

RESEARCH ARTICLE

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5'-UTR of *mals* increases the invasive capacity of *Salmonella enterica* serovar Typhi by influencing the expression of *bax*

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ABSTRACT **Aim:** An RNA-seq analysis recently identified a 236-nucleotide transcript upstream from *mals* in *Salmonella enterica* serovar Typhi. Here, we investigated its molecular characteristics and function. **Materials & methods:** RACE and northern blotting were used to determine the molecular characteristics of the sequence, and mutagenesis, microarray, immunoblotting and an invasion assay were used to investigate the functions of the transcript. **Results:** The transcript was identified as the *mals* 5'-untranslated region (UTR), which could influence the expression of the flagellar and SPI-1 genes and the invasion of HeLa cells by *S. Typhi*. Deletion of *bax* increased the expression of the invasion genes and the invasive capacity of *S. Typhi*, whereas the expression of the *mals* 5'-UTR reduced the expression of *bax*. **Conclusion:** The *mals* 5'-UTR reduces the expression of *bax* and increases the invasive capacity of *S. Typhi*.

Salmonella enterica serovar Typhi (*S. Typhi*) is a Gram-negative intracellular pathogen that causes human typhoid fever [1]. It is conceivable that the ability of *S. Typhi* to cause disease in humans correlates with its acquisition of virulence genes, designated 'Salmonella pathogenicity islands' (SPI). The invasion of nonphagocytic cells is a striking characteristic of the pathogenesis of *Salmonella*. A type III secretion system (T3SS) encoded by SPI-1 allows a variety of *Salmonella* effector proteins to be translocated into the host cell to manipulate various host cell signaling events [2]. The expression of SPI-1 is very tightly controlled during the pathogenic process.

Pathogens often adapt to changing environments by rapidly modulating the expression of their virulence genes during the course of infection. Many studies have already shown that bacterial gene expression is closely related to various proteins. As well as proteins, the regulatory activities of noncoding RNAs (ncRNAs) have attracted increasing attention as key regulatory components controlling gene expression in bacteria and eukaryotes [3]. ncRNAs are transcribed RNAs that are not translated into proteins, which range in size from 50 to 500 nucleotide(s) (nt). Vast numbers of ncRNA transcripts have been identified and characterized in diverse organisms [4,5]. They are classified into three classes according to their regulatory mechanism: *trans*-encoded small RNAs (sRNAs), *cis*-encoded antisense RNAs (asRNAs), and the 5'-untranslated regions of mRNAs (5'-UTRs). *Trans*-encoded sRNAs are located at distinct chromosomal locations and are only partially complementary to their target mRNAs. *Cis*-encoded asRNAs are transcribed from the DNA strand opposite the target gene, and form extensive regions of base-pairing with their target mRNAs. 5'-UTRs are encoded

KEYWORDS

• 5'-UTR • *bax* • invasiveness
• *mals* • *S. Typhi*

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as parts of mRNAs, as are T-boxes, riboswitches and other classes of translation or transcription attenuators that display alternative structures under different environmental conditions. When bound by metabolites, 5'-UTRs may mediate the attenuation of transcription [6] or translation initiation [7], or affect mRNA stability by changing their conformation [8,9]. Researchers have found that ncRNAs are involved in various physiological responses of bacteria, including metabolic regulation, responses to oxidative stress, and the coordination of virulence [10,11].

Initially, ncRNAs were predominantly detected in bacteria with computational approaches and comparative genomics, which rely upon ncRNA conservation among closely related species [12]. The application of RNA-seq and genomic tiling arrays has increased the identification and discovery of ncRNAs in various bacteria [13,14].

With an RNA-seq analysis of the transcriptome of *S. Typhi* under different environmental stresses, combined with bioinformatic tools, we have recently identified many putative novel ncRNAs in bacteria [15]. A 236-nt transcript partially encoded by the minus strand of *bax* was expressed and designated 'Asb' (antisense RNA of *bax*). The maximum expression of Asb is from 217 nt upstream from the start codon of *mals* and overlaps the 137-nt region downstream from the *bax* gene start codon, as shown by bioinformatic prediction (Figure 1). From genomic information for *S. Typhi* Ty2, *bax* has been described as a hypothetical protein gene and *mals* is a putative amylase gene. However, the structure and function of MalS and Bax have not yet been reported for *S. Typhi*. We were curious to know whether Asb is a functional transcript. In this study, we identified Asb as the 5'-UTR of the *mals* gene in *S. Typhi* and demonstrated that the *mals* 5'-UTR may function as a *cis* regulatory RNA, increasing the invasive capacity of *S. Typhi* by regulating the expression of the *bax* gene.

Materials & methods

• Bacterial strains & growth conditions

All the strains used in this study were isogenic derivatives of the wild-type *S. Typhi* GIFU 10007. The mutants and plasmids used in the study are listed in Table 1. Unless otherwise stated, the strains were grown in Luria–Bertani (LB) medium at 37°C. When necessary, the following antibiotics were added to the culture (final concentrations): ampicillin (100 µg/ml), gentamicin

(100 µg/ml), and kanamycin (50 µg/ml). Transcription from the *mals*-5'-UTR-regulated promoter was induced with L-arabinose (w/v 0.2%). Growth was monitored by measuring the optical density at 600 nm (OD₆₀₀).

• Construction of *mals* 5'-UTR & *bax* deletion mutants of *S. Typhi*

The oligonucleotides used in this study are listed in Table 2. A strain with a deletion of the *mals* 5'-UTR was constructed using the lambda Red recombinase method [16]. A kanamycin-resistance gene was amplified from the pET-28a-c(+) plasmid DNA with primers F1A/F1B. The *kan* resistance sequence in the amplified fragment was then flanked by 50-bp sequences complementary to either end of the *mals* 5'-UTR. *S. Typhi* containing plasmid pKD46 was transformed with the purified PCR product. The transformants were plated onto LB plates containing *kan*. The *mals* 5'-UTR deletion mutant was confirmed by PCR using primers F2A/F2B and F2A/F1B. The 3'-end of the *bax* gene (527 bp from the stop codon) was also deleted using the *bax*F1A/F1B primers. Deletion of this segment was confirmed by PCR using primers *bax*F2A/F2B and *bax*F2A/F1B. The strain in which the 5'-UTR of *mals* was deleted and the strain in which the 3'-end of *bax* was deleted were designated 'Δ*mals* 5'-UTR' and 'Δ*bax*', respectively.

• Construction of strains overexpressing the *mals* 5'-UTR

To construct the recombinant vector pBAD-*mals*-5'-UTR, a 240 bp fragment corresponding to the 5'-UTR of the *mals* gene was PCR amplified from the *S. Typhi* genomic DNA with primers PA and PB. The PCR product was digested with *Nco*I and *Hind*III and cloned into the *Nco*I/*Hind*III-digested vector pBAD/Myc-His A (Invitrogen, CA, USA). The wild-type *S. Typhi* and *bax* mutant strains were transformed with the resulting plasmid, pBAD-*mals*-5'-UTR, or the empty pBAD plasmid by electroporation to generate the strains designated 'WT-pBAD-5'-UTR', 'WT-pBAD', 'Δ*bax*-pBAD-5'-UTR' and 'Δ*bax*-pBAD', respectively. The plasmid inserts were verified by DNA sequencing.

• Construction of the Bax-3×FLAG fusion strain

DNA fragments (F1 and F2) specific to regions up- and downstream from the 3×FLAG tag, respectively, were amplified from the *S. Typhi*

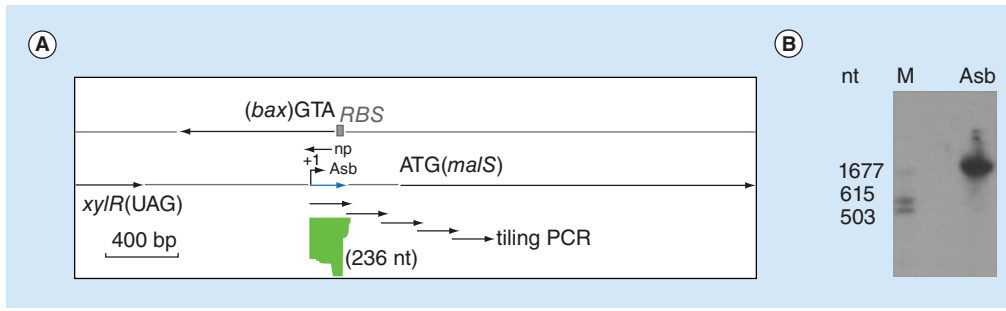


Figure 1. Genomic location and northern blot analysis of Asb expression. (A) Genomic context and expression of Asb. The maximum expression of Asb is indicated in green. The maximum expression peak is located in the 5'-UTR of *malS* and overlaps the partial upstream open reading frame region of *bax*. Arrows denote the direction and products of tiling PCR and transcription; np represents the specific probe used in the northern blot. The blue arrow represents the transcript Asb. +1 denotes the experimentally determined transcription start site of Asb. (B) Asb transcript detected in *Salmonella enterica* serovar Typhi wild-type strain by northern blot analysis using 5S-specific (lane M) and Asb-specific (lane Asb) cDNA probes, based on a BLAST search for the Asb sequences in GenBank.

M: Marker; nt: Nucleotide.

genomic DNA with the *baxP1A/B*, *baxP1A/B'*, and *baxP2A/B* primers. A *Bam*HI site was added to the 5'-termini of the *baxP1A* and *baxP2B* primers. To generate an 890 bp recombinant DNA fragment, PCR amplification was performed using the PCR products F1 and F2 as the template. This fragment was digested with *Bam*HI and ligated into the *Bam*HI-digested pGMB151 suicide plasmid. The *S. Typhi* wild-type strain was transformed with the construct. The *Bax*-3×FLAG fusion strain ligated to the 3×FLAG tag at the 3'-end of the *bax* gene was selected by PCR with primers *baxP1A* and *baxP2B* after selective incubation on LB plates with ampicillin and 5% (w/v) sucrose. The inserted DNA sequence was verified by DNA sequencing.

• RNA extraction

Overnight cultures of *S. Typhi* were diluted 100-fold with LB medium and shaken at 37°C at 250 rpm. To confirm the presence of Asb, total RNA was extracted when the OD₆₀₀ was 0.8. To overexpress the *malS* 5'-UTR, strains of *S. Typhi* and Δ *bax* carrying the empty pBAD plasmid or plasmid expressing *malS* 5'-UTR were grown in LB medium to an OD₆₀₀ of 0.4, and then 0.2% (w/v) of L-arabinose was added. Aliquots were taken at the indicated time intervals. Aliquots of the Δ *bax*-pBAD and Δ *bax*-pBAD-5'-UTR strains were taken after 30 min in the presence of L-arabinose. To extract the total RNAs, the cells were pelleted and the total RNAs were isolated

with TRIzol Reagent (Invitrogen), followed by DNase I treatment (Takara, Takara, Japan), and quantified with an ND-1000 spectrophotometer (NanoDrop Technologies, MA, USA).

• 5'- & 3'-rapid amplification of cDNA ends

5'-rapid amplification of cDNA ends (RACE) was performed using a 5'-Full RACE Kit (Takara), according to the manufacturer's instructions. In brief, 5 µg of extracted total RNA was treated in turn with calf intestine alkaline phosphatase (CIAP) and tobacco acid pyrophosphatase, and ligated to the 5' RNA adaptor using T4 RNA ligase. It was then reverse transcribed to the first-strand cDNA using a gene-specific primer (*malS*-5'-UTR-R) and reverse transcriptase. The outer PCR reaction was performed using this cDNA as the template with the 5'-RACE adaptor-specific primer (5-ASP-1) and the *malS*-5'-UTR-specific primer (5-R1). The inner PCR reaction was performed using the product of the outer PCR reaction as the template and the 5-ASP-2 and 5-R2 primers. The RACE-PCR products were cloned into the pGEM-T vector (Promega, WI, USA), and the transcription initiation site of the *malS* 5'-UTR was determined by DNA sequencing. 3'-RACE was used to determine the location of the 3'-end of the *malS* 5'-UTR, as previously described [17]. For the 3'-RACE, CIAP-treated total RNA was ligated to the 5'-phosphorylated 3'-RACE adaptor (3-AD), and then reverse transcribed using the *malS*-5'-UTR-specific

Table 1. Strains and plasmids used in this study.

Strain	Relevant characteristics	Source
<i>S. Typhi</i> GIFU 10007	Wild -type strain; z66 ⁺	Gifu University
TOP10	<i>E. coli</i> host strain of pBAD plasmid	Invitrogen
Δ <i>malS</i> -5'-UTR	GIFU10007 (Δ <i>malS</i> -5'UTR), Kan ^R	This work
Δ <i>bax</i>	GIFU10007 (Δ <i>bax</i>), Kan ^R	This work
WT-pBAD	GIFU10007 carrying pBADMyc-HisA empty plasmid	This work
WT-pBAD-5'UTR	GIFU 10007 carrying pBAD-5'UTR	This work
Δ <i>bax</i> -pBAD	Δ <i>bax</i> carrying pBADMyc-HisA empty plasmid	This work
Δ <i>bax</i> -pBAD-5'UTR	Δ <i>bax</i> carrying pBAD-5'UTR	This work
<i>E. coli</i> DH 5 α	<i>E. coli</i> host strain of T vector	Invitrogen
WT- <i>bax</i> -3 \times FLAG	GIFU 10007 carrying chromosomal fusional <i>bax</i> -3 \times FLAG	This work
Plasmids		
pBADMyc-hisA	P _{lacO} promoter; Amp ^R	Invitrogen
pBAD- <i>malS</i> -5'UTR	P _{lacO} promoter, 5'UTRinsert; Amp ^R	This work
pGEM-T vector	TA clone; Amp ^R	Promega
pET-28a-c(+)	Kan ^R	Laboratory collection
PKD46	Red helper plasmid; Amp ^R	Laboratory collection

primer (*malS*-5'-UTR-F) and the adaptor-specific primer (3-ASP) complementary to 3-AD. PCR amplification, cloning and sequencing were performed as described for 5'-RACE.

• Northern blotting analysis

To detect the *malS* 5'-UTR and *bax*, a *bax*-specific cDNA probe homologous to the 3'-end of *bax* that did not overlap the *malS* 5'-UTR was used. Total RNA (10–30 μ g) was separated on a 6% polyacrylamide gel containing 7 M urea and transferred to a HybondTM N⁺ membrane (GE Healthcare, UK) by electroblotting. The membrane was prehybridized in Hyb hybridization buffer (Innogen, China) at 42°C. The cDNA probes were end-labeled with [γ -³²P]-ATP using T4 polynucleotide kinase (Takara). The radiolabeled probes were incubated overnight at 42°C with the prehybridized membrane. The membrane was then washed twice with 2 \times SSC/0.05% SDS and twice with 0.1 \times SSC/0.1% SDS for 15 min each at room temperature. The membrane was exposed to X-ray film at -20°C and visualized by autoradiography.

• Quantitative real-time PCR assays

DNase I-digested total RNA (4 μ g) was reverse transcribed to cDNA using PrimeScript[®] Reverse Transcriptase (Takara) and the quantity of cDNA was monitored with SYBR[®] Premix Ex TaqTM II (Takara), according to the manufacturer's instructions on a C1000 Thermal Cycler

(Bio-Rad, CA, USA). The primers used in this quantitative real-time PCR (qRT-PCR) analysis are listed in **Table 2**. The target gene transcription in each sample was normalized to the 5S ribosomal RNA. Negative controls (no reverse transcriptase added) were included with each run to check for DNA contamination. To detect *bax*, a primer was designed to bind the 3'-end of *bax* but not overlap the region of the *malS* 5'-UTR. The relative amount of target RNA was determined by calculating the cycle threshold (Ct) values. Data were analyzed using $2^{-\Delta\Delta C_t}$, as previously described, to determine the fold change values [18].

• Microarray analysis

For the gene-expression profiling experiments, overnight cultures of wild-type and *malS* 5'-UTR mutant strains of *S. Typhi* were diluted (1:100) in LB medium and incubated with shaking at 37°C for 4 h. Total RNA was extracted from the cultures with the RNeasy Mini Kit (Qiagen, Germany), according to the manufacturer's instructions. The extracted RNA was digested with DNase I (Takara) to ensure that any DNA contamination was removed. The genomic microarray analysis was performed as described previously [19]. Each RNA sample (25 μ g) was reverse transcribed and the resulting cDNA was labeled as described previously [20]. A mixture of Cy3-labeled wild-type cDNA and Cy5-labeled *malS* 5'-UTR mutant cDNA, and another mixture of oppositely labeled cDNAs, were hybridized

separately onto the slides of a *Salmonella* genomic DNA microarray overnight at 45°C. Array scanning, image export and digital analysis were then performed as described elsewhere [21].

• **Motility assay**

Motility agar (LB medium with 0.3% agar) was used to characterize the motility phenotypes of the *malS* 5'-UTR mutant, *bax* mutant

Table 2. Oligonucleotides used in this study.

Primers	Sequence(5' →3')	Purpose
F1A	GAACTGCTCTTTGCACTATTGCGAGAATACTCTTGTTACTGCTAACCTGGGTCTGACGCTCAGTGGA	<i>malS</i> -5'UTR mutant construction
F1B	TGTTATCGTTAATAGTTGACATAGTTTCGGATTAAGACTATTCCCTGAGGTTTCGGCCTATTGGTTAA	
F2A	ATTTTGCTGGTGATG	
F2B	ATGGCTCATAACACCC	
baxF1A	TTACATCACCAGCCAAAATGCCGCGATTACCGCGACCGTAGGTCTGACGCTCAGTGGA	<i>bax</i> mutant construction
baxF1B	CTAAGGAGTATCGCGACCAGCATAGTGAAGCGTTGTTGTAGTTTCGGCCTATTGGTTAA	
baxF2A	TTCACCTCCTGATTTGCG	
baxF2B	ATGGCTCATAACACCC	
PA (<i>Nco</i> I)	ACACCATGGCCTGTTATAACTTGTCTCAGT	<i>malS</i> -5'UTR overexpression
PB (<i>Hind</i> III)	GCGAAGCTTTGCTTTTTAAGCCTATTTTTGC	Construction
<i>malS</i> -5'UTR-R	GGTGTGGAAATGTATCAATCCGA	5'-RACE
5-ASP-1	CATGGCTACATGCTGACAGCCTA	
5-ASP-2	CGCGATCCACAGCCTACTGATGATCAGTCGATG	
5-R1	TCCGACCAGGAGACCTAATGATA	
5-R2	ATGATATTGACTCCCATGCGACG	
3-AD	5'-phosphate-UUCACU GUUCUU AGC GGC CGC AUG CUC-idT -3	3'-RACE
3-ASP	GGCCGCTAAGAACAGTGAA	
<i>malS</i> -5'UTR-F	CGCCTTGTGGATAAGCTCG	
<i>malS</i> -5'UTR -NR	CGCAACACAAACGAGTCAAAAGTCCCACATAACTGAGACAAGT	Northern probe
<i>Bax</i> -NR	GCTCAGACGGCGACCAGCGTTCTGGTACTGTTTTGAGATCAGCCAATTACGGTCCG	
5s-qF	TTGTCTGGCGGCAGTAGC	
5s-qR	TTTGATGCCTGGCAGTTC	
<i>fliA</i> -FA	CTGTGGCAGCGTTATGTAC	Real-time PCR to verify the result of microarray analysis
<i>fliA</i> -FB	GCACTGCGTAAGTGGTAA	
<i>invF</i> -FA	CCGATAAATGGGTTTTGCTG	
<i>invF</i> -FB	TGGTTGACTGAGCGAGTAAA	
<i>prgH</i> -FA	GAACGGCTGTGAGTTTCCAT	
<i>prgH</i> -FB	GGCGAATCAGGATAAGCAAT	
<i>sigD</i> -FA	ATTGCTCCTGCGAGAACCTGG	
<i>sigD</i> -FB	CTTCTTGTGTTGTTGCTGGC	
BaxP1A(<i>Bam</i> HI)	CATGGATCCCGCTATAAAGTGAGCTGGTC	Western blot
BaxP1B	CTTTATAATCACCGTCATGGTCTTTGTAGTCCATATGCGCGGCGATCAGA	
BaxP1B'	TTATTTATCGTCGCATCTTTGTAGTCGATATCATGATCTTTATAATCACCGTCATGG	
BaxP2A	AAGATGACGACGATAAATAATTCTGTGTGCCGATGGCGCT	
BaxP2B (<i>Bam</i> HI)	TATGGATCCAAAGTCGAGCAGGTGCTGGACG	
AsbF1	CTTGTCTCAGTTATGTGGGACT	
AsbR1	TTCCCTGAGGTGCTTTTTAA	Tiling PCR
AsbR2	AATGAACCTCTCCCGAG	
AsbR3	ATGCAGCATGGCTGGTA	
AsbR4	GATCGGGTATCTACCGTT	
AsbR5	GCGAACCATCCCGAGC	

and wild-type *S. Typhi* strains. The plates were inoculated with a single colony from an LB agar plate and incubated at 37°C for 10 h. Motility was assessed by measuring the diameter of the motility circle. At least five replicate plates were observed under each condition.

• Invasion assay

An assay of the bacterial invasion of HeLa cells was performed as described previously [22,23]. HeLa cells were seeded into 24-well plates (Costar, USA) containing RPMI-1640 medium (Life Technologies, USA) supplemented with 10% FBS at a density of 2×10^5 cells per well and incubated for 16–h in 5% CO₂ at 37°C. They were infected at a multiplicity of infection of 20:1 with *S. Typhi* cultures grown to an OD₆₀₀ of 0.4. The cells were washed three times with phosphate-buffered saline 90 min after infection. They were then either lysed with 1 ml of 0.5% Triton X-100 per well to evaluate the level of adherence (T₀) or incubated in medium containing gentamicin (100 µg/ml) for a further 90 min to kill any remaining extracellular bacteria and to assess the level of invasion (T₉₀). The cells were then lysed as described above. The invasiveness of the bacteria was measured as described previously [19].

• Western blotting analysis

S. Typhi strains carrying the chromosomal 3×FLAG transcriptional fusions in the *bax* coding regions with an empty plasmid (pBAD) and a plasmid expressing the *malS* 5'-UTR (pBAD-5'-UTR) were grown at 37°C to an OD₆₀₀ of 0.4. *malS* 5'-UTR overexpression was induced by the addition of L-arabinose (0.2% w/v) to the cultures. Aliquots were taken before (0 min) or at 15 min, 30 min, 1 h, 2 h and 4 h after the addition of L-arabinose. Cell extracts were prepared by sonication. Protein samples were separated electrophoretically on 12% SDS-PAGE and wet transferred to PVDF membranes using a transfer apparatus (Bio-Rad). 3×FLAG and DnaK were detected with an anti-FLAG monoclonal antibody (CMC, Denmark diluted 1:5000) and an anti-DnaK monoclonal antibody (Enzo, USA diluted 1:8000), respectively, as the primary antibodies and goat anti-mouse immunoglobulin G antibody conjugated with horseradish peroxidase (diluted 1:5000) as the secondary antibody. Immunodetection was with Supersignal® West Pico (Thermo Scientific, USA) and the signals were detected with a chemiluminescence

imaging system (Clinx Science Instruments, China).

• Statistical analysis

The results are expressed as means ± standard deviation (SD). The significance of differences between groups was analyzed by Student's *t*-test or ANOVA. Differences were considered significant at $p < 0.05$.

Results

• *Asb* transcript is the 5'-UTR of the *malS* gene

The RNA-seq analysis of *S. Typhi* under stress conditions showed a 236-nt transcript encoded by the minus strand of *bax*, extending from 137 nt downstream to 99 nt upstream from the start codon of *bax* (Figure 1A). To confirm the existence of *Asb*, the total RNA from the *S. Typhi* wild-type strain, incubated to exponential phase, was extracted and a northern blotting analysis was performed using an *Asb*-specific ³²P-labeled cDNA probe. Surprisingly, a fragment of about 2000 nt was identified (Figure 1B). Following reverse transcription with N6 random primer, tiling PCR was performed with primers specific to the upstream and downstream regions of *Asb* to investigate the location of the sequence encoding *Asb*. Only downstream products overlapping the *malS* gene were found, and the longest was about 1000 bp (Figure 1A).

With a bioinformatic analysis using the RegRNA 2.0 software, the promoter of the *malS* gene of *S. Typhi* was predicted upstream from *Asb*. 5'-RACE with a primer specific to *Asb* was then performed to map the 5'-end of *Asb*. We found a transcription start site located 453 nt upstream from the *malS* start codon (marked with an arrow in Figure 1A). 3'-RACE was used to map the 3'-end of the identified *Asb*. This detected different end points, and the one that produced the longest transcript was located 1112 nt downstream from the *malS* start codon. This was confirmed with DNA sequencing. However, we could not find the different fragments identified in the 3'-RACE in the northern blotting analysis. These fragment transcripts may be the products of RNase activity on the full transcript of *malS*. Because *Asb* overlaps *malS* and is transcribed in the same direction as *malS*, we reasoned that the region of *Asb* complementary to the initiation region of *bax* is the 5'-UTR of the *malS* gene. We therefore renamed it '*malS* 5'-UTR'.

- **Gene-expression profiling regulated by *malS* 5'-UTR**

To gain insight into the function of the *malS* 5'-UTR, a *malS*-5'-UTR-deleted mutant was prepared that retained the SD sequence and open reading frame (ORF) structure of *malS*. Using a whole-genome microarray, the gene-expression profile of the *malS* 5'-UTR mutant was investigated to determine the influence of the *malS* 5'-UTR on gene expression in *S. Typhi*. A comparison of the global transcription profiles revealed that genes in about 50 gene clusters were differentially expressed in the *malS* 5'-UTR mutant and the wild-type strain (**Supplementary Material**, please see online at www.futuremedicine.com/doi/full/10.2217/FMB.15.12). These included genes involved in SPI-1 and invasion, virulence, chemotaxis and flagellar biosynthesis. Flagellum- and motility-associated genes, such as *flhDC*, *fliAZY*, *fliFGHJLMNOP*, *flgBCDEFHIJKL*, *motA* and *cheAWRBZ*, were upregulated in the *malS* 5'-UTR mutant. SPI-1 genes and invasion-associated genes including *prgHJK*, *iagAB*, *sipCDA*, *invFGEA*, *spaKINMOPQR*, *sigDE* and *sopE* were also upregulated in the *malS* 5'-UTR mutant. Some other ribosome-associated genes, including *rpsS*, *rpsH* and *rplF*, and the metabolism-associated genes *sdhDC* were downregulated. To confirm the findings of the microarray experiments, the four genes *fliA*, *invF*, *prgH* and *sigD* that were upregulated in the microarray assay were selected for qRT-PCR analysis. As in the microarray data, the expression of these genes was increased in the *malS* 5'-UTR mutant compared with that in the wild-type (**Figure 2**). These results indicate the possible involvement of the *malS* 5'-UTR in the motility and invasiveness of *S. Typhi*. In subsequent experiments, we focused on the major functions and mechanisms of the *malS* 5'-UTR in regulating the motility and invasiveness of *S. Typhi*.

- **Deletion of the *malS* 5'-UTR increases the motility of *S. Typhi***

Flagellar-based motility propels *Salmonella* through liquid environments and plays an important role in the virulence of the bacterium [24]. Given that flagellum-associated genes were upregulated when the *malS* 5'-UTR was deleted, a motility assay was performed by growing the bacterium on swimming plates. In accordance with the upregulated expression of the flagellum-associated genes in the microarray

analysis when *malS* 5'-UTR was deleted, the motility experiment displayed a significant increase in migration from the spot of inoculation in the mutant Δ *malS* 5'-UTR compared with the wild-type (**Figure 3**). These results suggest that the *malS* 5'-UTR is involved in the regulation of motility in *S. Typhi*.

- **Deletion of the *malS* 5'-UTR increases the invasiveness of *S. Typhi* into nonphagocytic cells**

The SPI-1 proteins are major virulence determinants contributing to *Salmonella* invasiveness [2,25]. The microarray analysis described above revealed the enhanced expression of invasion-associated genes when the *malS* 5'-UTR was deleted. This suggests that the *malS* 5'-UTR may play a role in the invasion of nonphagocytic cells by *Salmonella*. To investigate this possibility, we compared the capacity of the wild-type *S. Typhi*, Δ *malS* 5'-UTR and Δ *bax* strains to invade HeLa cells *in vitro*. As shown in **Figure 4**, the deletion of the *malS* 5'-UTR significantly increased bacterial invasion compared with that of the wild-type strain. These results suggest that the effect of the *malS* 5'-UTR on Bax may negatively regulate the invasive capacity of *S. Typhi*.

- **Deletion of *bax* may increase the expression of invasion-associated genes**

To investigate the possibility that the *malS* 5'-UTR affects the expression of *bax* by overlapping the region between the two genes, a *bax* mutant was prepared in which the *malS* 5'-UTR overlapping region was intact. A transcriptome analysis of the *bax* mutant was performed with a *Salmonella* genomic microarray. The results indicated that the expression of most invasion-associated genes, including *invF*, *sigD* and *prgH*, was upregulated in the *bax* mutant compared with that in the wild-type *S. Typhi* (data not shown). A qRT-PCR analysis of the expression levels of *invF*, *sigD* and *prgH* was consistent with the microarray data (**Figure 4A**). The invasion of HeLa cells by the *bax* mutant was also investigated. The invasive capacity of the *bax* mutant was stronger than that of the wild-type (**Figure 4B**). However, the result of the microarray assay showed that there was no obvious change in the expression of the flagellar genes. A motility assay then showed that the *bax* mutant displayed reduced motility compared with the wild-type, although the difference was not significant (**Figure 3**). Because the expression of

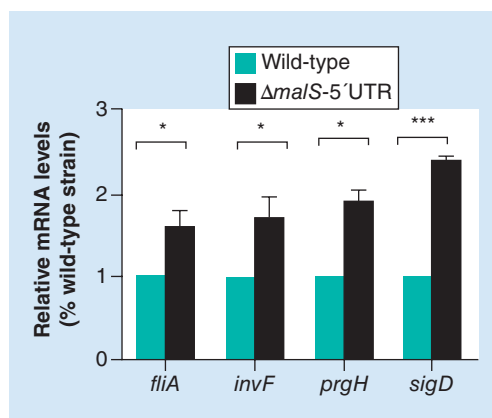


Figure 2. Detection of *fliA*, *invF*, *prgH* and *sigD* gene expression levels. *Salmonella enterica* serovar Typhi wild-type and $\Delta mals$ 5'-untranslated region strains were cultured in LB medium at 37°C for 4 h. Total RNA was extracted and the expression levels of the *fliA*, *invF*, *prgH* and *sigD* genes were determined with quantitative real-time PCR. 5S rRNA was used as the internal control. Bars represent the means \pm standard deviation of three independent experiments. Student's *t*-test was used to calculate whether the differences between groups were significant. * $p < 0.05$, *** $p < 0.001$.

invasion-associated genes and the invasion of HeLa cells increased in both the *mals* 5'-UTR and *bax* mutants, the *mals* 5'-UTR probably influences the invasiveness of *Salmonella* by interfering with the expression of *bax*. However, the *mals* 5'-UTR may regulate motility through another pathway rather than through its effect on *bax* expression.

• Overexpression of the *mals* 5'-UTR reduces the expression of *bax*

Researchers have found that ncRNAs can increase or reduce the expression of their target genes by influencing the stability of the target mRNAs [26]. To clarify the effect of the *mals* 5'-UTR on the expression of *bax*, its possible mRNA target, the wild-type strain was transformed with the recombinant plasmid pBAD-*mals*-5'-UTR, which expressed a 240 nt transcript of the *mals* 5'-UTR in the strain after induction with L-arabinose. After the overexpression of the *mals* 5'-UTR, the level of *bax* mRNA was investigated with northern blot and qRT-PCR. As shown in Figure 5A & B, in the WT-PBAD-5'-UTR strain, the *bax* mRNA level decreased gradually with increasing induction

time, probably as a result of ribonuclease activity. qRT-PCR showed that the overexpression of the *mals* 5'-UTR in the wild-type reduced the *bax* mRNA level within 20 min compared with that in the wild-type strain carrying the empty pBAD plasmid. Thus, the qRT-PCR results are consistent with the results of the northern blot analysis, indicating that the *mals* 5'-UTR negatively affects *bax* mRNA levels.

The effect of *mals* 5'-UTR overexpression on the expression of Bax protein was also assessed. For this purpose, a Bax-3 \times FLAG fusion strain was constructed, which was transformed with the pBAD-*mals*-5'-UTR plasmid. Western blot with anti-FLAG-tag and anti-DnaK antibodies was used to investigate the levels of Bax and the internal control DnaK. Before the induction of *mals* 5'-UTR expression, the Bax level was high; within 30 min of induction, the Bax level was significantly reduced. Clearly, overexpression of the *mals* 5'-UTR severely reduced Bax synthesis as the induction time increased. Cells carrying the empty vector alone maintained constant levels of Bax protein during the same period (Figure 5C). The pattern of Bax protein accumulation was similar to the accumulation of *bax* mRNA. Altogether, these results indicate that the expression of *bax* is negatively affected by the *mals* 5'-UTR.

• Effect of *mals* 5'-UTR overexpression on the invasiveness of *S. Typhi*

To gain further insight into the mechanism of the *mals* 5'-UTR affecting bacterial invasion, the *mals* 5'-UTR was overexpressed in the *bax* mutant by transforming bacteria with an arabinose-inducible expression plasmid. The mRNA levels of *invF*, an SPI-1 regulatory gene, were measured with qRT-PCR and the bacterial invasion of HeLa cells was also examined. After the *mals* 5'-UTR was overexpressed in the *bax* deletion mutant, *invF* mRNA levels and the bacterial invasion of HeLa cells were both increased compared with those in the mutant strain containing the empty plasmid (Figure 6). These findings suggest that the effect of the *mals* 5'-UTR on invasion may not be completely through *bax*. It is possible that the invasiveness of the bacterium is affected in other ways when the *mals* 5'-UTR is deleted.

Discussion

It has become clear that many bacterial ncRNAs play essential regulatory roles in the gene

expression underlying various cellular processes and physiological responses through extremely diverse mechanisms [27,28]. The advent of RNA-seq has led to the discovery of numerous ncRNAs in bacteria, some of which are associated with bacterial invasion. For instance, one of the island-encoded sRNAs, *IsrM*, is important in the invasion by *Salmonella* of nonphagocytic cells [29]. Recently, we applied the RNA-seq technology to a transcriptomic analysis of *S. Typhi*, and found large numbers of putative ncRNAs, some of which were identified [15,30].

In this study, we focused on a 236-nt transcript fragment acquired in an RNA-seq analysis of *S. Typhi*, which is transcribed from the upstream region of the *malS* gene and is complementary to part of the *bax* mRNA. A northern blotting analysis with a cDNA probe specific for the transcript showed that the whole transcript is about 2000 nt long. Because at that time, RNAs were interrupted during RNA-seq and each sequencing length was only 40 nt, it was very difficult to obtain the full information for long RNAs. However, tiling PCR after reverse transcription showed that this fragment may cover most of the *malS* mRNA, which has a theoretical 2028-bp ORF in *S. Typhi* (data not shown). By combining the results of northern blot, RACE, tiling PCR and a bioinformatic analysis of the promoter, we inferred that the transcript fragment is actually the 5'-UTR of the *malS* gene.

Several 5'-UTRs of mRNAs have been shown to be functional units in bacteria [8,9]. To determine the function of the *malS* 5'-UTR, we created a mutant in which the 236-nt fragment of the *malS* 5'-UTR was deleted, whereas the SD box and the ORF structure of *malS* were retained intact. The deletion of the *malS* 5'-UTR did not affect the expression of the *malS* gene, which was estimated with qRT-PCR (data not shown), which was consistent with our expectation. We monitored the phenotype of the mutant and found that the growth rate was slightly lower than that of the wild-type (data not shown), whereas the motility of the $\Delta malS$ 5'-UTR mutant was increased (Figure 3). The motility of bacteria relies on their flagella. The transcription of flagellar genes is organized into a regulatory hierarchy of class I, II and III genes [31]. *FliA* alone or with *FlhDC* activates the expression of the class III genes, which encode the filament protein and motor proteins [32]. *FliC* is the major component of the flagellum and the deletion of *fliC* reduces

bacterial motility. The flagellar rotation velocity is mediated by some of the flagellar motor proteins, including *MotA*. Microarray data revealed that the expression of *flhC*, *fliA*, *fliC* and *motA* was upregulated in the *malS* 5'-UTR mutant (see **Supplementary Material**). This suggests that the *malS* 5'-UTR participates in the regulation of *S. Typhi* motility. The bacterial gene-expression regulation is complex, and various factors may synergistically act and form network. Besides

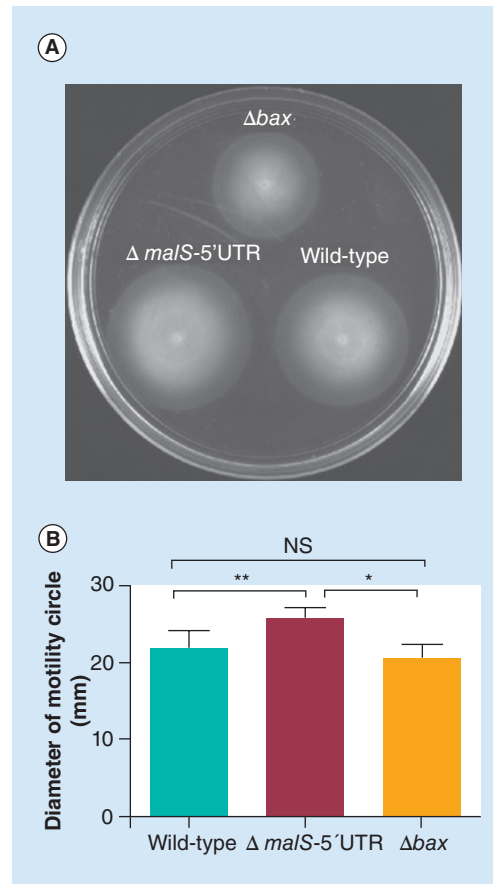


Figure 3. Motility assay of the wild-type, $\Delta malS$ 5'-untranslated region and Δbax strains.

Bacteria were stabbed into plates containing 0.3% agar. After incubation for 10 h, the diameter of the bacterial zone was measured. (A) Images showing the migration of each *S. Typhi* strain. (B) Graphical representation of the motility of different *Salmonella enterica* serovar Typhi strains. The results are the mean values of three independent experiments. ANOVA was used to calculate whether the differences between groups were significant.

* $p < 0.05$; ** $p < 0.01$.

NS: Not statistically significant; UTR: Untranslated region.

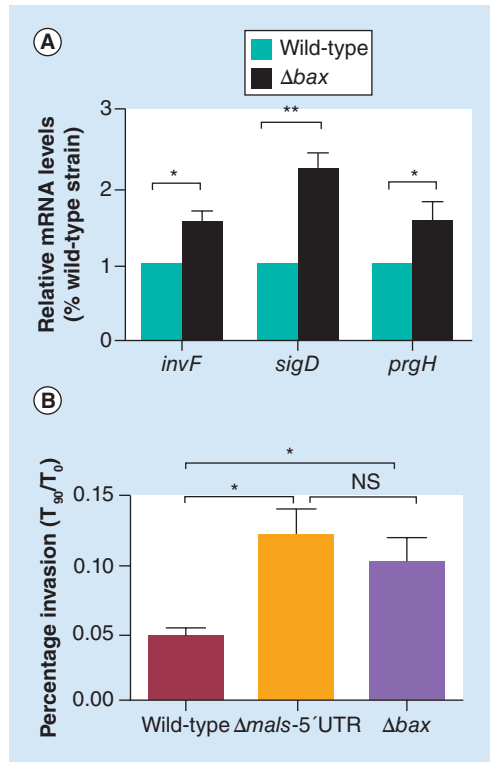


Figure 4. Interaction of wild-type, $\Delta mals$ 5'-untranslated region and Δbax strains with HeLa cells. (A) mRNA levels of invasion-associated genes (*invF*, *sigD* and *prgH*) in wild-type and Δbax strains. Wild-type and Δbax strains were cultured as described in Figure 2. RNA was extracted and transcription was determined with quantitative real-time PCR. 5S rRNA was used as the internal control. Bars represent the means \pm standard deviation of three independent experiments. Student's *t*-test was used to calculate whether the differences between groups were significant. **(B)** *Salmonella enterica* serovar Typhi invasiveness of HeLa cells was investigated by comparing the number of bacteria at 90 min with the number at 0 min. The value of T_{90}/T_0 represents the bacterial capacity for invading HeLa cells. The results are the mean values of three independent experiments. ANOVA was used to calculate whether the differences between groups were significant. * $p < 0.05$; ** $p < 0.01$. NS: Not statistically significant; UTR: Untranslated region.

flagellar gene expression, bacterial motility may be influenced by some other things, such as assemble of flagellin, motor rotation. So, it is possible that motility change is weaker than the change of flagellar gene expression.

Considering *bax* expression can be influenced by the *mals* 5'-UTR, we also investigated the expression of the flagellum-associated genes with qRT-PCR in the *bax* mutant, but not see the result similar with that in *mals* 5'-UTR mutant. In the motility experiment, the circle of motility of the Δbax mutant tended to be smaller than the control value, but the difference was no statistically significant. So, we think that the effect on motility of *mals* 5'-UTR is not dependent on the Bax.

Flagellar gene expression is tightly linked to the regulation of SPI-1 gene expression in *Salmonella* and contributes to the bacterial invasion of enteric epithelial cells [33]. The T3SS is essential for the virulence of many pathogens, including *Salmonella* [34]. The SPI-1 T3SS contains some invasion genes that allow bacteria to invade epithelial cells. The deletion of the *mals* 5'-UTR enhanced the bacterial invasion capacity for HeLa cells (Figure 4B). This is consistent with the microarray results described above. The microarray data showed the obvious upregulation of many SPI-1 genes involved in bacterial invasiveness, including *prgH*, *sipC* and so on in the $\Delta mals$ 5'-UTR mutant. Our study raises the possibility, for the first time, that the *mals* 5'-UTR plays an important role in the process of host cell infection by *S. Typhi*.

The *mals* gene, originally identified in *E. coli* as a member of the complex maltose regulon, is related to the uptake and decomposition of maltodextrins. The ORF of *mals* encodes a periplasmic α -amylase and is regulated by MalT, the activator of the maltose system in *E. coli* [35,36]. The homology of the MalS and Bax ORFs is 94% and 84%, respectively, when the *E. coli* proteins are compared with those of *S. Typhi*. Bax is regarded as a hypothetical protein, or a putative endo- β -N-acetylglucosaminidase in *E. coli*, but as a putative exporter of amidase in *S. Typhi*. However, the structure and function of MalS and Bax have not yet been reported in *S. Typhi*. Because the *mals* 5'-UTR-encoding region overlaps a portion of the *bax* gene and the *mals* 5'-UTR might influence Bax function, we investigated the function of Bax in this study. A *bax* mutant was prepared, in which the deleted region was outside the region overlapping the *mals* 5'-UTR. Invasion experiments revealed that the *bax* mutant had a greater invasive capacity than the wild-type (Figure 4B). Although the mechanism underlying the effect of Bax

on the invasiveness of the bacterium requires further investigation, it is possible that the *malS* 5'-UTR interferes with *bax* expression to regulate the invasion of host cells by *S. Typhi*.

To confirm the effects of the *malS* 5'-UTR on the expression of *bax*, a Bax-3×FLAG fusion strain of *S. Typhi* was constructed and the overexpression of the *malS* 5'-UTR was mediated by the recombinant plasmid pBAD-*malS*-5'-UTR in the fusion strain. The overexpression of the *malS* 5'-UTR led to significant reductions in *bax* mRNA and Bax protein, implying that the expression of the *malS* 5'-UTR reduces the stability of *bax* mRNA and affects the translation of Bax protein (Figure 5). It is possible that the transcription of the target mRNA is inhibited by ncRNA through steric hindrance [37]. Alternatively, the binding of the ncRNA to the target mRNA could expose a

ribonuclease-sensitive site, leading to the degradation of the target mRNA. Therefore, it is highly likely that the *malS* 5'-UTR increases the invasive capacity of the bacterium by reducing the expression of Bax protein, a negative invasiveness factor in *S. Typhi*.

If the effect of the *malS* 5'-UTR on the bacterial invasion of HeLa cells is dependent on *bax* alone, the overexpression of the *malS* 5'-UTR in the *bax* mutant would cause no obvious change in the invasive capacity of the mutant. However, in this study, we found that the transcriptional level of *invF* and the bacterial invasion of HeLa cells increased significantly after the *malS* 5'-UTR was overexpressed in the *bax* mutant. Therefore, besides *bax*, the *malS* 5'-UTR may also affect the expression of invasion-associated genes and the invasiveness of the bacterium into HeLa cells

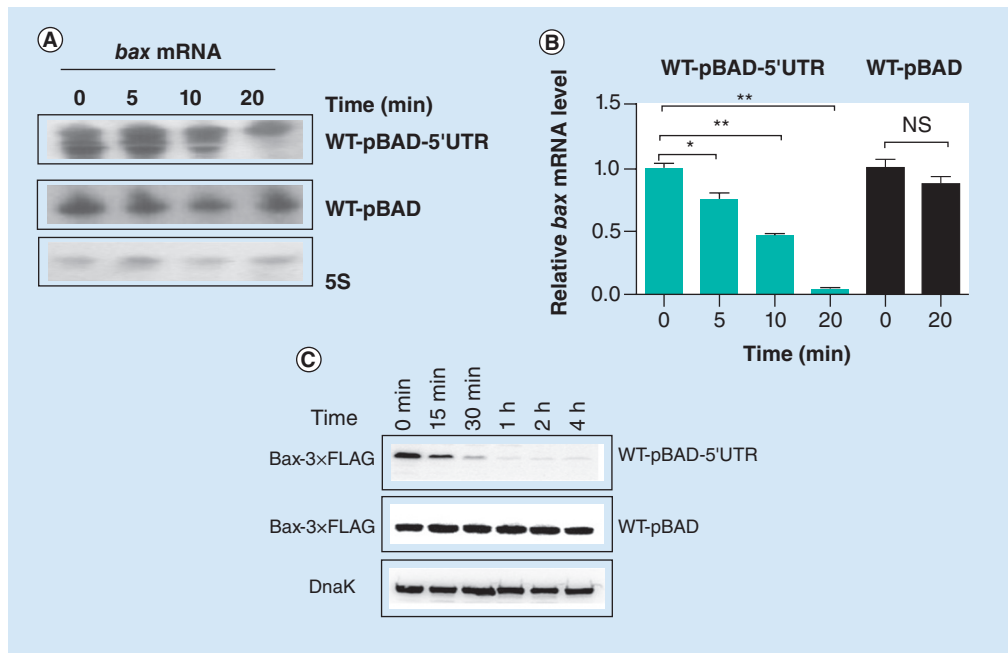


Figure 5. Analysis of the effect of *malS* 5'-untranslated region overexpression on Bax expression. Strains WT-pBAD-5'-UTR and WT-pBAD were grown to an OD_{600} of 0.4 and induced with L-arabinose (0.2% final concentration). Samples were removed after the addition of L-arabinose at the time indicated and RNA was extracted and examined by northern analysis and quantitative real-time PCR, 5S rRNA was used as the internal control. *bax* mRNA levels were measured with (A) northern blot and (B) quantitative real-time PCR analysis. (C) Protein samples were separated in 12% SDS-PAGE and reacted with antibodies against the FLAG sequence or DnaK. Bax protein levels were assessed with western blot. The expression of bacterial DnaK was used as the internal control. Each lane was loaded with 40 μ g of total protein. Bars represent the means \pm standard deviation of three independent experiments. Student's *t*-test was used to calculate whether the differences between groups were significant.

* $p < 0.05$; ** $p < 0.01$.

NS: Not statistically significant; UTR: Untranslated region; WT: Wild-type.

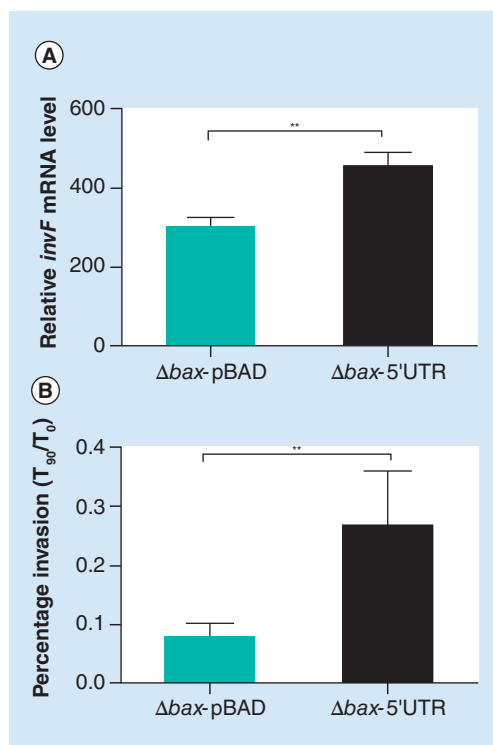


Figure 6. Expression of *invF* and invasive capacity in the *bax* mutant after overexpression of *malS* 5'-untranslated region.

The *bax* mutant of *S. Typhi* was transfected with pBAD-5'UTR or pBAD empty plasmid. (A) The mRNA levels of *invF* were measured with qRT-PCR, 5S rRNA was used as the internal control. (B) Interaction of Δbax -pBAD and Δbax -pBAD-5'UTR strains with HeLa cells. *S. Typhi* invasion of HeLa cells was investigated by comparing the number of bacteria at 90 min with the number at 0 min. The value T_{90}/T_0 represents the bacterial capacity for invading HeLa cells. Bars represent the means \pm standard deviation of three independent experiments. Student's *t*-test was used to calculate whether the differences between groups were significant.

* $p < 0.05$; ** $p < 0.01$.

UTR: Untranslated region.

in other ways. Therefore, the mechanism by which the *malS* 5'-UTR affects bacterial invasion may predominantly involve the inhibition of *bax* expression and an increase in bacterial motility. It is very likely that the effect of the *malS* 5'-UTR on *bax* expression occurs in *cis* at the posttranscriptional level. However, it is also possible that the *malS* 5'-UTR regulates the expression of many genes in *trans*, including flagellar genes, and this assumption

is supported by the results of the microarray (see **Supplementary Material**). Therefore, other unknown factors may participate in the network of gene-expression regulation involving the *malS* 5'-UTR. This warrants further detailed research.

Conclusion & future perspective

In conclusion, we have demonstrated that the *malS* 5'-UTR is transcribed in *S. Typhi* and increases bacterial invasiveness by affecting the expression of *bax*, identified as a negative effector of invasion for the first time in this study. However, the mechanisms underlying the inhibition of bacterial cell invasion by Bax and the effect of the *malS* 5'-UTR on the expression of flagellar-associated genes and the motility of *S. Typhi* remain unclear. Further studies are required to clarify the mechanism of *bax*-affected invasion.

The expression of SPI-1 is affected by a complex regulatory network in *S. enterica*. The deletion of the *malS* 5'-UTR results in a global dysregulation of SPI-1 protein expression. However, the exact mechanism remains unclear. We wish to clarify how the *malS* 5'-UTR affects SPI-1 expression. Bacterial ncRNAs are regulators that modulate gene expression by hybridizing to the target mRNA. We identified some mRNAs in the microarray affected by *malS* 5'-UTR. We will determine the target mRNAs that are its major targets, and use point mutations to determine whether the interaction between them is direct or indirect. It would be interesting to investigate the expression patterns of *malS* 5'-UTR and ncRNAs at different stages of infection, under condition resembling those in the gastrointestinal tract, and to determine whether *malS* 5'-UTR and ncRNAs act coordinately during infection. To confirm the effects of *malS* 5'-UTR-mediated cell invasion, we will also introduce the *malS* 5'-UTR mutation into other *Salmonella* serovar strains. It is anticipated that an in-depth study of ncRNA biology will shed new light on *Salmonella* pathogenicity.

Author contributions

Conceived and designed the experiments: M Gong, X Huang. Experiments were performed by M Gong, S Xu, Y Jin and Y Zhang. Data was analyzed by M Gong, X Zhang and X Sheng. Contributed reagents/materials/analysis tools: M Gong, Z Wang, Y Zhu, B Ni, H Zhang. Wrote the manuscript: M Gong, I Dadzie, X Huang.

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discussed in the manuscript apart from those disclosed.

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Financial & competing interests disclosure

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

EXECUTIVE SUMMARY**Asb transcript is the 5'-untranslated region of the *malS* gene**

- A 236-nt transcript strongly expressed in *Salmonella enterica* serovar Typhi (*S. Typhi*) was designated 'Asb.' We confirmed the region of Asb complementary to the initiation region of *bax* to be the 5'-untranslated region (UTR) of the *malS* gene with a bioinformatic analysis, RACE, northern blot and tiling PCR.

Gene-expression profiling regulated by *malS* 5'-UTR

- A whole-genome microarray and quantitative real-time PCR analysis showed that the expression of flagellar and invasion-associated genes was upregulated in the *malS* 5'-UTR mutant compared with that in the wild-type.

Deletion of the *malS* 5'-UTR increases the motility of *S. Typhi*

- A motility experiment showed a significant increase in the migration of the mutant $\Delta malS$ 5'-UTR from the spot of inoculation compared with that of the wild-type.

Deletion of the *malS* 5'-UTR increases the invasiveness of *S. Typhi* into nonphagocytic cells

- Absence of the *malS* 5'-UTR significantly increased the bacterial invasive capacity in HeLa cells compared with that of the wild-type strain.

Deletion of *bax* may increase the expression of invasion-associated genes

- The deletion of *bax* may increase the expression of invasion genes and the invasive capacity of *S. Typhi*.

Overexpression of the *malS* 5'-UTR reduces the expression of *bax*

- The overexpression of the *malS* 5'-UTR led to significant reductions in *bax* mRNA and Bax protein.

Effect of *malS* 5'-UTR overexpression on the invasiveness of *S. Typhi*

- After the *malS* 5'-UTR was overexpressed in the *bax* deletion mutant, *invF* mRNA levels and the bacterial invasion of HeLa cells both increased compared with those of the mutant strain containing the empty plasmid.

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