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Characterization of the yeHUT Two-Component Regulatory System of Salmonella enterica Serovar Typhi and Typhimurium

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Abstract

Proteins exhibiting hyper-variable sequences within a bacterial pathogen may be associated with host adaptation. Several lineages of the monophyletic pathogen Salmonella enterica serovar Typhi (S. Typhi) have accumulated non-synonymous mutations in the putative two-component regulatory system yeHUT. Consequently we evaluated the function of yeHUT in S. Typhi BRD948 and S. Typhimurium ST4/74. Transcriptome analysis identified the cstA gene, encoding a carbon starvation protein as the predominantly yeHUT regulated gene in both these serovars. Deletion of yeHUT had no detectable effect on the ability of these mutant Salmonella to invade cultured epithelial cells (S. Typhi and S. Typhimurium) or induce colitis in a murine model (S. Typhimurium only). Growth, metabolic and antimicrobial susceptibility tests identified no obvious influences of yeHUT on these phenotypes.

Introduction

Enteric bacteria have evolved a complex lifestyle involving survival in a variety of niches, including the environment and the intestinal lumen of various animal species. Therefore bacteria must regulate global gene expression in response to their environment, gathering information in part through specialized two-component regulatory systems that sense environmental changes. These systems largely operate by exploiting signal-sensing domains on histidine kinase (HK) sensory proteins linked to cognate response regulators (RR) that can modulate gene expression [1]. The regulons associated with many two-component systems have been defined and in some cases some of their environmental stimuli elucidated [2]. However, others remain poorly characterized.

Orthologues of the candidate two-component regulatory system yeHUT are found in almost all enteric bacteria, including plant and animal pathogens [3]. Detectable autophosphorylation of YehU and phosphotransfer to YehT has been shown to occur in Escherichia coli [4]. In E. coli, yeHUT regulates yjY, a gene that encoded an inner membrane protein belonging to the carbon starvation protein superfamily. yeHUT may be involved in the stationary phase control network as YjY is strongly induced at the onset of this growth phase [3].
However, detailed phenotypic analysis of derivatives harboring mutations in the *yehUT* system identified no other obvious differences with otherwise isogenic wild type *E. coli* [3,5]. However, overexpression of *yehT* in *E. coli* conferred resistance to compounds such as crystal violet, deoxycholate [6], and beta-lactam antibiotics [7].

*Salm抵押* enterica* serovar Typhi* (S. Typhi) is a human restricted enteric bacterial pathogen that causes typhoid [8]. S. Typhi is a monophyletic pathogen that likely crossed into the human population once thousands of years ago [9]. Thus, all S. Typhi have a common ancestor and genetic variation in this pathogen is limited and largely characterized by extensive loss of gene function in the form of pseudogene accumulation [10]. Pseudogenes are defined as genes that are potentially inactivated by mutations including nonsense single nucleotide polymorphisms (SNPs), frame-shifts and truncation by deletion or rearrangement [10-13]. Whole genome sequence of multiple S. Typhi isolates has revealed its remarkable clonal nature with very few non-synonymous SNPs [9,10]. However, Holt et al. [10] identified several genes containing more than six substitutions when compared to the S. Typhi CT18 genome, deviating from the expected Poisson distribution in terms of the accumulation of non-synonymous SNPs, and these proteins may therefore be under selection. In S. Typhi, the *yehUT* operon has accumulated relatively more non-synonymous mutations in a number of lineages of the phylogenetic tree compared to other genes, suggesting this operon is potentially undergoing adaptive evolution [9,10]. These observations stimulated us to conduct a more in depth investigation of the *yehUT* loci in an attempt to identify a phenotype in *Salmonyella*.

**Materials and Methods**

**Ethics**

Ethical approval for the use of serum samples in this study was granted by the Life and Health Sciences Ethical Review Committee of the University of Birmingham, UK. Informed written consent was obtained from all participants.

For the mouse experiments wild type C57BL/6 mice 8-10 weeks of age were maintained according to UK Home Office Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 and the Code of Practice for the housing and care of animals used in scientific procedures. The protocols (Home Office Project licence number: 80/2596) were approved by the Animal Welfare and Ethical Review Body at the Wellcome Trust Sanger Institute. Surgery was performed under isofluorane anaesthesia to eliminate suffering.

**Bacteria used for mutant construction**

For safety reasons an attenuated derivative of S. Typhi Ty2 (BRD948 ΔaroA, ΔaroC, ΔhtrA) was used throughout as the parental isolate for the construction of mutant derivatives [14]. S. Typhimurium ST4/74, originally isolated from a calf with salmonellosis, was used as the parent strain for mutant derivative construction [15].

**Informatics analyses**

The domain architecture of YehU/YehT was analyzed using InterPro [16]. Transmembrane alpha helical segments were predicted with the TMHMM webserver [17]. The *yehUT* genes of *S. Typhi* Ty2 (Accession number AE014613) and *S. Typhimurium* SL1344 (Accession number FQ312003) were analyzed using the Genome browser Artemis [18]. The ST4/74 strain is the older designation of SL1344 [19]. The analysis required a completed genome sequence with annotation that is available for SL1344 (Accession number FQ312003), but not strictly for ST4/74. The amino acid sequences of YehU/YehT between *S. Typhi* Ty2 (Accession number AE014613) and *S. Typhimurium* SL1344 (Accession number FQ312003) were compared using ClustalW2 [20,21]. The YehU/YehT protein sequences were also compared with the two-component regulatory system of EnvZ/OmpR using ClustalW2 [20,21]. Non-synonymous SNPs in the *yehUT* genes of *S. Typhimurium* SL1344 (Accession number FQ312003) were identified by comparison to *S. Typhi* CT18 (Accession number: AL513382) using ClustalW2 [20,21].

**Growth conditions**

Experimental analysis determined that *yehU* was expressed during growth in minimal media, pH 7.0, with glucose or acetate as a carbon source (Accession number GSE2456). Therefore, for the SDS-PAGE and Western blot analysis *S. Typhi*, *S. Typhimurium* and their mutant derivatives were grown (where stated) in Luria-Bertani (LB) broth containing different salt concentrations (low salt, 0.09M NaCl; standard, 0.17M NaCl), in SPI-2 inducible conditions as described in [22], or in SPI-2 inducible conditions including either 0.5% glucose or 0.25% acetate [23]. Cultures were shaken at 37°C overnight and standardized to an OD600 of 1.0.

In all other experiments strains were grown in low salt LB broth. All S. Typhi cultures were supplemented with a mixture of aromatic amino acids (aro-mix; 4μg/ml phenylalanine, 4μg/ml trytophan, 1μg/ml paraaminobenzoic acid and 1μg/ml dihydrobenzoic acid) and 4μg/ml tyrosine. Cultures were routinely shaken at 200rpm and incubated overnight at 37°C in a non-baffled flask. All mutant strains were grown with 30μg/ml kanamycin (Roche), 20μg/ml chloramphenicol (Sigma) or 30μg/ml ampicillin selection where appropriate.

**Construction of the ΔyehUT mutant derivatives**

The Δ*yehUT* mutant derivatives of *S. Typhi* BRD948 and *S. Typhimurium* ST4/74 were constructed using the λ red-recombinase system, described previously [24]. Bacteria were transformed with pSIM18 carrying the λ red-recombinase genes [25]. PCR amplification of the *cat* or *aph* genes using *pKD3* or *pKD4* (5ng/μl) respectively, with appropriate primers (Table S1 in File S1) that contain the priming sites GTGTAGGCTGGAGCTGCTTCG (forward) and CATATGAAATATCCTCCTTAG (reverse), homologous to regions on the template plasmids. S. Typhi targeting primers were designed using the Ty2 genome sequence [26] and *S. Typhimurium* targeting primers were designed using the SL1344 genome sequence [27] as references (Table S1 in File S1). PCR conditions used were: 94°C for 2 min; 30 cycles of
94°C for 30s; 55°C for 30s; and 72°C for 2.5 min; final elongation 72°C for 7 min [24]. The PCR product (3-5ng) was transformed into S. Typhi pSIM18 and S. Typhimurium pSIM18, which had been grown in hygromycin broth to an OD600 of 0.4 and induced to express a red recombinase with heating to 42°C for 15 minutes. Mutant clones that were able to grow on the appropriate antibiotic selective media were cultured overnight at 37°C.

Phage P22 lysates for S. Typhimurium were made on the transformants and used to transfer the antibiotic resistance gene into a fresh S. Typhimurium strain background. Plasmid pSIM18 was cured from S. Typhi and single colonies that grew on LB with appropriate antibiotic but failed to grow on hygromycin agar were selected as mutant clones. The FRT (FLP recognition target)-flanked antibiotic resistance cassette was then eliminated with pCP20, as previously described [28].

Unless stated otherwise mutations constructed as part of this work were designed to minimize polar effects unless otherwise stated.

In S. Typhimurium, mutations in yehUT were complemented by co-transducing wild type yehUT gene with a closely linked aph gene that conferred kanamycin resistance to transductants, into the chromosome of S. Typhimurium ΔyehUT::cat using phage P22. Bacterial colonies that grew on LB with kanamycin and failed to grow on LB with chloramphenicol were selected. PCR was then carried out to confirm that we had directly replaced the yehUT deletion with an intact yehUT copy (Table S2 in File S1).

In S. Typhi, the wild type yehUT coding sequence was PCR-amplified using primers (Table S2 in File S1). This DNA fragment was then digested by restriction enzymes XbaI and KpnI and ligated to the low copy plasmid pWKS30 [29]. The resulting vector was introduced into the mutant S. Typhi ΔyehUT::cat by electroporation to produce the complement strain S. Typhi ΔyehUT::cat pWKS30:: yehUT::aph.

Construction of yehT::FLAG

A FLAG epitope tag peptide was added to the end of the yehT response regulator using a modification of the standard chromosomal gene disruption protocol [30]. A C-terminal FLAG translational fusion of yehUT was constructed by PCR amplification of pSUB11 using primers gtaagccgctcgtatctgaaagtttaaagagggatggtctgGACTACAAA GACCATGACGG and ccttggctgtctcttgcaaaacagatctcttttaATATG AATATCC TCCTTAG (reverse) (Table S1 in File S1). This PCR product was transformed into S. Typhi BRD948 and S. Typhimurium ST4/74 containing pSIM18.

Western blotting

Cultured bacterial cells were centrifuged and resuspended in 300μL of sterile PBS and immediately mixed with 300μL of Laemmli 4X lysis buffer (5% SDS; 10% 2-mercaptoethanol; 40% glycerol; 0.005% bromophenol blue; 250mM 16:24 Tris HCl pH 6.8). Suspensions were heated at 100°C for 5 minutes. Extracted proteins were separated by SDS-PAGE and visualized by Coomassie-blue staining (Sigma) to ensure equal gel loading of proteins. Bacterial proteins resolved by SDS-PAGE were transferred to a nitrocellulose membrane (Invitrogen) and detected with mouse anti-FLAG M2 monoclonal antibodies conjugated to horseradish peroxidase (HRP) (1:5000 (Sigma)), which were consequently bound to HRP-conjugated rabbit anti-mouse antibodies (1:5000 (Dako)). Detection was performed by chemiluminescence (Amersham ECL system, GE Healthcare)[30]. Proteins of the appropriate parental strain were used as a negative control (S. Typhi BRD948 or S. Typhimurium ST4/74) and S. Typhimurium SL1344 fillA FLAG was constructed as described for the yehT::FLAG and used as a positive control (27kDa).

DNA microarray analysis (Accession number GSE50825)

The mRNA transcriptional profiles of S. Typhi and S. Typhimurium and derivatives harboring ΔyehU, ΔyehT and ΔyehUT mutations were analyzed in at least three individual experiments. Overnight bacterial cultures grown in low salt LB shaking at 37°C were diluted to 1 in 100ml of LB broth and grown to an OD600 of 0.3 (mid-log phase). Total RNA of ~1X10^8 cfu was extracted following treatment with 1mg/ml lysozyme for 5 minutes and purification using the RNeasy mini kit (Qiagen), following the manufacturer's instructions.

RNA quantity and quality was analyzed using the 2100 Bioanalyzer (Agilent) and 25ng/μL of RNA was used to generate cDNA. This was labeled with a single-colored dye probe (Cy3) and hybridized to the appropriate custom microarrays (Agilent). The arrays were scanned using the DNA high-resolution microarray scanner (Agilent). The raw data was normalized by the RMA method [31] and differentially expressed genes were identified using the “Limma” Package in R (Bioconductor) [32]. Those genes with at least two-fold difference in expression and an adjusted p-value of <0.05 (using the Benjamini-Hochberg method) were considered to be differentially expressed.

qRT-PCR

Genes that were significantly differentially expressed in the DNA microarray experiments were tested using qRT-PCR. Using RNA extracted and purified in the different DNA microarray experiments, cDNA was synthesized using QuantiTect Reverse Transcription kit (Qiagen) with random primers, according to the manufacturer’s instructions. PCR was conducted using 20ng/μL cDNA template in a 15μl reaction, with 0.5μM of each primer set (Table S3 and S4 in File S1) and Taq polymerase mix (Qiagen). PCR conditions used were: 95°C for 2 min; 40 cycles of 94°C for 15s; 60°C for 30s; 72°C for 30s; 95°C for 15s, 60°C for 1 min; and 95°C for 15s (Qiagen). The qRT-PCR was performed with a StepOne Plus machine (Applied Biosystems) using SYBR Green Dye (Qiagen). Fold change in gene expression was normalized to expression of the housekeeping gene recA using the delta-delta Ct method [33].

Invasion assay

Human laryngeal epithelial (HEP-2) cells were obtained from American Type Culture Collection (ATCC) 10801 University Boulevard Manassas, VA 20110, USA. The cells were maintained in DMEM-F12/L-Glut/FBS as described [34].
Bacteria for this invasion assay were first grown during the day by shaking in low salt LB at 220 rpm for 6 hours (at 37°C) followed by inoculating 0.5mls of this culture into 45mls fresh low salt LB in a 50ml falcon tube and left to grow statically overnight at 37°C [35]. Confluent monolayers of HEp-2 cells were inoculated at a dose of 10^6 cfu of bacteria. Infected monolayers were then incubated for 1 hour in a tissue culture incubator at 37°C, washed twice with Phosphate Buffer Saline (PBS), and then overlaid with tissue culture medium supplemented with 100μg/ml gentamicin. After 90 minutes, the cell culture was washed three times with PBS and lysed with 1% Triton X-100 in PBS to release intracellular bacteria. An aliquot of this suspension was used to determine the number of intracellular bacteria by plating serial dilutions onto LB agar plates. The bacterial counts of the control strains were compared with their respective ΔyehUT mutant derivatives.

Vi phage assay

S. Typhi BRD948 and its mutant derivative S. Typhi ΔyehUT were tested for sensitivity (plaque morphology) and susceptibility (bacterial counts) to Vi phage types II and VII [36]. The strains were grown in 3ml of LB left shaking overnight at 37°C. Molten 0.35% L-agar was cooled to 42°C, and 3ml aliquots were added to Falcon tubes containing 10μl of dilutions of Vi phage type II or VII (from 10^-1 to 10^-7) and 150μl of the bacterial culture; the mixtures were poured immediately onto L-agar plates. The plates were left overnight at 37°C. The next day plaque morphology and count was compared.

Serum bactericidal assays with human sera

Killing of S. Typhi, S. Typhi ΔyehUT, and S. Typhi ΔyehUT::cat pWKS30::yehUT::aph and S. Typhimurium, S. Typhimurium ΔyehUT and S. Typhimurium ΔyehUT::cat yehUT::aph with fresh, human serum were assessed as previously described [37]. Bacteria in log growth at a concentration of 1 x 10^7 cfu/ml were added at a 1:10 dilution to 100% human serum to give a final concentration of 1 x 10^6 cfu/ml and incubated at 37°C for 180 min on a rocker plate at 20rpm. Sera heat-inactivated at 56°C for 30 min were used as a control. Samples were taken at 45, 90 and 180-minute intervals. The concentration of Salmonella was determined by serial dilutions and plating on LB agar.

Mouse experiments

Acute infection mouse model. Mixed competitive inhibition experiments were performed using groups of ten isofluorane-anaesthetized wild type C57BL/6 mice, which were orally inoculated with 10^8 cfu of mixed cultures of S. Typhimurium ΔphoN::aph and S. Typhimurium ΔyehUT::cat in 200μl of sterile PBS. The phoN gene is dispensable for the intestinal and systemic phases of murine salmonellosis [38]. Therefore it was deemed an appropriate chromosomal insertion site for the aph gene required to distinguish the control S. Typhimurium from the mutant derivatives [39,40]. All mice were weighed daily and monitored for signs of illness. For bacterial quantification tissue from the mesenteric lymph nodes (MLN), cecum, ileum, spleen and liver from the mice were collected on day 5-post infection and homogenized in 5ml of sterile PBS. Viable bacteria in the tissues were quantified by serial dilutions and plating onto LB agar containing the appropriate antibiotics to select for the Salmonella. Statistical tests were performed by the Wilcoxon signed-ranked test using Prism software (GraphPad Software Inc.) on the mixed competitive inhibition data. Differences between data sets where p < 0.05 were considered statistically significant.

Streptomycin-pretreated colitis mouse model. A streptomycin experiment was performed using three groups of five mice. Mice were pre-treated with 50mg of streptomycin in 200μl of sterile water 24 hours prior to infection [41]. One group of five mice were naïve and the other two groups of five mice were anaesthetized using isofluorane and orally inoculated with 10^7 cfu of either S. Typhimurium ΔphoN::aph or S. Typhimurium ΔyehUT::cat, in 200μl of sterile PBS. All mice were weighed daily and monitored for signs of illness. All mice were culled on Day 3 of infection or mice exceeding 20% total weight loss were culled prior to Day 3 in accordance with UK Home Office guidelines. The ceca of the mice were collected, weighed and homogenized in 5ml of sterile PBS for bacterial quantification, qRT-PCR of inflammatory cytokines and pathological examination. Statistical analyses were performed on the data. Bacterial quantification in which viable bacteria was quantified by serial dilutions on LB agar containing the appropriate antibiotics to select for the Salmonella.

qRT-PCR of inflammatory cytokines: RNA was isolated from 3mm-long cecal sections using RNeasy Mini kit (Qiagen) according to manufacturer’s instruction. RNA integrity and quantity were then assessed using Agilent 2100 Bioanalyzer (Agilent Technologies). cDNA was then synthesized using QuantiTect Reverse Transcription kit (Qiagen) with random primers, according to the manufacturer’s instructions. All TaqMan qRT-PCR primers and probes were designed by Primer Express 3.0 (Applied Biosystems) to span exon-exon junction to avoid genomic DNA amplification. PCR was conducted using 5ng/μl cDNA template in a 25μl reaction, with 20μM of each cytokine primer set (Table S5 in File S1) and 10μM appropriate probe, according to manufacturer’s instructions (Thermo Scientific). The Gapdh gene was used as the endogenous control. The qRT-PCR was performed with a StepOne Plus machine (Applied Biosystems) using ABSolute Blue qRT-PCR Rox mix (Thermo Scientific). Relative gene expression was determined by normalization to Gapdh copy numbers [42].

Histopathology: To evaluate intestinal inflammatory disease histopathology, we fixed cecal segments in 4% paraformaldehyde and stained 5 μm-thick paraffin sections in hematoxylin and eosin according to standard protocols. Scoring of intestinal inflammation was performed in a blinded manner by a clinical consultant histopathologist with comparative pathology expertise, as follows: submucosal edema: mild-1, moderate-2, severe-3; submucosal cellular inflammation: mild-1, moderate-2, severe-3; mucosal cellular inflammation: mild-1, moderate-2, severe-3; crypt abscesses: absent-0, present (occasional/mild)-1, present (moderate/severe)-2; mucosal ulceration: absent-0, present (focal/mild)-1, present (moderate/severe)-2.
Phenotyping microarrays (BIOLOG)

Phenotype microarrays (PM) of metabolism of carbon sources (PM 1 to 2), nitrogen sources (PM 3), phosphorus and sulfur sources (PM 4), biosynthetic pathway substrates (PM 5), and peptide nitrogen sources (PM 6 to 8), osmotic/ionic response (PM 9), pH response (PM 10) and bacterial chemical sensitivity (PM 11 to 20) were performed according to manufacturer’s instructions. (Biolog Inc. Hayward, California, USA) [43]. S. Typhi BRD948 and S. Typhimurium ST4/74 were used as reference strains for comparison with their respective ΔyehUT mutants (S. Typhi ΔyehUT and S. Typhimurium ΔyehUT). PM micro tier plates were incubated at 37°C for 24 hours in the Omnilog (Biolog Inc.) and each well monitored for redox indidor change representing kinetic respiration. Tests were performed in triplicate and the kinetic data analyzed using Omnilog PM software (Biolog Inc.). Data was exported from the Biolog File Manager, and further analysis was conducted in R. Data was transformed in to “signal values” [44], and the Bioconductor package Limma [32] was used to test for differences in dye reduction. Benjamini-Hochberg corrected p-values were used to determine statistical significance, controlling for a false discovery rate of 5%.

Minimum inhibitory concentrations (MICs) of the ΔyehUT mutants of S. Typhi and S. Typhimurium were determined using Epsilometer test (E test) strips against antibiotics amoxicillin, tetracycline, ciprofloxacin, ceftriaxone, and meropenem (Oxoid, Hampshire, UK), trimethoprim-sulfamethoxazole and azithromycin (BioMérieux, SA, Marcy-L’Etoile, France). Semi-confluent growth was achieved using 0.5 McFarland organism bacterial suspensions [45] on IsoSensitest agar (Oxoid, Hampshire, UK) after 24 hours incubation aerobically at 37°C. Since the mutant strains were not clinical isolates parental strains were used as the controls to see if the deletion of yehUT had an effect on antibiotic susceptibility.

Results

Informatics analysis of the YehUT system

In S. Typhi Ty2 the yehU and the yehT genes are encoded on a 2,349 bp long fragment with a 4 bp overlap between the yehU and yehT open reading frames and are likely part of an operon. Neighboring genes include yehV, yehW, and yehX upstream of yehU, and yehS and yehR downstream from yehT. The genes yehX, yehW, yehU, yehT and yehS are located on the forward strand, whilst t0694, yehV, t0699 and yehR lie on the reverse strand (Figure 1) [26,46]. The function of these genes is not currently known, although our sequence comparison-using Interpro indicated that yehV and yehS encode a putative transcriptional regulator and a conserved hypothetical protein, respectively [16].

The domain architecture of YehU and YehT was predicted from a consensus definition from Interpro [16]. These results predicted four domains in YehU (sensor, GAF, histidine phosphotransfer (DHp) and kinase domains) and two in YehT, (receiver and cell envelope-related transcriptional attenuator (LytR) domains) (Figure 1). The sensor domain is predicted to contain at least 5 transmembrane helices [17]. GAF domains are so-called because they are found in cGMP-specific phosphodiesterases, adenylyl cyclases and transcriptional activators, including formate hydrogen lyase system activators (FhlA), and although their precise function is not clear they may be involved in ligand binding and protein-protein interaction [47]. The LytR domain named after the Bacillus subtilis LytT and Staphylococcus aureus LytR response regulators contains a specific DNA-binding motif and is commonly found in a number of bacterial transcriptional regulators [48]. The YehU/ YehT system was compared to EnvZ/OmpR and revealed that YehU does not have a histidine residue at position 243 as in the sensor kinase EnvZ [49].

YehT expression in different media

In order to study the function of the YehUT system we initially determined the optimal conditions in which the YehT protein of S. Typhi and S. Typhimurium was expressed. To this end a S. Typhi and S. Typhimurium derivative was constructed that directed the expression of a YehT fusion protein fused at the C-terminal with the epitope-tag FLAG. Whole protein preparations from these derivatives cultured in five alternative media indicated that YehT was expressed in all conditions. In S. Typhi, YehT was expressed at relatively higher level in low salt LB (0.09M NaCl) than standard LB (0.17M NaCl). In a further experiment, expression was also slightly higher in a minimal medium designed to induce SPI-2, compared to the same medium additionally containing either glucose or acetate (Figure 2). However, in S. Typhimurium the overall level of expression of YehT was relatively low in all five culture media (Figure 3). Therefore, low salt LB was selected as the best growth media for determination of yehUT dependent transcription analysis and elucidation of the phenotype of ΔyehUT for both S. Typhi and S. Typhimurium.

Identification of yehUT regulated genes

In order to elucidate the role of yehUT on global gene expression, we performed transcriptome profiling based on microarray analysis of mRNA purified from S. Typhi and otherwise isogenic mutant derivatives harboring non-polar deletions of yehUT or either one of these two genes. S. Typhi and all mutant derivatives, including ΔyehUT S. Typhi grew at comparable rates (data not shown). Analysis of differentially expressed genes relative to the average expression level of each gene across all arrays was performed and genes that showed a statistically significant change in expression in mutant compared to S. Typhi control strain were identified (Accession number GSE50825) [32]. The differentially expressed genes were grouped into functional classes based on genome annotation [50]. Transcriptome analysis of the S. Typhi transcriptome compared to the ΔyehU, ΔyehT and ΔyehUT mutant derivatives provided evidence of relative down
Figure 1. A The yehUT operon with surrounding genes. The operon is 2349bp in length and located between 782210 to 784611 bp in S. Typhi Ty2 (Accession number AE014613) and between c2251460 to c253861 bp in S. Typhimurium SL1344 (Accession number FQ312003). The surrounding genes include yehV, t0694, yehW, yehX (upstream of yehU) and yehS, t0699 and yehR (downstream from yehT). B The domain organisation of the YehU and YehT proteins. YehU protein comprises of five transmembrane (TM) alpha helical segments predicted using TMHMM webserver [17]. The intracellular signalling component consists of a GAF domain, a DHp (dimerization/histidine-containing phosphotransfer) domain that is connected to an ATP (adenosine triphosphate)-dependent Kinase domain. YehT protein consists of a Receiver domain and a LyTR-homologous DNA binding domain [16]. The phosphorylation sites are indicated (H, Histidine; D, Aspartate). The SNP positions identified in yehU and yehT genes are indicated below the proteins by grey boxes [10].

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Figure 2. SDS-PAGE and western blot analysis of epitope-tagged YehT protein from S. Typhi BRD948. Proteins from whole cell bacterial lysates were transferred after electrophoretic separation in a 12% SDS-PAGE gel (Invitrogen) onto a nitrocellulose membrane (Invitrogen) and probed with anti-FLAG M2 monoclonal antibodies (Sigma). Lanes: (M) protein molecular weight markers (SeeBlue Plus2 Pre-stained standard; Invitrogen); (-) proteins extracted from S. Typhi BRD948 (negative control); (+) proteins extracted from S. Typhimurium SL1344 ΔfliA (positive control, 27.4kDa); FLAG proteins extracted from S. Typhi ΔyehT-tagged (27.4kDa). Bacteria was grown in low salt LB; normal salt LB; SPI-2 inducing; SPI-2 + 0.5% glucose; SPI-2 + 0.25% acetate overnight, shaking at 37°C.

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regulation in each of the mutants of three genes: cstA1 (t4582), encoding a carbon starvation protein, and t4581 and t4580 annotated as conserved hypotheticals (Table 1).

The cstA1 gene was the most differentially expressed in all mutant derivatives compared to S. Typhi. This gene is located in the same chromosomal region as t4581 and t4580 on the reverse strand of the S. Typhi Ty2 genome: cstA1 (t4582) is situated at c4734842 bp to c4736992 bp, with t4581 95 bp and t4580 309 bp downstream, respectively [18]. Previous RNA-sequencing data has shown that these genes are transcribed in vitro at 37°C during mid-log phase [51]. The cstA1 gene was identified as a putative carbon starvation protein using the NCBI database [52,53]. Furthermore, the gene is predicted to encode a membrane protein involved in peptide utilization to avoid carbon starvation as the bacterium enters stationary phase [54]. This protein appears to be conserved in the Enterobacteriaceae, including Salmonella species [54]. cstA has been described as a cAMP-CRP (cyclic adenosine monophosphate-catabolite regulation protein) dependent gene in E. coli when the concentration of cAMP rises during carbon starvation activating the gene [55].

Both NCBI and Pfam databases identified t4581 as encoding a hypothetical protein of unknown function, although structural modeling suggested a domain that might bind nucleic acids [52-54]. Gene t4580 is predicted to encode a putative GTP-binding protein (YjaA), using NCBI [52,53]. In E. coli the crystal structure supports a GTP-dependent function, but the biological role of the protein remains unclear [56].

Transcriptome analysis of the S. Typhi ΔyehUT mutant displayed not only dysregulation of cstA1, t4580 and t4581, but also an additional 12 genes: ten genes were down regulated and two up regulated (Table 1). These genes were not differentially expressed in either of the single mutants. There was relative down regulation of a number of Salmonella Pathogenicity Island (SPI) associated genes, including spaM, spaN, and spaL located on SPI-1, pipB located in SPI-5, and the SPI-7 encoded gene sopE [57]. Other down regulated genes included t4221, a member of the AraC family transcriptional regulator family and t4220, a GerE family regulatory protein. Metabolic genes relatively down regulated in the S. Typhi ΔyehUT mutant included ppc, which encodes phosphoenolpyruvate carboxylase, an enzyme involved in the oxidation of carbohydrates via the tricarboxylic acid cycle [58] and yiiD, a galactoside O-acetyltransferase, which in E. coli may assist cellular detoxification by acetylatyng non-metabolizable pyranosides and thereby preventing their reentry into the cell [59]. The two genes that were up regulated in the double mutant were the nth gene, which encodes endonuclease III involved in the DNA repair system [60,61] and t1038, which encodes an unknown hypothetical protein.

The differential expression of several genes, including cstA1, t4581, t4580, spaM, spaN, spaL and sopE, were validated using qRT-PCR. The overall pattern of up and down regulation of genes was identical to the microarray results (Table S6 in File S1; Figure 4a). Complementation of the ΔyehUT mutant with plasmid-encoded yehUT restored expression of the genes, although many of the genes were then relatively over-expressed, which is most likely a result of the high copy number of plasmids containing yehUT (Table S6 in File S1; Figure 4b).

Phenotypic analysis of the yehUT deletion mutant derivatives of S. Typhi

S. Typhi BRD948 and its ΔyehUT mutant derivative were phenotypically analyzed using serum bactericidal assays (Graph B in Figure S1), tissue invasion assays with Hep2 cells (Graph A in Figure S2), and a Vi phage sensitivity assay (Figure S3), but no significant differences were observed. There was no metabolic phenotype identified for S. Typhi ΔyehUT using phenotypic Biolog microarrays, which monitors a range of different growth conditions, including various substrates, ionic concentrations (pHs) and osmolarities. Furthermore, no difference in MIC was observed between S. Typhi and the ΔyehUT mutant derivative when tested against a range of antibiotics commonly used to treat Salmonella infections, including amoxicillin, ceftriaxone, ciprofloxacin, trimethoprim-sulfamethoxazole, meropenem and azithromycin.
Properties of a yehUT deletion mutant derivative of S. Typhimurium

The two-component regulatory system yehUT is also present in S. Typhimurium which allowed us to use this serovar for a comparison against S. Typhi and as a surrogate in vivo model of typhoid fever. Informatics analysis of the S. Typhimurium SL1344 genome identified one non-synonymous SNP in yehT and four non-synonymous SNPs in yehU compared to S. Typhi CT18 (Table S7 in File S1) [18]. The consensus encoded polypeptides are almost identical in both serovars with only four amino acid differences in YehU and two amino acid difference in YehT identified between S. Typhi Ty2 and S. Typhimurium SL1344, with no difference observed between predicted protein structures [20,21,54].

Transcriptome analysis of mRNA prepared from S. Typhimurium and the yehUT mutant derivatives, provided evidence for the comparative down regulation of the same four genes identified in similar experiments with S. Typhi: cstA1 (SL1344_4463), cstA2 (SL1344_0588) and hypothetical proteins SL1344_4462 and SL1344_SPAB_05703 (Accession number GSE50825) (Table 2). Three out of the four genes (cstA1, SL1344_4462 and SL1344_SPAB_05703) were confirmed by qRT-PCR to be relatively down regulated in the ΔyehUT mutant (Table S6 in File S1; Figure 5a). Expression of these genes was restored by complementation of the ΔyehUT mutant (Table S6 in File S1; Figure 5b). On further analysis these genes are found in the same region in the genome with the cstA1 gene located at c4808148bp to c4810298 bp on the reverse strand, SL1344_4462 situated 95bp downstream of cstA1 on the same strand and SL1344_SPAB_05703 overlapping upstream of cstA1 on the forward strand (4810246 bp to 4810350 bp) [18].

Table 1 (continued).

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Table 1. DNA microarray analysis of S. Typhi ΔyehUT compared to S. Typhi BRD948, grown in low salt LB at mid-log phase.
**Discussion**

S. Typhi is a Gram-negative organism that causes the disease typhoid fever and is spread fecal-orally by ingestion of contaminated food or water [8]. Unlike most *S. enterica* serovars, *S. Typhi* is a human restricted pathogen that entered the human population once relatively recently. Thus, all *S. Typhi* can be mapped phylogenetically back to a common ancestor and the genomes of collections of *S. Typhi* exhibit limited genetic variation [10]. That the *yehUT* operon of *S. Typhi* and *S. Typhimurium*...
Typhimurium ΔyehUT mutant. A The relative expression levels of the 4 genes found to be differentially expressed on microarray analysis of the S. Typhimurium ΔyehUT mutant when compared to S. Typhimurium in low salt LB, at mid-log phase, at 37°C (Table S6 in File S1). The 4 genes examined are listed on the y-axis. Grey boxes < 1.0 indicates down regulated genes and grey boxes >1.0 indicates up regulated genes relative to S. Typhimurium. B qRT-PCR analysis showing relative expression levels of genes after complementation of the ΔyehUT mutant compared to S. Typhimurium (Table S6 in File S1).

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Figure 5.

Typhi was found to contain an unusually high number of SNPs compared to the rest of the genome was therefore of potential significance in the evolution of this pathogen [10]. In addition, large scale transposon mutagenesis (TraDIS) data obtained by investigation of S. Typhimurium mutant survival during infection of mice, chickens, pigs and cattle showed that a single insertion in the yehT gene had a significant attenuation in calves (www-tradis.vet.cam.ac.uk). Consequently we combined transcriptome analysis and phenotypic assays to gain a better understanding of this system in Salmonella.

The transcriptome analysis for both S. Typhi and S. Typhimurium containing ΔyehU, ΔyehT or ΔyehUT revealed relative down regulation of cstA1 associated with the response to carbon starvation along with two neighboring hypothetical genes: t4580 and t4581 of S. Typhi; SL1344_4462, and SL1344_SPAB_05703 of S. Typhimurium. These three genes are situated within the same region of the genome of both serovars. The cstA gene was previously shown in E. coli to be positively regulated by the cyclic AMP (cAMP) and cAMP receptor protein (CRP) complex and to be involved in peptide utilization during carbon starvation [55,62,63]. In E. coli, expression was increased upon exhaustion of succinate, glycerol, or glucose from the medium, or upon entering stationary phase in LB [62]. CstA has been implicated as a peptide transporter [63], supported by sequence analysis that revealed the CstA protein consists of 701 amino acids and a hydrophobicity profile suggestive of an integral membrane protein [64]. A CstA homologue YjiY (>96% identity) was also up regulated when yehT was over expressed in E. coli [3]. Furthermore, YjiY, like CstA, may take part in amino acid/peptide utilization [3]. The remaining 31 genes differentially expressed in the E. coli study did not match the genes differentially expressed in Salmonella. However, Kraxenberger et al. [3] utilized over expression of the yehT gene compared to our mutant derivatives, which may account for at least some of the difference in gene expression profiles.

S. Typhi ΔyehUT exhibited differential expression of a further 12 genes compared to the ΔyehU and ΔyehT mutant derivatives, including genes involved in metabolism (yidD, ppc) pathogenicity (spaM, spaN, spal, pipB) and regulation (AraC, GerE). This suggested that yehUT might modulate metabolism or virulence functions of S. Typhi; hence we went on to test the mutant in various biochemical and virulence assays. The difference in gene expression observed between the derivatives harboring individual and double gene deletions in S. Typhi might be indicative of cross-regulation between yehUT and another two-component regulatory system compensating for the lone loss of the sensor kinase or response regulator in the yehUT system [65,66].

An independent study reported relative dysregulation of 26 genes in an S. Typhi GIFU10007ΔyehUT mutant derivative under hypotonic conditions [67]. This dysregulation is somewhat more extensive than the three genes (cstA1, t4580, t4581) we identified using a ΔyehT mutant derivative of S. Typhi BRD948. The number of genes differentially expressed (26 genes) is more compatible with the analysis obtained from our ΔyehUT mutant (15 genes), with four genes (spaN, spaM, spal, pipB) apparently dysregulated in both studies. It is noteworthy that the cstA1 gene was not differentially expressed to a statistically significant level in their study and yet was the top dysregulated gene here in both S. Typhi and S. Typhimurium yehUT mutant derivatives under isotonic conditions. These differences in transcriptome profiling of yehUT may be due to differences in the experimental conditions or to the use of different parental strains.

Genetic regulation of the yehUT operon is not well characterized. Differential regulation of yehU or yehT was not seen in a previous study in which we identified genes by
microarray and RNA-seq of S. Typhi BRD948 that exhibited OmpR-dependent transcription, during mid log phase, in LB media [68]. However, another study showed that OmpR positively regulated expression of yehUT in S. Typhi GIFU10007 at low osmolarity [67]. One explanation for this discrepancy is that OmpR only induces yehUT expression when the osmolarity is lowered.

It has been proposed that YehU activates YehT by phosphorylation of a conserved histidine residue at position 382 [3]. In E. coli mutations in the putative histidine residue of YehU involved in phosphorytransfer (position 382) and YehT (position 524) impacted expression of yijY, a component of the yehUT regulon, suggesting an important role for phosphorylation at these sites [3]. A previous study has shown that auto-phosphorylation of YehU and phosphotransfer to YehT were barely detectable under all conditions tested [4]. However, YehU was shown to have no identifiable F box, which is a component of the histidine protein kinase domain involved in ATP binding, and probably also catalysis and phosphotransfer. This property may explain this reduction in phosphorylation [69]. In silico analysis predicted a histidine phosphotransfer (DHp) domain component of YehU, but the conserved histidine residue at 382 was not seen in second sensor kinase EnvZ on alignment comparison suggesting that these regulators have distinct mechanisms of signal transduction.

There are two copies of cstA gene homologues with a comparable identity of 61.9% in the genomes of both S. Typhi and S. Typhimurium serovars: cstA1 (S. Typhi H582; S. Typhimurium SL1344_4463), which was differentially expressed in our microarray analysis and cstA2 (S. Typhi i2268; S. Typhimurium SL1344_0588), which was confirmed not to be dysregulated [20,21]. In a previous study we demonstrated that OmpR negatively regulates cstA2 in both S. Typhi and S. Typhimurium, using DNA microarray analysis, but exerts no effect on cstA1 [68]. A clearer understanding of the regulatory networks of both cstA1 and cstA2 may provide clues to how Salmonella is continually fine tuning its adaption to its host and environment.

Other than within the transcriptome profile, we were unable to identify a phenotype for S. Typhi or S. Typhimurium harboring mutations in yehUT. Despite the transcriptome analysis of S. Typhi ΔyehUT revealing dysregulation of a number of SPI genes yehUT mutant derivatives were able to invade cultured epithelial cells at rates comparable to control and showed no difference the mouse infection models compared to the parental strain. This is supported by studies in E. coli in which a phenotype for ΔyehUT mutant was also not detected [3,5].

The yehUT operon of S. Typhi may be undergoing either adaptive evolution due to host or environmental stresses or possibly a form of relaxed evolution as evidenced by the accumulation of a high number of non-synonymous SNPs. Further investigation into the biological complexity of these individual SNPs within S. Typhi needs to be undertaken to answer these questions more fully.

Supporting Information

Figure S1. Resistance to killing by human serum of (A) Salmonella Typhimurium ST4/74, S. Typhimurium ST4/74 ΔyehUT and S. Typhimurium ΔyehUT::cat :: yehUT::aph, and (B) S. Typhi BRD 948, S. Typhi ΔyehUT and S. Typhi ΔyehUT ::cat pWKS30::yehUT::aph. Deletion of yehUT had no detectable impact on the resistance to antibody-mediated, complement dependent killing of either S. Typhimurium (A) or S. Typhi (B) (p>0.05 for all time points). Complementation of the yehUT locus for both S. Typhimurium and S. Typhi mutant derivatives also had no detectable effect on resistance to serum killing. Data for each line represent the mean of the results from 10 healthy adult donors done in triplicate. The initial concentration of bacteria in the assay is approximately 1x10^8 cfu/ml. Negative values show a decrease in the number of viable bacteria over time. Error bars represent SEM.

Figure S2. Comparison of the ability of (A) S. Typhi BRD 948 and S. Typhi ΔyehUT, and (B) S. Typhimurium ST4/74 and S. Typhimurium ST4/74 ΔyehUT to invade HEp-2 cells. For each isolate three experiments were conducted with bacteria grown by shaking in low salt LB for 6 hours at 37°C followed by inoculating 0.5mls of this culture into 45mls fresh low salt LB in a 50ml falcon tube. These samples were left to grow statically overnight at 37°C. Each column represents the mean and SEM of numbers of viable bacteria recovered from three separate wells. In (A) the data was normalized to S. Typhi BRD948 and the invasiveness of S. Typhi ΔyehUT was not obviously affected (p=0.38). In (B) the data was normalized to S. Typhimurium ST4/74 and the invasiveness of S. Typhimurium ΔyehUT was also not obviously affected (p=0.33).

Figure S3. Vi phage assays of S. Typhi BRD948 and S. Typhi ΔyehUT.
Plaque morphology and plaque counts (pfu/ml) were assessed after overnight Vi phage type II and VII infections of both S. Typhi BRD948 and S. Typhi BRD948 yehUT. There was no significant difference between S. Typhi and S. Typhi yehUT in terms of plaque morphology or counts for either Type II (p=0.41) or Type VII (p=0.66) phage infections.

Figure S4. Mixed competitive inhibition mouse experiment of S. Typhimurium ST4/74 and S. Typhimurium ΔyehUT.
The competitive index (CI) ratio of output (ΔyehUT mutant/S. Typhimurium ST4/74) / input (ΔyehUT / S. Typhimurium ST4/74) was calculated for bacterial colony-forming units (CFU) in tissue from mesenteric lymph nodes (MLN), cecum, ileum, spleen and liver of five C57BL/6 mice orally inoculated with 10^8 CFU of S. Typhimurium ST4/74 and the ΔyehUT derivative of this parental isolate. This experiment was performed twice. Mean (central bars) and 95% confidence intervals (error bars) are shown. Results showed that there were no significant differences in bacterial counts between S.
Typhimurium ST4/74 and S. Typhimurium ΔyehUT in the MLN (p=0.5), cecum (p=0.91), ileum (p=0.50), spleen (p=0.04) and liver (p=0.004).

Figure S5. Cecal bacterial counts of S. Typhimurium ST4/74, S. Typhimurium ΔyehUT and S. Typhimurium ΔyehUT::cat :: yehUT::aph, in the streptomycin-pretreated colitis mouse experiment. CFUs in tissue from cecum of five C57BL/6 mice pretreated with 10^3 CFU of either (a) S. Typhimurium ST4/74, (b) S. Typhimurium ΔyehUT or (c) S. Typhimurium ΔyehUT::cat :: yehUT::aph. This experiment was performed three times. Mean (central bars) and SEM (error bars) are shown. The results showed that there were no significant differences between S. Typhimurium ST4/74 and the ΔyehUT derivative (p=0.16) and between ΔyehUT and ΔyehUT::cat :: yehUT::aph (p=0.62).

(EPS)

Figure S6. Histopathological scoring of cecal segments of S. Typhimurium ST4/74, S. Typhimurium ΔyehUT and S. Typhimurium ΔyehUT::cat :: yehUT::aph in the streptomycin-pretreated colitis mouse experiment. Histopathological scoring of hematoxylin and eosin stained mouse cecal segments after five C57BL/6 mice were pretreated with 50mg of streptomycin and then 24 hours later orally inoculated with 10^3 CFU of either (a) S. Typhimurium ST4/74, (b) S. Typhimurium ΔyehUT or (c) S. Typhimurium ΔyehUT::cat :: yehUT::aph. This experiment was performed three times. Mean (central bars) and SEM (error bars) are shown. The results showed that there were no significant differences between S. Typhimurium ST4/74 and the ΔyehUT derivative (p=0.5), in the streptomycin-pretreated colitis mouse experiment; Table S6. qRT-PCR analyses of differentially expressed genes of the S. Typhimurium ΔyehUT mutant; Table S7. SNPs identified by informatics analysis of the yehUT genes of the S. Typhimurium SL1344 genome (Accession number: FQ312003). Coordinates correspond to S. Typhi CT18 finished genome sequence (Accession number: AL513382).

(EPX)

Author Contributions
Conceived and designed the experiments: GD RAK DJP CAM KS LJK KEH. Performed the experiments: VWK TMW PJH SJK LK LFM LE DJP. Analyzed the data: VWK RB CJM LB AJP MJA. Contributed reagents/materials/analysis tools: GD KS CJM RB CAM PJH LB. Wrote the manuscript: VWK DJP RAK GD. Designed the software used in analysis: LB.
References


yeHUt of S. Typhi and S. Typhimurium


