI-*Hin*dIII product from pBP100 in pWSK29, Ap^R	This study
pBP160	1.1kb 5′-flanking DNA of STM1490(χ3769) in pGEM-T, Ap^R	This study
pBP161	1.0kb 3′-flanking DNA of STM1490(χ3769) in pGEM-T, Ap^R	This study
pBP196	2.1kb DNA ligated 5′-flanking and 3′-flanking of STM1490(χ3769) in pGEM-T, Ap^R	This study
pBP354	1.2kb *Hin*dII Km^R gene from pBSL86 in pBP196, Ap^R, Km^R	This study
pBP365	Derivative of pMEG375, recombinant suicide plasmid for ΔSTM1490 of χ3769, Ap^R, Cm^R, Km^R	This study
pBP462	1.8kb STM1490 H229Y *Bam*HI-*Hin*dIII product from pBP115 in pWSK29, Ap^R	This study
pBP463	1.8kb STM1490 C246W *Bam*HI-*Hin*dIII product from pBP115 in pWSK29, Ap^R	This study

included in the culture medium with the following concentrations (μg/ml) : L-cysteine, 110; L-histidine, 22.

General DNA manipulations

DNA manipulations were carried out as described by Sambrook *et al*. [9]. DNA preparation, ligation, restriction analysis, transformation and electrophoresis were carried out as described by Sambrook *et al*. [9]. To move DNA library contained in *S. typhimurium* to *S. typhi* strain, P_{22}HT*int* mediated general transduction was performed [10]. PCR amplification was employed to obtain DNA fragments for cloning and verification of chromosomal detection of the mutation.

Construction of a recombinant suicide plasmid for the chromosomal deletion of genes *STM1490*

The 5′-flanking region of *STM1490* gene was amplified by PCR using *S. typhi* Ty2 chromosomal DNA as a template with using a pair of primers [5′-aagcttagcgatcagcaggcgacg-3′ and 5′-ggatccctgcaagcaacgcgccgaag-3′]. The 1.1 kb amplified DNA from *S. typhi* chromosomes was cloned into the pGEM-T vector (Promega), resulting in pBP160 (Table 1). With application of the same approach, the 1.0 kb 3′-flanking DNAs of *STM1490* gene from *S. typhi* Ty2 was amplified by PCR with use of a primer set [5′-ggatccgttgattcgtctattacag g-3′ and 5′-aagcttttacgccaacattatttgtc-3′] and cloned into the pGEM5Z-T vector (Promega), resulting in pBP161 (Table 1). To construct the recombinant DNAs for *STM1490* gene deletion, the 1.0 kb *Hin*dIII-*Bam*HI DNA fragment isolated from pBP161 was ligated into the plasmid pBP160 digested with the same enzyme, resulting in the plasmid pBP196 (Table 1). A 1.2 kb *Bam*HI kanamycin (Km) resistance gene fragment isolated from pBSL86 [11] was inserted at *Bam*HI site located in the middle between 5′-flanking and 3′-flanking

DNA of pBP196, resulting in pBP354. The 3.3 kb *Bam*HI-*Bam*HI fragment isolated from pBP354 was cloned into a suicide vector pMEG375, resulting in the recombinant suicide plasmid pBP365 (Table 1).

Results

Screening of *S. typhimurium* genes complementing *S. typhi* Ty2 cysteine requirement

The cysteine auxotrophy of *S. typhi* may caused by lack of cysteine metabolism associated gene(s). Therefore we hypothesized that introduction of *S. typhimurium* genomic library into *S. typhi* can identify gene(s) providing *S. typhi* cysteine autotrophy. The genomic library was moved from *S. typhimurium* to *S. typhi* by bacteriophage P_{22}HT*int* mediated general transduction. Although P_{22}HT*int* bacteriophage is specific for *S. typhimurium*, it can still inject DNA contained in phage particle to *S. typhi*. Total eleven transductants harboring plasmid could grow on the cysteine free minimal agar containing ampicillin. Based on the restriction enzyme analyses of the isolated plasmids, the plasmids were divided into four groups (Table 2). The plasmids pBP71, pBP75, pBP79 and pBP81 were picked as the group representative. Nucleotide sequence of pBP71 was the same as that in pBP75, indicating group I and group Ⅱ contains the same genes. Thus the plasmids were re-grouped into 3 categories, and the plasmids pBP71, pBP79 and pBP81 were chosen as a representative clone of each group. Although all three plasmids provided cysteine autotrophy function to *S. typhi* strain, the *S. typhi* containing pBP79 or pBP81 grow very slowly in the cysteine free minimal media. However, the strain harboring pBP71 exhibited apparent growth on cysteine-free minimal media. *S. typhi* Ty2 derivatives grew also in the CMM media with methionine, suggesting that biochemical reactions involved in the conversion of methionine to cysteine are normal in *S. typhi*.

Table 2. Grouping of plasmids isolated from genomic library based on complementing activity to *S. typhi* cysteine requirement

(A)

Group	Plasmid
GroupI	pBP71, pBP72, pBP73, pBP77, pBP78, pBP80
GroupII	pBP74, pBP75, pBP76
GroupIII	pBP79
GroupIV	pBP81

(B)

Medium* \ Strains*	χ3769	χ3769 [pBR322]	χ3769 [pBP71]	χ3769 [pBP79]	χ3769 [pBP81]
LB	++**	++	++	++	++
CMM	-	-	++	+	+
CMM + cysteinde	++	++	++	++	++
CMM + methionine	++	++	++	++	++
CMM + $MgCl_2$	-	-	++	+	+

*Each strains were incubated identically for 48 hr at 37°C on CMM (Fe^-) medium containing ampicillin. But χ3769 (wt) was incubated no ampicillin.

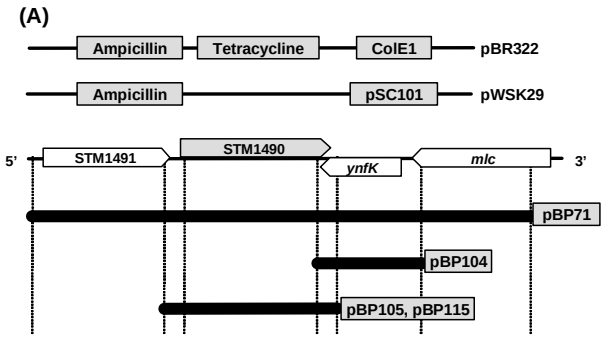
**Relative growth rate: -, no growth; +, weakly growth; ++, growth

Identification of genes involved in the *S. typhi* cysteine autotrophy in the plasmid pBP71

To identify and analyze genes contained in DNA insert of pBP71, nucleotide sequencing was performed. Nucleotide sequence analyses suggested that the insert DNA contains three ORFs (Fig. 1A); Ynfk (putative dethiobiotin synthease), STM1490 (putative chloride channel protein) and STM1491 (ABC-type proline/glycine betaine transport). Because the DNA contained in pBP71 harbors a part of ORF of STM1491 and *mlc*, we ignored those genes for the further characterization. To determine genetic limits for cysteine prototrophy, the DNA segment corresponding YnfK or STM1490 were PCR amplified and cloned into pBR322, resulting in pBP104 or pBP105, respectively (Fig. 1A). The *S. typhi* containing pBP104 did not grow on the cysteine-free CMM. Interestingly, *S. typhi* contain pBP105 grew on the cysteine-free CMM, indicating that *STM1490* gene complements cysteine auxotrophy (Fig. 1B). Because *STM1490* allele is exist in *S. typhi* chromosome, there is a chance that the complementation activity for pBP105 derived by the effect of gene dosage due to multicopy plasmid. To examine this possibility the *STM1490* DNA fragment was cloned into pWSK29 vector which contains low copy number replication origin of pSC101, resulting in pBP115. *S. typhi* containing pBP115 grew on the cysteine-free CMM, indicating gene dosage independent complementation (Fig. 1B).

Constructions of a *S. typhi* mutant carrying *STM1490* deletion

A recombinant suicide plasmid pBP365 (Table 1) was used to construct a *S. typhi* Δ*STM1490* mutant by allelic exchange. The pBP365 was conjugally transferred from *E.*



(B)

	CMM	CMM (no sulfate)
χ3769	–	–
χ3769[pBR322]	–	–
X3769[pWSK29]	–	–
X3769[pBP71]	+	+
X3769[pBP104]	–	–
X3769pBP105]	+	+
X3769[pBP115]	+	+

Fig. 1. Converting cysteine autotrophy in *S. typhi* Ty2 by STM1490, a putative chloride channel protein. (A) Plasmid used for complementation tests. The pBP71 contain three ORFs; Ynfk, STM1490 and STM1491. The pBP104 contains Ynfk. The pBP105 contains replication origin of pBR322 and maintains number of high copy, whereas pBP115 replicates to origin of pSC101, a low copy number plasmid. (B) Analyses of growth of *S. typhi* Ty2 complementated by DNA segments from *S. typhimurium* LT2. Each strain were incubated for 48 hr at 37°C on CMM medium (Cys^- and Fe^-) containing ampicillin (100 g/l) or none sulfate.

coli χ7213 to *S. typhi* χ3769. Through series of steps required for the mutant selection and screening, a Δ*STM1490* mutant was selected and designated as a *S. typhi* CK44 (Table 1). The mutation was confirmed genetically by comparison of the amplified DNA size with various primer sets (Fig. 2).

Amino acid substitution in STM1490

Comparison of amino acids sequences of STM1490 from *S. typhi* and other enteric including *E. coli*, *Sigella*, and *S. typhimurium* revealed amino acid difference at two distinct places, 229th and 246th. The *S. typhi* STM1490 at 229th and 246th were Y and W instead of H and C as seen in *S. typhimurium* STM1490, respectively (Fig. 3). We replaced these two amino acids in *S. typhimurium* STM1490 with those in *S. typhi* STM1490. Overlapping PCR method described in a previous study [12] was applied to introduce

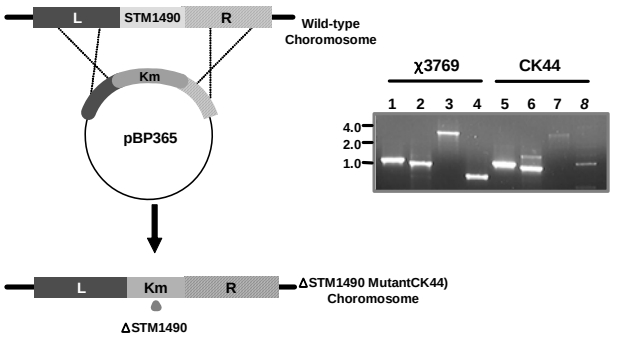


Fig. 2. Construction of *STM1490* deletion mutant in *S. typhi*. Homologous recombination between two homologous regions, *S. typhi* chromosome and the recombinant suicide plasmid pBP365 resulted in the *STM1490* mutant *S. typhi* CK44. Genetic confirmation of *STM1490* deletion on the chromosome of the strain *S. typhi* Ty2. DNA fragments were PCR amplified from the chromosomal DNA of χ3769 and CK44 (Δ*STM1490*). Each PCR product was 0.7 kb (amplified from χ3769) and no amplified (amplified from CK44). Lanes: 1 and 4, 5′ franking DNA of *STM1490*; 2 and 5, 3′ franking DNA of *STM1490*; 3 and 6, 5′-flanking-*STM1490*-3′ flanking DNA; 4 and 8, STM1490 *orf*

Fig. 3. Alignments of *Sallmonella* STM1490. As amino acid sequence of STM1490 compared with *E. coli*, *Shigella flexneri*, *S. typhimurium* and *S. typhi*, STM1490 derivative *E. coli* and *S. flexneri* were longer than others about 10 amino acids. STM1490 of *S. typhimurium* was different H229Y and C246W in *S. typhi*.

a point mutation (Fig. 4A). The mutated *STM1490* genes were cloned into pBR322 plasmid, resulting in pBP462 (H229Y) and pBP463 (C246W). The nucleotide changes in both pBP462 and pBP463 were confirmed by DNA sequence analysis. Plasmids pBP462 or pBP463 were introduced into the CK44 strain. The wild-type and CK44 strains were failed growth on the cysteine-free minimal media, but the CK44 harboring pBP115 grew on the same minimal media. The *S. typhi* CK44 carrying pBP462 or pBP463 grew well on the CMM agar or M9 agar media without cysteine supplement. This result suggests that single amino acid change does not influence to the function of STM1490 (Fig 4B). Replacing amino acids at both 229th and 246th of *S. typhi* STM1490 remains to be done for the

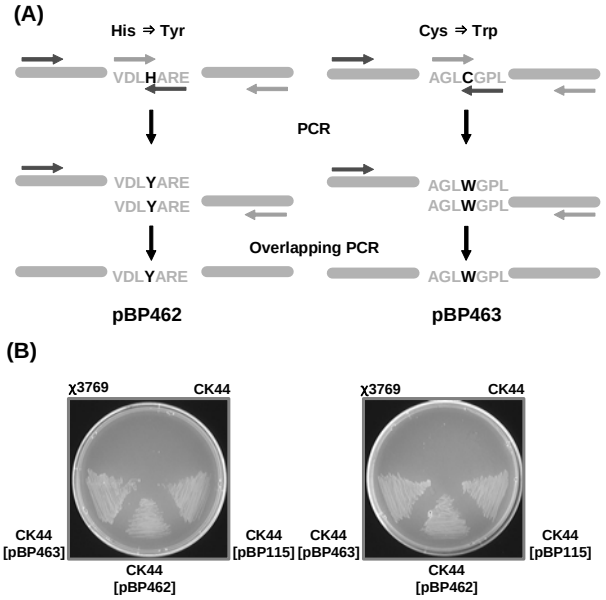


Fig. 4. Introduction of point mutation in the STM1490. (A) A brief scheme for the amino acid substitution. Two amino acid at 229H and 246C seen in *S. typhimurium* STM1490 was replaced to 229Y and 246W as seen in *S. typhi* STM1490. Overlapping PCR method was applied to introduce mutations. The recombinant DNAs carrying substituted amino acids, H229Y and C246W, were cloned into pBR322, resulting in pBP462 or pBP463, respectively. (B) Complementation assay for cysteine requirement. Both minimal agar media (CMM for left penal and M9 for right penal) was inoculated with bacteria as indicated, and incubated for 24 hr at 37°C. All agar media is cysteine-free.

definitive conclusion of role of two amino acids difference. Additionally, examination of promoter activity for the transcription of STM1490 is required to explain clearly the complementing activity of STM1490 gene.

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초록 : *Salmonella typhi*의 시스테인 영양요구성에 관여하는 유전자의 동정 및 특성 연구

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오랜 기간 동안의 연구에도 불구하고 사람에 특이적으로 장티푸스를 유발하는 *S. typhi*는 실험동물을 대상으로 하는 감염모델이 확립되어 있지 않기 때문에 *S. typhi*의 병원성 유발기작에 관한 정보는 부족하다. *S. typhi* Ty2 균주는 최소배지에서 시스테인의 영양요구성을 지닌다. 본 연구에서는 시스테인 영양요구성이, *S. typhi* Ty2 균주가 실험동물에서 균체형성을 하는데 어떤 영향을 미치는지를 조사하기 위해 시스테인 영양요구성을 상보할 수 있는 유전자를 찾고자 하였다. *S. typhimurium*의 genomic library로 형질전환된 *S. typhi* 균들 중 시스테인을 함유하지 않은 최소배지에서 생육을 하는 3개의 형질전환 균주를 선별하였으며, 이들 중 2개는 *S. typhi*의 시스테인 영양요구성을 아주 약하게 상보하였고 하나는 명확하게 시스테인의 영양요구성을 상보하였다. 이 클론에 포함되어져 있는 3개의 ORF의 시스테인 영양요구성을 분석한 결과, *STM1490*을 가진 클론이 *S. typhi*의 시스테인 영양요구성을 상보하였다. 비록 *S. typhi*에도 *STM1490*에 해당하는 유전자가 존재하지만 *S. typhimurium*의 STM1490에 해당하는 ORF와 비교하였을 때 2개의 아미노산 잔기가 서로 달랐다. 이들의 차이가 시스테인 영양요구성을 보이는 것은 아닌지 확인하기 위해 Overlapping PCR을 통해 *S. typhimurium*의 STM1490 아미노산(H229Y, C246W)을 치환하였다. 아미노산을 바꾼 돌연변이체도 역시 시스테인 영양요구성을 상보할 수 있어서 아미노산의 차이는 아닌 것을 확인되었으므로 그 외의 다른 인자들이 영양요구성에 관여하는 것으로 추정되었고 후속연구에서 그 인자를 찾을 수 있을 것으로 생각된다.