Role of two-component sensory systems of *Salmonella enterica* serovar Dublin in the pathogenesis of systemic salmonellosis in cattle

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Salmonella enterica serovar Dublin (S. Dublin) is associated with enteritis, typhoid and abortion in cattle. Infections are acquired by the oral route, and the bacteria transit through varied anatomical and cellular niches to elicit systemic disease. *S. Dublin* must therefore sense and respond to diverse extrinsic stimuli to control gene expression in a spatial and temporal manner. Two-component systems (TCSs) play key roles in such processes, and typically contain a membrane-associated sensor kinase (SK) that modifies a cognate response regulator. Analysis of the genome sequence of *S. Dublin* identified 31 conserved SK genes. Each SK gene was separately disrupted by lambda Red recombinase-mediated insertion of transposons harbouring unique sequence tags. Calves were challenged with a pool of the mutants together with control strains of defined virulence by the oral and intravenous routes. Quantification of tagged mutants in output pools derived from various tissues and cannulated lymphatic vessels allowed the assignment of spatial roles for each SK following oral inoculation or when the intestinal barrier was bypassed by intravenous delivery. Mutant phenotypes were also assigned in cultured intestinal epithelial cells. Mutants with insertions in *barA*, *envZ*, *phoQ*, *ssrA* or *qseC* were significantly negatively selected at all enteric and systemic sites sampled after oral dosing. Mutants lacking *baeS*, *dpiB* or *citA* were negatively selected at some but not all sites. After intravenous inoculation, only *barA* and *phoQ* mutants were significantly under-represented at systemic sites. The novel role of *baeS* in intestinal colonization was confirmed by oral co-infection studies, with a mutant exhibiting modest but significant attenuation at a number of enteric sites. This is the first systematic analysis of the role of all *Salmonella* TCSs in a highly relevant model of enteric fever. Spatial roles were assigned to eight *S. Dublin* SKs, but most were not essential for intestinal or systemic infection of the target host.

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INTRODUCTION

Salmonella enterica is a facultative intracellular pathogen of worldwide importance. Over 2500 serovars exist, and these may be divided into three broad classes on the basis of host specificity (Uzzau *et al.*, 2000); in healthy outbred adults, ubiquitous serovars (e.g. Typhimurium) produce acute but self-limiting enteritis in diverse species, whereas host-specific serovars are associated with severe systemic disease in a single species, which may not involve diarrhoea (e.g. Gallinarum in poultry). Host-restricted serovars are primarily associated with systemic disease in one host, but may cause disease in a limited number of other species (e.g. Dublin in cattle and humans). The molecular mechanisms

underlying the differential host range and virulence of *S. enterica* serovars are incompletely understood.

S. enterica serovar Dublin (*S. Dublin*) is a significant pathogen of cattle (Wallis & Barrow, 2005) and encounters diverse environments during translocation from the bovine intestines. Following ingestion by cattle and exposure to the acid barrier, bile, mucous, intestinal cryptidins and other innate defences, *Salmonella* enters enterocytes and intestinal M cells and is initially confined to a *Salmonella*-containing vacuole (SCV) (Bolton *et al.*, 1999; Frost *et al.*, 1997). In the SCV, the bacteria are exposed to nutrient-limiting conditions, low pH, cationic peptides and reactive oxygen and nitrogen species. *Salmonella* counteracts fusion of the SCV with the late endocytic pathway, and thus contact with degradative enzymes, in a manner dependent on type III secretion system (T3SS)-2. Within 12 h of invasion of the bovine ileal epithelium, *Salmonella* clears

Abbreviations: Amp, ampicillin; CI, competitive index; Kan, kanamycin; MLN, mesenteric lymph node; Nal, nalidixic acid; RR, response regulator; SCV, *Salmonella*-containing vacuole; SK, sensor kinase; T3SS, type III secretion system; TCS, two-component system.

the enterocyte layer and is found within MHC class II⁺ cells with features of antigen-presenting cells (Monaghan *et al.*, 2001). From here, bacteria transit via an unknown route that may involve host cell necrosis to mesenteric lymph nodes (MLNs). By cannulation of blood and lymph vessels serving the bovine distal ileum we have established that *S. Dublin* translocates in a predominantly cell-free niche via efferent lymph, in contrast to observations in murine typhoid models (Paulin *et al.*, 2002; Pullinger *et al.*, 2007). From lymph, the bacteria may be deposited in blood at the thoracic duct; however, the precise mode of spread to visceral organs remains ill-defined. It may be inferred, however, that translocation via MLNs and associated lymphatic vessels is significant in the pathogenesis of systemic salmonellosis in cattle, as signature-tagged clones arriving at the liver and spleen are the same ones in the same proportions as those migrating via MLNs and efferent lymph (Pullinger *et al.*, 2007), in contrast to observations with *Yersinia pseudotuberculosis* in mice (Barnes *et al.*, 2006). In the liver, *S. enterica* serovar Typhimurium (*S. Typhimurium*) has been reported inside hepatic phagocytes, albeit after intravenous dosing of inbred mice (Sheppard *et al.*, 2003). In order to survive and spread through these varied compartments, host-restricted *S. enterica* serovars must be able to sense and respond to the changing niches that they encounter.

In common with other bacterial pathogens, *Salmonella* is equipped with a plethora of systems to sense and respond to extrinsic signals. These include chemotaxis systems to sense nutrients and promote their assimilation, quorum-sensing systems to survey population density, and regulatory systems to modulate gene expression in response to environmental cues. Of those implicated in pathogenesis, many comprise two components (Beier & Gross, 2006): a sensor kinase (SK) that autophosphorylates at a conserved histidine residue on receipt of a specific signal, and a response regulator (RR) to which the phosphate moiety is transferred from the cognate SK(s). Phosphorylation of the RR alters its activity, leading to altered expression of genes under its direct or indirect control.

Genome-wide mutagenesis followed by screening of pools of mutants by different routes has implicated some two-component system (TCS) genes of *S. Typhimurium* in systemic infection in mice (e.g. *phoPQ*, *citB*, *envZ*, *pmrB*, *ssrA* and *ssrB*) (Chaudhuri *et al.*, 2009; Lawley *et al.*, 2006; Santiviago *et al.*, 2009). Signature-tagged transposon mutants lacking *citA*, *dpiA*, *ssrA* and *ssrB* have also been found to be impaired in intestinal colonization of farm species (Carnell *et al.*, 2007; Morgan *et al.*, 2004). Furthermore, *S. Typhimurium* mutants with deletions in 83 regulatory genes, including 11 TCS genes, have been individually assessed in mouse models and *ssrAB*, *phoPQ* and *envZ/ompR* implicated in virulence following intra-gastric and intraperitoneal inoculation (Yoon *et al.*, 2009). A defined *cpxA* mutant of *S. Typhimurium* is also attenuated in mice (Humphreys *et al.*, 2004).

Resolvase-*in vivo* expression technology has indicated that the *PhoPQ* and *PmrAB* TCSs are activated during infection of mice, but that genes regulated by these TCSs are differentially expressed in different anatomical compartments (Merighi *et al.*, 2005), indicating that *Salmonella* uses its TCSs to adapt to changing niches as infection spreads. Contextual regulation of virulence-associated loci is vital in pathogenesis, as evidenced by the attenuating effect of constitutive activation of T3SS-2 in the murine typhoid model (Coombes *et al.*, 2005b). However, our knowledge of the role of sensory systems in *Salmonella* pathogenesis derives almost exclusively from surrogate rodent- or cell-based assays. It is becoming clear that both cell- and host-specific virulence factors are deployed by *Salmonella*. For example, *Salmonella* mutants with defects in the *PhoPQ* TCS exhibit reduced replication in immortalized epithelial and macrophage-like lines, yet overgrow in fibroblasts (Cano *et al.*, 2001) and a subpopulation of CD18-negative lamina propria cells in the murine intestine (Tierrez & Garcia-del Portillo, 2005). A requirement therefore exists to probe the role of sensory systems in the enteric and systemic phases of *Salmonella* infection in target hosts.

The aim of this study was to assign spatial roles to the sensory systems of *S. Dublin* during infection of its natural bovine host. Our approach was to analyse the draft *S. Dublin* genome sequence to predict TCSs, then to construct mutants with defects in each of the predicted SKs by lambda Red recombinase-mediated integration of PCR amplicons harbouring unique oligonucleotide tags. Hybridization of labelled tags amplified from inocula or bacteria recovered from animals can be taken as a measure of the abundance of the mutants (Hensel *et al.*, 1995). A novel lymphatic cannulation model was then used to follow the translocation of tagged mutant and control strains from the distal ileal lumen to draining MLNs and associated efferent lymph vessels to determine whether SKs play niche-specific roles in the pathogenesis of systemic salmonellosis in cattle. We also bypassed the intestinal barrier by intravenous administration of pooled mutants. These approaches have previously revealed that systemic translocation of *S. Dublin* in calves requires T3SS-1 (Pullinger *et al.*, 2007) and a serovar Dublin-specific island (Pullinger *et al.*, 2008), but not other virulence-associated or serovar-specific loci.

METHODS

Bacterial strains and culture conditions. *S. Dublin* 3246 (SD3246) and a nalidixic acid-resistant derivative that is fully virulent in calves (SD3246 Nal^R) have been previously described (Paulin *et al.*, 2002). The strains used in this study were cultured in Luria-Bertani (LB) medium at 37 °C with shaking at 130 r.p.m. Antibiotics were added where appropriate at the following concentrations: kanamycin (Kan), 50 µg ml⁻¹; nalidixic acid (Nal), 20 µg ml⁻¹; ampicillin (Amp), 100 µg ml⁻¹. Plasmid pKD46 (Datsenko & Wanner, 2000) was obtained from the *E. coli* Genetic Stock Center, Yale University.

Identification and *in silico* analysis of putative SK genes in *S. Dublin*.

When this study was initiated, the only available *S. Dublin* genome sequence was not closed or annotated. We therefore searched an *S. Typhimurium* genome sequence for SK genes, and then determined whether the *S. Dublin* strain CT02021853 draft sequence contained orthologous genes. The *S. Typhimurium* LT2 sequence was interrogated using the J. Craig Venter Institute (JCVI) Comprehensive Microbial Resource (<http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi>). Keyword searches using the strings 'sensor kinase' and 'two component system' identified 27 TCS SK genes. These had been assigned to one of two COGs (Clusters of Orthologous Genes), either COG0642 or COG3290. The LT2 sequence was then interrogated for other genes assigned to these two COGs. BLASTN searches were carried out using the predicted SK gene sequences to determine whether similar sequences were present in the draft *S. Dublin* CT02021853 genome sequence at *coliBASE* (<http://xbase.bham.ac.uk/colibase/>) and under GenBank accession number CP001144.

Construction and validation of signature-tagged *S. Dublin* mutants.

Thirty-one uniquely tagged mini-Tn5Km2 mutants of SD3246 Nal^R with insertions in putative SK genes were created by lambda Red recombinase-mediated integration of linear PCR products essentially as described by Pullinger *et al.* (2007). The linear DNA fragments were prepared by amplification of mini-Tn5Km2 transposons with compatible signature tags by PCR using primers which incorporate 40 nt gene-specific homology extensions (Table 1). Amplicons were designed to replace an internal portion of the gene with the tagged transposon. Products were *DpnI*-digested, purified using spin columns, electroporated into SD3246 Nal^R harbouring plasmid pKD46, and induced to express the γ , β and *exo* genes with 0.2% (w/v) L-arabinose at 30 °C. Transformants with resistance to Nal and Kan were selected at 37 °C and confirmed to have lost pKD46 by analysis of sensitivity to Amp. Insertion sites were confirmed by PCR using the gene- and transposon-specific primers listed in Table 1. All mutations were transduced into the archived SD3246 Nal^R strain using bacteriophage P22/int to reduce the likelihood that phenotypes were a consequence of second-site mutations. Growth kinetics of SD3246 Nal^R wild-type and SK mutant strains were analysed following inoculation of LB medium with $\sim 10^3$ c.f.u. from overnight cultures by measuring OD₆₀₀ every 30 min for 18 h using a Bioscreen C real-time spectrophotometer (Thermo).

Validated SK mutants were stored at -80 °C in 15% (v/v) glycerol in a 96-well microtitre plate. In addition, 12 previously characterized *S. enterica* mutants were added to the mutant bank as controls. These included three calf-virulent tagged SD3246 Nal^R strains (27E4, 27E2 and 27F11) as positive controls, three chicken-virulent but calf-avirulent tagged *S. enterica* serovar Gallinarum (*S. Gallinarum*) SG9 strains (12D10, 12B6 and 14C3) as negative controls, three SD3246 Nal^R T3SS-1 mutants (*spaR*, *sipC* and *sipA*) and three SD3246 Nal^R T3SS-2 mutants (*ssaJ*, *sseC* and *sseD*) (Pullinger *et al.*, 2007).

Experimental animals. Animal experiments were conducted according to the requirements of the Animals (Scientific Procedures) Act 1986 (license 30/2485) with the approval of the local Ethical Review Committee. Friesian bull calves were fed twice daily on a diet of reconstituted dried milk and unmedicated weaner nuts, with access to water *ad libitum*. Calves aged 25–30 days were used for the experiments and were confirmed to be culture-negative for *Salmonella*, as described by Paulin *et al.* (2002).

Screening of signature-tagged mutants following oral or intravenous inoculation of calves. The 43 tagged *S. enterica* mutants were cultured statically in LB medium with Nal and Kan overnight at 37 °C. The mutants were pooled, and an aliquot removed for preparation of 'input pool' genomic DNA, as described by Hensel *et al.* (1995). Two calves that had been starved overnight

were orally inoculated with the pool (1.6×10^9 c.f.u. per calf) in reconstituted milk, and the calves were then fed as normal. Three days post-inoculation, a laparotomy was performed under terminal general anaesthesia and the distal ileal loop exteriorised. Efferent lymph vessels were cannulated as described by Pullinger *et al.* (2007), and lymph was collected for 2 h. Lymph, blood, bile and tissue collected at post mortem examination (ileal mucosa, ileal MLNs, liver and spleen) were homogenized in 0.9% (w/v) saline where necessary, and serial 10-fold dilutions were plated onto MacConkey agar containing Nal and Kan. Three calves were separately injected intravenously with the pool [5.5×10^6 c.f.u. per calf in 0.9% (w/v) saline] and humanely killed 3 days post-inoculation. Samples were collected, homogenized and plated as for orally inoculated animals.

Where recoveries in excess of 3000 colonies were obtained from samples collected at post mortem examination, colonies were pooled for preparation of 'output pool' genomic DNA. PCR amplification of radiolabelled tags from input and output pools and dot blot hybridizations were performed as described by Morgan *et al.* (2004). For each sample from each calf, duplicate dot blot hybridizations were performed. The intensity (volume) of the signal of each spot was quantified using a Storm PhosphorImager (GE Healthcare) and expressed as the mean output:input ratio.

Screening of mutants in INT407 cells. The relative ability of the 43 mutants to invade and replicate in INT407 cells, a human intestinal epithelial cell line, was simultaneously assayed. INT407 cells were seeded at 5×10^6 cells per flask in Eagle's minimal essential medium (EMEM) supplemented with 10% (v/v) fetal calf serum (complete EMEM) in T25 tissue culture flasks. The cells were incubated at 37 °C in a humidified 5% CO₂ atmosphere until confluent. The monolayers were washed once with PBS and 10 ml volumes of complete EMEM were added to each flask 30 min prior to addition of bacteria. The 43 mutants were grown individually overnight in LB broth at 25 °C and 130 r.p.m., then subcultured 1:10 into fresh LB broth without antibiotics, and incubated at 37 °C and 130 r.p.m. for 2 h. An equal volume of all the cultures was pooled, and an aliquot was retained for preparation of input DNA. The pooled bacteria were diluted to approximately 1×10^6 c.f.u. ml⁻¹ in complete EMEM, and 10 ml of this was added to each of six flasks. After incubation for 90 min, the monolayers were washed three times with PBS and incubated for a further 2 h in complete EMEM containing 150 µg gentamicin ml⁻¹. The monolayers were then washed once with PBS and the medium replaced with fresh complete EMEM containing 10 µg gentamicin ml⁻¹. At 24, 48 and 72 h post-inoculation, two of the flasks were removed from the incubator, and the cells were washed three times with PBS then lysed by addition of 0.5 ml per well of PBS containing 1% (v/v) Triton X-100. After 5 min at room temperature, 4.5 ml PBS was added, and the samples were transferred to suitable tubes. These samples were diluted in PBS to 10⁻¹, 10⁻² and 10⁻³, and 300 µl volumes were spread onto 10 LB agar plates containing Kan and Nal, and incubated overnight at 37 °C. For each flask, ~ 3000 – $10\,000$ colonies were pooled for preparation of output pool genomic DNA, and their relative proportions determined as for the calf experiments described above.

Determination of competitive indices (CIs) in calves. Bacteria were grown in LB broth overnight at 37 °C. Wild-type and $\Delta baeS::$ mini-Tn5Km2 mutant strains were mixed in equal numbers ($\sim 1.6 \times 10^9$ c.f.u. per calf) in 50 ml reconstituted milk, and used to inoculate six 4-week-old calves orally. Faecal samples were collected daily. Wild-type and mutant bacteria were enumerated in faeces and tissue samples were collected at post mortem examination 3 days post-infection and CIs calculated as described by Pullinger *et al.* (2007). CIs were also determined 3 days following intravenous injection of a 1:1 ratio of wild-type and $\Delta baeS::$ mini-Tn5Km2 mutant strains in 2 ml sterile 0.9% saline into three 4-week-old calves ($\sim 5 \times 10^6$ c.f.u. per calf).

Table 1. Primers used to construct and confirm SK mutants in SD3246 NaI^R

P1 and P2 primers were used for amplification of tagged transposons with gene-specific homology extensions. P3 primers were used as gene flanking primers in combination with the transposon-specific primers P6 (5'-cctaggcgccagatctg-3') and/or P10 (5'-tcctctagatgcacctgc-3') to check the mutants.

SK	Locus tag	Primers and sequences (5'-3')
<i>arcB</i>	SeD_A3688	arcB-P1: gataagatggagcggcgtaaggttcagcttgataaccaacctgtctcttgatcagatctgg arcB-P2: cacgttcagttcaatatcttcgaccagcagaacatgcagcctgactcttatacacaagtgcg arcB-P3: cctacgaacagtggctgg
<i>baeS</i>	SeD_A2476	baeS-P1: cttgccacctgcacgtgctgctgatcagatgacattggctgtctcttgatcagatctgg baeS-P2: gcggtgctggcaagctgggttaagtcctgcgccaatttccctgactcttatacacaagtgcg baeS-P3: gcaacgcgtaatatgtagcg
<i>barA</i>	SeD_A3278	barA-P1: gcctatccgtaatatggtgaataaccgttgaccgcattgcctgtctcttgatcagatctgg barA-P2: ttaacgtcaactccagccctttatcatgcgacgagtgagcctgactcttatacacaagtgcg barA-P3: tcacgcgaatatggtatg
<i>copS</i>	SeD_A1170	copS-P1: cgcgctgagcaaaagaattaacgtatcgggatgatatgacgctgtctcttgatcagatctgg copS-P2: ccagctcttcaatattatcgacaaggcttggatgactcctgactcttatacacaagtgcg copS-P3: agcgacccgaatttctgc
<i>cpxA</i>	SeD_A4456	cpxA-P1: cagcgggtattactggtgacctctgaaggacgcgtgatcgtgtctcttgatcagatctgg cpxA-P2: ggtcattgatcatgctgtccagacgctgcgttcggtttctgactcttatacacaagtgcg cpxA-P3: atcttcgcatcttctgg
<i>creC</i>	SeD_A5001	creC-P1: aggattattctcgtggaatgacgtctggttacgctgcgtgtctcttgatcagatctgg creC-P2: ccatcggctgagcgcttaatgtgataacgccatttccagctgactcttatacacaagtgcg creC-P3: gaatgcgtttgctgctgg
<i>dcuS</i>	SeD_A4699	dcuS-P1: gcgctgtttgttttgcgtgctgttggtccatctgatcctgtctcttgatcagatctgg dcuS-P1: cttcgtaatgtccaccacgtgcgaccagcatgactcagctgactcttatacacaagtgcg dcuS-P3: agcaaaccgatctcttcc
<i>dpiB</i>	SeD_A0726	dpiB-P1: ggacggattttgactatgtggtgataggcgatcgccactcctgtctcttgatcagatctgg dpiB-P2: gcggtgcgaggctctcaacatactgtttaatctgggttagctgactcttatacacaagtgcg dpiB-P3: tcttcagcgtaatgacc
<i>envZ</i>	SeD_A3870	envZ-P1: agcctccagcagtttaataaggttctggtctacgaagtcctgtctcttgatcagatctgg envZ-P2: accgtagcagatagtagtaataaactgttcgataatggcgctgactcttatacacaagtgcg envZ-P3: tgccaatctccagcatcc
<i>glnL</i>	SeD_A4395	glnL-P1: ctacgcaaaactgttcgggacgccgttacctgaactgttctgtctcttgatcagatctgg glnL-P2: aggttgctgtagcctgtgcgctgttcaatgatgactttggctgactcttatacacaagtgcg glnL-P3: gcagcggttaactggaagg
<i>hydH (zraS)</i>	SeD_A4580	hydH-P1: ttccagccgcttttgcgtatgagaggtcacggatgagggctgtctcttgatcagatctgg hydH-P2: cagttcgagcaattcgttaccaccgggttcaaacggctgctgactcttatacacaagtgcg hydH-P3: gagtcgactgttacctgc
<i>kdpD</i>	SeD_A0811	kdpD-P1: gatctcgatcaggtgatcgtgcgctggaagagcccgagctgtctcttgatcagatctgg kdpD-P2: agccagaccgtacgttttatcgcgcttttcagcggcaggctgactcttatacacaagtgcg kdpD-P3: atacgcgcatatccagc
<i>narQ</i>	SeD_A2846	narQ-P1: taccctacgacgtattcgccagcaggtcgttcgccgctgctgtctcttgatcagatctgg narQ-P2: agtggagttcgccatagaccgtgtcctgcatcgtcactggctgactcttatacacaagtgcg narQ-P3: acgaatgcagagctatcg
<i>narX</i>	SeD_A1557	narX-P1: gtcagcgcgactttaccaacgtgccaatatcagtgagcgtgtctcttgatcagatctgg narX-P2: catacaagagagcagctgggcaatagaatcatgcagttcgtgactcttatacacaagtgcg narX-P3: tgctctctaccgctatcg
<i>pgtB</i>	SeD_A2755	pgtB-P1: ctttcgacgctgcttgaggcgagtaggtagtagtacctgtctcttgatcagatctgg pgtB-P2: cggctggttaatttcatgcgacgctgggtcatggtctggctgactcttatacacaagtgcg pgtB-P3: aacacgacggtacgtttg
<i>phoQ</i>	SeD_A2136	phoQ-P1: taaaaccacctttctgttctgcgcggcgaaagcaacctgctgtctcttgatcagatctgg phoQ-P2: cgtctccgattgagcatttccggtgatgatcttcaagcctgactcttatacacaagtgcg phoQ-P3: aatgatcgtcggtctggc
<i>phoR</i>	SeD_A0436	phoR-P1: ggggctacgtcggcggatgacaacgggcagaatattctcctgtctcttgatcagatctgg phoR-P2: cttcagctccacgaccgcagcatcatcggtacatccactgactcttatacacaagtgcg phoR-P3: ctggaacggctgtcatgg

Table 1. cont.

SK	Locus tag	Primers and sequences (5'–3')
<i>pmrB</i> (<i>basS</i>)	SeD_A4686	pmrB-P1: cgtattaccgctccgctcgccgaactgcaaaaagagctggctgtctcttgatcagatctgg pmrB-P2: acagagttgtctggcgctttccagcatggtgtcagctcctgactcttatacacaagtgcg pmrB-P3: atgtagttgggtgatgcg
<i>rscC</i>	SeD_A2615	rscC-P1: tctattctgtgctgtagatttagtctggagcgtattcgctgtctcttgatcagatctgg rscC-P2: tgaagttacgctgtacgccggtccacctggaagaaggctgactcttatacacaagtgcg rscC-P3: gatagccgtccatgttcg
<i>rstB</i>	SeD_A1872	rstB-P1: ttgctctatctgatcgcgacgcaactgcggaaattccgctgtctcttgatcagatctgg rstB-P2: gaacgcgacgccagacgttcgaaactggagccattatcgctgactcttatacacaagtgcg rstB-P3: agagtgtacgatggcgag
<i>ssrA</i>	SeD_A1949	ssrA-P1: gctttcctttataccaacccatgccgggtttactggagctgtctcttgatcagatctgg ssrA-P2: atactatctaattcatccagtcgtgtgtgtaaacgtgctgactcttatacacaagtgcg ssrA-P3: tccgttctatccgagatg
<i>tctE</i>	SeD_A3083	tctE-P1: tcgactctattacaaagtcgacccctccgaaaggctgtctcttgatcagatctgg tctE-P2: gtaataaccagcaaccagcaccagggtcagcaaccagctgactcttatacacaagtgcg tctE-P3: cgctcatcgctgcagac
<i>torS</i>	SeD_A4216	torS-P1: taagcctcgcgctcattagtcaggtggagcaaccaatacctgtctcttgatcagatctgg torS-P2: aagacgcgctaaccagcctgtttcggttttcgatggcgctgactcttatacacaagtgcg torS-P3: gaacgtcgatttcgctc
<i>ttrS</i>	SeD_A1956	ttrS-P1: cgcgattgatgctcagcggtttggcggtatttattaggcgtgtctcttgatcagatctgg ttrS-P2: ggaaacggcgtgattaagctcatgggcaaacccggaggctgactcttatacacaagtgcg ttrS-P3: agggacgtggaatatcgg
<i>uhpB</i>	SeD_A4178	uhpB-P1: ccactgcctgggtttctggcactatctaccagcactacctgtctcttgatcagatctgg uhpB-P2: tttgcaacgactggttcagctcgcgacagcgtgaatgcctgactcttatacacaagtgcg uhpB-P3: tgacagacgcgaacacgc
<i>qseE</i>	SeD_A2940	qseE-P1: gtgtatcaaagccagcgtaagcgtatagcgatatgtggctgtctcttgatcagatctgg qseE-P2: ctctgtggcactgtctaccagtttccggttgtaatacgaagcctgactcttatacacaagtgcg qseE-P3: cctgcattcgacgaatac
<i>qseC</i>	SeD_A3529	qseC-P1: ttgcacaatggctatctgaaggatgacaacgacctgtggctgtctcttgatcagatctgg qseC-P2: caccatcatggagtgatgcggctaaaagctgattaagcctgactcttatacacaagtgcg qseC-P3: acaggcttaagagtagcg
<i>yojN</i>	SeD_A2613	yojN-P1: taccgttgaaagatctctcttcggctttaaagagtcacacctgtctcttgatcagatctgg yojN-P2: gtaatatttgcaggttaagatgcggcaacagatgatcggtgactcttatacacaagtgcg yojN-P3: tgctgctggtaacgatg
<i>citA</i>	SeD_A0058	A0058-P1: cgctgtataacgctaaaagctacgtatcggtgcgcaaaaggctgtctcttgatcagatctgg A0058-P2: atcgtcttgttgcaaaagctaataaccagccctgaggcctgactcttatacacaagtgcg A0058-P3: tggttgccagacactcg
<i>cusS</i>	SeD_A0662	cusS-P1: tttagcgtggcggagggatcaacgcgataaaaacgatcgctgtctcttgatcagatctgg cusS-P2: taaaaccattactcttcggataaaaagcggacgaccagctgactcttatacacaagtgcg cusS-P3: tgctggctagtatacg
<i>cheA</i>	SeD_A1323	cheA-P1: cgagagtaaaactgcgcatgtactttcaggtgaaagccctgtctcttgatcagatctgg cheA-P2: aaggcgttttccggcatttcaatccgtggtcgagactgctgactcttatacacaagtgcg cheA-P3: gatgaagctgacgagctg

Statistical analysis. The dot blot results were analysed by comparing output to input signal intensities using paired Student's *t* tests. For the CI experiments, the Mann–Whitney non-parametric test was used to determine whether the output ratios differed significantly from the input ratios. *P* values of ≤ 0.05 were considered significant.

RESULTS AND DISCUSSION

Identification and *in silico* analysis of putative SK genes in *S. Dublin*

Thirty putative SK genes were identified in the *S. Typhimurium* LT2 genome. This is similar to the number in

E. coli (Mizuno, 1997; Zhou *et al.*, 2003). Based on BLASTN analysis, all 30 of these genes had close homologues in the draft *S. Dublin* CT02021853 genome sequence. These SKs and their cognate RRs are listed with their known or predicted functions in Table 2. A further histidine kinase involved in chemotaxis and encoded by *cheA* was also conserved. Two of the predicted SKs, RcsC and YojN (also called RcsD), are components of the same atypical regulatory system. RcsC is the membrane SK, RcsB is the RR, and YojN is an additional histidine-containing phosphotransfer protein that may be used as an intermediary in the phosphotransfer (Takeda *et al.*, 2001). Similarly, the TorSR system has a third component, TorT, thought to be a periplasmic intermediate (Baraquet *et al.*, 2006).

Table 2. Two-component sensory systems of *S. enterica* and their predicted functions

SK	RR	Predicted function	References
ArcB	ArcA	Resistance to reactive O ₂ /N, redox sensor	Lu <i>et al.</i> (2002)
BaeS	BaeR	Envelope stress, increases multidrug resistance	Nishino <i>et al.</i> (2007)
BarA	SirA	Control of T3SS-1, motility, biofilm formation, carbon storage	Mondragón <i>et al.</i> (2006); Teplitski <i>et al.</i> (2003)
CopS	CopR	Unclear (possibly copper/silver efflux)	Espariz <i>et al.</i> (2007)
CpxA	CpxR	Envelope stress, protein degradation	Humphreys <i>et al.</i> (2004)
CreC	CreB	Carbon source-responsive	Cariss <i>et al.</i> (2008)
DcuS	DcuR	Fumarate respiration (anaerobic)	Janausch <i>et al.</i> (2002)
DpiB	DpiA	Unclear (possibly citrate utilization)	
EnvZ	OmpR	Cell envelope stress, osmolarity sensor, affects membrane permeability, nutrient availability and flagellar expression	Bang <i>et al.</i> (2000); Higashitani <i>et al.</i> (1993); Shin & Park (1995)
GlnL	GlnG	Nitrogen assimilation	Zimmer <i>et al.</i> (2000)
HydH	HydG	Zinc/lead efflux	Leonhartsberger <i>et al.</i> (2001)
KdpD	KdpE	Potassium transport, osmolarity sensor	Polarek <i>et al.</i> (1992)
NarQ	NarP	Nitrate- and nitrite-responsive, anaerobic respiration	Stewart (2003)
NarX	NarL	Nitrate- and nitrite-responsive, anaerobic respiration	Stewart (2003)
PgtB	PgtA	Phosphoglycerate transport	Niu <i>et al.</i> (1995)
PhoQ	PhoP	Mg ²⁺ uptake, peptide resistance, macrophage survival	Groisman & Mouslim (2006)
PhoR	PhoB	Inorganic phosphate uptake/production	Conlin <i>et al.</i> (2001); Wanner & Latterell (1980)
PmrB	PmrA	LPS modification, peptide resistance	Gunn (2008)
RcsC	RcsB	Capsule synthesis in response to peptidoglycan stress	Laubacher & Ades (2008)
YojN		Capsule synthesis in response to peptidoglycan stress	Laubacher & Ades (2008)
RstB	RstA	Iron transport	Jeon <i>et al.</i> (2008)
SsrA	SsrB	Control of T3SS-2 and effectors	Cirillo <i>et al.</i> (1998); Geddes <i>et al.</i> (2007); Hensel <i>et al.</i> (1998); Shea <i>et al.</i> (1996)
TctE	TctD	Tricarboxylate transport	Winnen <i>et al.</i> (2003)
TorS	TorR	Trimethylamine N-oxide respiration	Baraquet <i>et al.</i> (2006)
TtrS	TtrR	Tetrathionate respiration	Hensel <i>et al.</i> (1999)
UhpB	UhpA	Glucose 6-phosphate transport	Island <i>et al.</i> (1992)
QseE	QseF	Epinephrine, phosphate and sulphate sensor	Reading <i>et al.</i> (2009)
QseC	QseB	Quorum-sensing and host stress hormone sensing	Rasko <i>et al.</i> (2008)
CitA	CitB	Citrate utilization	Yamamoto <i>et al.</i> (2009)
CusS	CusR	Copper sensing	Pontel & Soncini (2009)
CheA	CheY	Chemotaxis	Jones <i>et al.</i> (1992)

Analysis of the draft *S. Dublin* CT02021853 sequence indicated that two predicted SK genes may be pseudogenes based on frameshift mutations when compared with *S. Typhimurium* LT2. These are *dpiB* (SeD_A0726) and *qseE* (SeD_A2940). Such gene decay may be significant in the evolution of host-restricted serovars relative to ubiquitous counterparts (McClelland *et al.*, 2004). We recently determined the draft genome sequence of the *S. Dublin* strain used in this study (SD3246) using the Illumina platform (data not shown). Analysis of these two loci showed that *dpiB* contains the same frameshift mutation in SD3246; however, the frameshift mutation in *qseE* was not present. In addition, the CusS SK is predicted to be very short in both strains of *S. Dublin* and in *S. Typhimurium* LT2, and is likely to be inactive. In *E. coli*, CusSR regulates a copper efflux system that is absent in *Salmonella* (Pontel & Soncini, 2009). All the other SK genes were found to be complete in the draft SD3246 sequence (data not shown).

Targeted sequencing will be required to establish that the polymorphisms detected in draft sequences are genuine.

Construction and characterization of SK mutants

All 31 SK genes of SD3246 NaI^R were disrupted by replacement of internal regions with uniquely tagged mini-Tn5Km2 transposons as described in Methods. We have previously reported an absence of polar effects of such insertions in the *Salmonella* *siiA*–*F* operon (Morgan *et al.*, 2007); however, we acknowledge that polar effects at the level of transcription or translational coupling of SK and RR genes cannot be excluded. In the first instance we sought to evaluate the role of every SK with minimal use of the target host and further studies will be needed to unravel the basis of signal transduction.

The growth kinetics of 30 SK mutants in LB medium were indistinguishable from those of the wild-type (data not

shown). However, the SD3246 *Nal^R arcB* mutant had a longer doubling time (~2.3 h compared with 1.3 h for the wild-type under the assay conditions), consistent with the phenotype of *arcAB* mutants of *E. coli* K-12 (Oshima *et al.*, 2002). The fate of the 31 SK mutants and 12 control strains was then analysed following oral or intravenous inoculation of calves to evaluate the importance of the SKs during enteric and systemic phases of disease.

Phenotype of *S. Dublin* SK mutants following oral inoculation of calves

Three days after oral inoculation of calves, the composition of inocula and output pools recovered from ileal mucosa, associated MLNs, efferent lymph, liver and spleen was analysed as described in Methods. Recoveries from blood and bile were too low (<3000 colonies) to infer that mutants were absent owing to attenuation as opposed to by chance (Barrow, 1983). Where recoveries in excess of 3000 colonies were obtained, reduced signal intensity for a given tag in the output relative to the input indicates that the mutant from which it derives is negatively selected *in vivo* (Hensel *et al.*, 1995). Representative dot blots of tags

amplified from the output pools of one calf relative to the inoculum are shown in Fig. 1. Blots were highly reproducible, with minimal inter-animal variation and no evidence of random loss of mutants indicative of bottleneck effects. The intensity (volume) of each of the spots was quantified and the mean output volume:input volume calculated from duplicate dot blots from two calves per site. This ratio provides a measure of the extent of positive or negative selection of mutants relative to the inoculum. Mean output:input ratios for all mutants at all sites are recorded in Table 3. Paired Student's *t* tests were used to determine whether mutants were significantly under- or over-represented in the output pools relative to the inoculum. Significant ratios below 1 (denoting negative selection at $P \leq 0.05$) are shaded dark grey in Table 3, whereas significant ratios above 1 (denoting positive selection relative to the inoculum at $P \leq 0.05$) are shaded light grey.

Tagged *S. Gallinarum* SG9 strains 12D10 and 12B6 (positions A1 and A2) were recovered from all sites in significantly lower numbers than the input pools (Table 3), consistent with the attenuation of *S. Gallinarum* SG9

Fig. 1. Representative dot blot hybridizations showing the fate of a pool of tagged *S. enterica* wild-type and SK mutant strains following oral inoculation of a calf. Positions A1–A3, chicken-virulent *S. Gallinarum* (SG9) mutants; positions A4–A6, calf-virulent *S. Dublin* (SD3246 *Nal^R*) mutants; positions A7–A9, T3SS-1 mutants of SD3246 *Nal^R*; positions A10–A12, T3SS-2 mutants of SD3246 *Nal^R*; positions B1–D7, SK mutants as indicated in the table below the dot blots. The locations of hybridization signals for the five SK mutants that were significantly negatively selected are indicated. Output:input ratios of the signal intensities from duplicate blots of this kind from two calves inoculated orally and three calves inoculated intravenously are recorded in Table 3.

Table 3. Mean output:input ratios of mutants screened in calves by oral and intravenous inoculation, and in INT407 cells

Values represent the mean output:input ratios for each site (calf screens) or time interval (INT407 cells). For each site sampled in calves dosed by the intravenous route, duplicate blots were analysed from three calves ($n=6$). Duplicate blots were analysed from two calves dosed orally ($n=4$), and duplicate blots were analysed from two flasks of INT407 cells at each time point ($n=4$). The output to input volumes were compared using paired t tests, and P values ≤ 0.05 were considered significant. Dark-grey shading indicates mutants that were significantly under-represented in the output pool relative to the input. Light-grey shading indicates mutants that were significantly over-represented in the output pool relative to the input.

Mutant	Intravenous		Oral					INT407		
	Liver	Spleen	Ileal mucosa	MLN	Lymph	Liver	Spleen	24 h	48 h	72 h
12D10	0.40	0.18	0.10	0.10	0.09	0.08	0.07	0.11	0.20	0.14
12B6	0.16	0.10	0.16	0.15	0.14	0.12	0.13	0.09	0.15	0.12
14C3	4.73	0.75	0.99	0.86	1.01	0.76	0.87	0.79	1.12	0.60
27E4	1.35	1.65	1.23	0.97	0.86	0.81	0.69	0.96	1.16	1.05
27E2	1.14	1.54	1.40	0.91	1.05	0.84	0.87	0.31	0.63	0.52
27F11	1.18	1.23	1.34	1.12	0.67	0.62	1.01	0.42	0.84	0.41
<i>spaR</i>	1.01	1.18	0.49	0.54	0.37	0.41	0.50	0.12	0.15	0.22
<i>sipC</i>	0.51	0.76	0.07	0.08	0.05	0.07	0.08	0.05	0.05	0.72
<i>sipA</i>	0.86	0.72	0.12	0.14	0.11	0.13	0.16	0.23	0.28	0.20
<i>ssaJ</i>	0.63	0.60	0.60	0.63	0.50	0.71	0.68	0.16	0.25	0.36
<i>sseC</i>	0.67	1.04	0.81	1.00	0.70	0.70	0.82	0.48	1.44	1.38
<i>sseD</i>	0.44	1.90	0.15	0.24	0.10	0.10	0.13	0.37	1.19	4.65
<i>arcB</i>	1.10	0.73	1.30	1.06	0.77	0.71	0.69	0.24	0.39	0.32
<i>baeS</i>	0.86	0.97	0.72	0.59	0.55	0.31	0.62	0.31	0.34	0.29
<i>barA</i>	0.54	0.44	0.18	0.15	0.11	0.13	0.12	0.04	0.11	0.05
<i>copS</i>	0.89	0.83	1.73	1.48	1.45	1.09	1.66	0.34	0.40	0.32
<i>cpxA</i>	0.96	0.78	2.18	1.48	1.02	1.20	0.93	0.26	0.34	0.36
<i>creC</i>	1.16	1.00	1.40	0.92	0.80	1.14	0.85	1.17	1.28	1.69
<i>dcuS</i>	1.41	2.11	1.67	1.46	1.39	0.97	1.38	2.66	1.02	0.85
<i>dpiB</i>	1.46	1.74	0.77	0.66	0.53	0.56	0.88	0.60	0.38	0.34
<i>envZ</i>	0.66	0.75	0.40	0.51	0.35	0.43	0.54	0.30	0.27	0.50
<i>glnL</i>	1.28	1.37	1.29	1.72	2.48	3.85	3.74	0.72	0.51	0.47
<i>hydH</i>	1.23	1.40	2.20	2.06	1.85	2.11	2.24	0.78	0.44	0.43
<i>kdpD</i>	1.15	1.30	1.00	0.89	0.80	0.95	1.02	1.09	0.83	1.12
<i>narQ</i>	1.02	0.83	1.88	1.46	0.83	1.06	0.83	0.57	0.80	0.78
<i>narX</i>	1.38	1.68	2.99	2.72	2.53	1.31	1.91	0.84	0.94	0.86
<i>pgtB</i>	0.74	1.46	1.84	1.56	2.26	1.38	1.53	0.57	0.89	0.59
<i>phoQ</i>	0.28	0.30	0.27	0.24	0.22	0.21	0.23	1.68	2.35	5.92
<i>phoR</i>	1.20	1.67	2.68	2.49	2.56	2.06	1.83	0.69	1.46	1.52
<i>pmrB</i>	1.22	1.69	3.70	2.29	2.38	1.25	2.00	2.58	2.50	3.03
<i>rscC</i>	0.95	1.14	1.89	1.64	1.26	0.97	1.36	0.89	0.80	0.85
<i>rstB</i>	1.13	1.21	1.29	1.31	0.91	1.13	1.32	0.38	0.28	0.57
<i>ssrA</i>	0.77	1.13	0.16	0.41	0.17	0.19	0.23	0.59	1.28	5.51
<i>tctE</i>	1.16	0.78	1.20	1.29	1.13	1.18	1.54	0.87	1.00	1.94
<i>torS</i>	1.23	0.66	1.13	1.03	0.72	0.64	0.73	0.44	0.45	0.45
<i>ttrS</i>	1.20	1.49	3.22	2.40	2.23	1.08	1.83	2.88	1.83	2.46
<i>uhpB</i>	1.42	1.35	1.56	1.42	1.14	0.95	1.01	1.12	1.80	1.79
<i>qseE</i>	1.58	1.67	1.66	1.93	1.82	2.29	1.47	0.54	0.49	0.60
<i>qseC</i>	0.53	0.75	0.74	0.70	0.44	0.59	0.50	2.75	2.93	2.48
<i>yojN</i>	0.84	1.10	2.36	2.15	2.18	2.17	2.57	3.65	3.20	2.49
<i>citA</i>	0.92	0.88	1.13	0.86	0.66	0.65	0.55	2.15	1.12	2.07
<i>cusS</i>	0.75	0.69	1.85	1.27	1.26	0.94	1.41	0.81	1.02	1.54
<i>cheA</i>	1.18	1.17	1.44	1.15	0.94	0.73	0.95	7.14	7.56	13.48

relative to *S. Dublin* SD3246 after inoculation of calves by this route (Paulin *et al.*, 2002), and previous data obtained with signature-tagged mutants (Pullinger *et al.*, 2007). All of the tagged virulent *S. Dublin* controls (positions A4–A6) colonized enteric and systemic sites after oral inoculation of calves, as reported previously (Pullinger *et al.*, 2007), but

none was more than twofold under- or over-represented. The *S. Dublin* T3SS-1 mutants (*spaR*, *sipC* and *sipA*; positions A7–A9) were significantly under-represented at all sites following inoculation by the oral route (Table 3), consistent with the well-established role of T3SS-1 in invasion of the intestinal mucosa (Lostroh & Lee, 2001)

and with our previous data in this model (Pullinger *et al.*, 2007). The T3SS-2 mutants (*ssaJ*, *sseC* and *sseD*; positions A10–A12) in general were under-represented from many sites, particularly the secretion apparatus *ssaJ* mutant, though the attenuation was less marked than that of T3SS-1 mutants. As anticipated, all three T3SS-2 mutants were negatively selected in the liver and spleen relative to the input, consistent with the known role of T3SS-2 in the pathogenesis of *S. Dublin* infection in calves (Bispham *et al.*, 2001; Pullinger *et al.*, 2007). These internal controls lend confidence that negative selection of SK mutants is a measure of attenuation.

Statistical analysis indicated that *barA*, *phoQ*, *qseC*, *envZ* and *ssrA* mutants were significantly under-represented at all enteric and systemic sites sampled after oral dosing. Mutants lacking *baeS* or *dpiB* were also under-represented at all sites sampled, though not significantly so in the ileal mucosa (*baeS* and *dpiB*) or spleen (*dpiB*). The *citA* mutant was significantly under-represented in lymph, liver and spleen samples, but not those of ileal mucosa or MLNs.

The negative selection of the *barA* mutant at all sites after oral dosing of calves is consistent with the role of the BarA (SirA) TCS in control of T3SS-1, motility, biofilm formation and carbon storage (Mondragón *et al.*, 2006; Teplitski *et al.*, 2003). T3SS-1 is vital for enteric and systemic virulence of SD3246 in this model (Pullinger *et al.*, 2007), and studies using the murine typhoid model indicate a role for *barA* in systemic virulence of *S. Typhimurium* (Chaudhuri *et al.*, 2009; Santiviago *et al.*, 2009; Yoon *et al.*, 2009), though intestinal colonization was not analysed in these studies. The finding that an *S. Dublin phoQ* mutant is negatively selected at all sites after oral dosing of calves is similarly consistent with the known role of PhoPQ in control of type III secretion (Lostroh & Lee, 2001). PhoPQ responds to pH and divalent cation limitation and also influences Mg^{2+} uptake, cationic peptide resistance, resistance to oxidative stress and plasmid virulence gene expression (Groisman & Mouslim, 2006). PhoPQ has been identified as an important virulence factor for *S. Typhimurium* in mice (Chaudhuri *et al.*, 2009; Santiviago *et al.*, 2009), including after intragastric dosing (Yoon *et al.*, 2009).

The magnitude of the attenuation of the SD3246 NaI^R *qseC* mutant was less than that observed for *barA* or *phoQ* mutants but is consistent with data from this laboratory in which an *S. Typhimurium qseC* mutant exhibited modest attenuation when tested by co-infection of calves with the parent strain (Pullinger *et al.*, 2010). In an independent co-infection study, deletion of *qseC* was associated with a significant reduction in intestinal colonization of pigs (Bearson & Bearson, 2008). The function of the QseBC TCS in *Salmonella* is unclear. QseBC regulates genes that affect antimicrobial peptide resistance (*pmrAB*, *cptA*) and resistance to a variety of drugs (*mdaB*) and reactive oxygen compounds (e.g. *katE*, STM1731, *dps*); however, mutations in *qseBC* do not affect antimicrobial susceptibility (Merighi *et al.*, 2009). In addition, QseBC has been reported to affect

motility and virulence in response to bacterial autoinducers and host stress-related catecholamines (Bearson & Bearson, 2008; Rasko *et al.*, 2008), though we and others have questioned this (Merighi *et al.*, 2009; Pullinger *et al.*, 2010). It has also been reported that mutations in *qseBC* affect invasion of epithelial cells *in vitro* (Bearson & Bearson, 2008; Merighi *et al.*, 2009; Moreira *et al.*, 2010), though no consistent effect on *Salmonella* pathogenicity island 1 invasion genes has been observed (Merighi *et al.*, 2009; Pullinger *et al.*, 2010).

EnvZ/OmpR has previously been established to influence *S. Typhimurium* virulence in mice inoculated by the intragastric and intraperitoneal routes (Santiviago *et al.*, 2009; Yoon *et al.*, 2009). EnvZ senses changes in osmolarity, leading to phosphorylation of its cognate RR OmpR, and subsequent alterations in the ratio of two porins (OmpC and OmpF) that influence cell permeability and nutrient availability. Studies have also connected OmpR with flagellar expression (Bang *et al.*, 2000), fatty acid transport (Higashitani *et al.*, 1993) and stationary phase acid tolerance (Bang *et al.*, 2000). It was recently shown that when *E. coli* colonizes the mouse gut it adapts by the rapid acquisition of point mutations within EnvZ/OmpR, which fine-tunes its activities and confers a selective advantage due to its effects on permeability and flagellin expression (Giraud *et al.*, 2008). It remains to be determined whether such adaptive mutations occur during *Salmonella* infection.

The SsrA/SsrB TCS, which is unique to *Salmonella*, regulates the expression of T3SS-2 effectors. Such effectors facilitate the intracellular survival of bacteria within SCVs in a variety of cell types. T3SS-2 is a major virulence factor in murine typhoid models (Cirillo *et al.*, 1998; Geddes *et al.*, 2007; Hensel *et al.*, 1998; Shea *et al.*, 1996), and mutant screens have shown that *ssrA* and *ssrB* mutants are attenuated in mice (Chaudhuri *et al.*, 2009; Lawley *et al.*, 2006; Santiviago *et al.*, 2009; Yoon *et al.*, 2009). In calves, the *S. Dublin* T3SS-2 is required for intestinal colonization, induction of enteritis and systemic virulence (Bispham *et al.*, 2001). Signature-tagged *S. Dublin* mutants that lack genes encoding structural components of T3SS-2 are negatively selected at enteric and systemic sites 4 days after oral inoculation (Pullinger *et al.*, 2007). Our data are also consistent with the finding that *S. Typhimurium* T3SS-2 mutants, including *ssrA* and *ssrB* mutants, are attenuated for intestinal colonization 3–5 days after oral inoculation of calves (Morgan *et al.*, 2004), and with the attenuation of an *S. Typhimurium ssaR* mutant 5 days after inoculation into bovine ligated ileal loops (Coombes *et al.*, 2005a). *In vitro*, T3SS-2 expression can be induced in nutrient-limited conditions at low pH (Hansen-Wester & Hensel, 2002). Interestingly, although T3SS-2 was previously thought to be induced in the environment of the SCV, *in vivo* expression studies in mice have shown that T3SS-2 expression is induced at very early times in the ileal lumen, and that this is independent of active bacterial invasion of the epithelium (Brown *et al.*, 2005), suggesting that conditions such as low pH in the stomach are a signal

for T3SS-2 expression. Transcription of *ssrB* is uncoupled from that of *ssrA* (Feng *et al.*, 2003); therefore, it is likely that SsrB is expressed in the *ssrA* mutant and it may still be activated by factors other than SsrA, as has been described, for example, for SlyA, PhoP and OmpR (Bijlsma & Groisman, 2005; Feng *et al.*, 2004; Yoon *et al.*, 2009).

In comparison, less is known about the role of other SKs that significantly influenced the colonization of some, but not all, sites by *S. Dublin* after oral inoculation of calves. BaeSR responds to envelope stresses such as perturbations in the redox potential in the envelope, and increases multidrug and metal resistance through the induction of the drug efflux systems *acrD* and *mdtABC* (Nishino *et al.*, 2007). Overexpression of BaeR leads to increased resistance to deoxycholate, a component of bile (Nishino *et al.*, 2007), suggesting that this activity might be important in survival in the intestine. However, the SD3246 Nal^R *baeS* mutant grew as well as the parent strain when plated on MacConkey agar plates that contained 0.5% (w/v) bile salts and also on LB agar containing 3 or 10% (w/v) bovine bile (data not shown). To the best of our knowledge, this is the first report to implicate BaeS in virulence.

The *citA* and *dpiB* genes are each encoded adjacent to, and are annotated as regulators of, citrate utilization operons in *Salmonella*. In *Klebsiella pneumoniae* the CitAB TCS induces expression of genes required for citrate catabolism under anaerobic conditions in the presence of citrate and sodium ions (Yamamoto *et al.*, 2009). During anaerobiosis, *S. Typhimurium* is also capable of growth on citrate by a Na⁺-dependent pathway, forming acetate, formate and carbon dioxide as products (Bott, 1997). Therefore in the anaerobic environment of the intestine, CitAB potentially activates *Salmonella* citrate fermentation genes and confers a competitive advantage by providing an additional carbon and energy source. Consistent with a role for CitAB *in vivo*, a *citB* mutant was negatively selected following intraperitoneal inoculation of mice (Lawley *et al.*, 2006), and a *citA* mutant of *S. Typhimurium* (then designated STM0053) was attenuated for intestinal colonization upon screening signature-tagged *S. Typhimurium* mutants in calves and chicks (Morgan *et al.*, 2004), but not pigs (Carnell *et al.*, 2007). The role of DpiB is less clear, in part as *S. Dublin* *dpiB* has a frameshift predicted to result in a truncated protein of 406 amino acids (compared with 533 residues in *S. Typhimurium*). As the C terminus is predicted to contain a catalytic ATP-binding domain, further studies will be required to determine whether *S. Dublin* DpiB is active.

The calf oral challenge model not only confirmed attenuating defects reported in the murine typhoid model (above), but also supported the absence of virulence defects for mutants lacking *copS*, *cpxA*, *glnL* or *yojN* when tested by intragastric or intraperitoneal infection of mice (Yoon *et al.*, 2009) and for an *S. Typhimurium* *qseE* mutant in calves (Pullinger *et al.*, 2010). The *arcB* mutant, which exhibited slower growth *in vitro*, efficiently colonized the bovine

gut, consistent with the lack of statistically significant attenuation in orally dosed mice of *arcA* mutants of *S. Typhimurium* (Yoon *et al.*, 2009) and *S. enterica* serovar Enteritidis (Lu *et al.*, 2002). It is noteworthy that several mutants were enriched at enteric and systemic sites relative to the input pool (in particular *glnL*, *hydH*, *narX*, *pgtB*, *phoR*, *pmrB*, *ttrS*, *qseE* and *yojN* mutants). The extent to which this is due to enhanced fitness as opposed to competition dynamics within a pool where other mutants are attenuated requires further studies by separate or co-infection of calves.

Phenotype of *S. Dublin* SK mutants following intravenous inoculation of calves

Three days after intravenous instillation of the mutant pool, representative output pools (>3000 colonies) were obtained from the liver and spleen of all three calves. Recoveries from enteric sites, blood and bile were too low and variable to permit robust analysis. The output:input volume ratios for the liver and spleen samples were analysed by pairwise Student's *t* test and are recorded in Table 3.

Tagged virulent *S. Dublin* strains efficiently colonized the liver and spleen, and *S. Gallinarum* SG9 mutants were, in general, significantly under-represented at these sites, as expected (Paulin *et al.*, 2002; Pullinger *et al.*, 2007). Strain 14C3 was enriched in the liver, albeit not significantly (Table 3). T3SS-1 mutants were not significantly attenuated by this route, with the exception of the *sipC* mutant in the spleen, consistent with the function of T3SS-1 in intestinal invasion and the systemic virulence of such mutants after intravenous or parenteral dosing of mice (Chaudhuri *et al.*, 2009; Santiviago *et al.*, 2009). The *ssaJ* T3SS-2 mutant was significantly under-represented in the liver and spleen, but whilst the *sseC* and *sseD* mutants were negatively selected in the liver the difference was not statistically significant. No attenuation of the *sseC* and *sseD* mutants was detected in the spleen. The relatively weak phenotype of *S. Dublin* T3SS-2 mutants after intravenous dosing of calves is consistent with previous data from our laboratory, in which an *sseD* mutant was recovered by enrichment from the liver and spleen [maximum log₁₀(c.f.u. g⁻¹) value of 2 at 7 days post intravenous dosing, compared with a log₁₀(c.f.u. g⁻¹) value of 3.5 for the parent strain] (Bispham *et al.*, 2001). Consistent with the weak and variable phenotype of T3SS-2 mutants, no significant role was detected for the SPI-2 regulator SsrA.

Only two SK mutants of *S. Dublin* proved to be significantly attenuated in both the liver and spleen (*barA* and *phoQ*). These were also significantly under-represented in the liver and spleen after oral dosing (Table 3), albeit that the mutants were also impaired in their ability to colonize enteric sites. The PhoPQ system is well established as playing a role in systemic virulence in murine typhoid models (Groisman & Mouslim, 2006). The extent of negative selection of the *barA* mutant after intravenous

dosing was less pronounced than following oral inoculation, but the phenotype is consistent with attenuation of *barA* mutants after intravenous dosing of mice with a mutant library (Chaudhuri *et al.*, 2009) and after delivery by the intraperitoneal route (Santiviago *et al.*, 2009; Yoon *et al.*, 2009).

Phenotype of *S. Dublin* SK mutants in INT407 cells

Representative output pools were obtained following infection of INT407 intestinal epithelial cells at 24, 48 and 72 h post-infection. As anticipated, T3SS-1 mutants were significantly under-represented at all time intervals, consistent with the role of T3SS-1 in invasion (Table 3). Results for the T3SS-2 mutants were less clear, with *sseC* and *sseD* mutants (and the *ssrA* regulator) being under-represented at 24 h, but enriched at later time points. Mutation of *cheA*, which produces a smooth-swimming non-chemotactic phenotype, resulted in marked over-representation in INT407 cells, consistent with the hyperinvasive phenotype of an *S. Typhimurium cheA* mutant in HEp-2 cells (Jones *et al.*, 1992).

The SD3246 Nal^R *barA*, *baeS*, *envZ* and *dpiB* mutants were significantly under-represented in INT407 cells, consistent with the attenuation of such mutants in calves dosed by the oral route (Table 3). Similarly, *pmrB*, *ttrS* and *yojN* mutants were enriched in the outputs from both INT407 cells and calves. In contrast, however, *phoQ* and *qseC* mutants attenuated after oral dosing of calves were over-represented in INT407 cells, and several mutants that showed no attenuation in calves were significantly under-represented in INT407 cells (particularly *arcB*, *copS*, *cpxA*, *torS* and *qseE*). Observations made in surrogate cell-based assays must therefore be approached with caution, as phenotypes are not reliably reproduced in target animal models.

BaeS contributes to virulence in calves following oral inoculation

As described above, the SD3246 Nal^R *baeS* mutant was attenuated in the oral inoculation screen, where output:input ratios ranged from 0.31 to 0.72. These attenuation scores were not as low as those obtained for mutants such as *phoQ* (0.21–0.27) or *barA* (0.11–0.18) which have well-established roles in virulence. To confirm the role of BaeS in the virulence of *S. Dublin* in calves, the phenotype of the *baeS* mutant relative to the parent strain was assessed by co-infection studies. CIs were determined at enteric and systemic sites 3 days following oral infection (Fig. 2). The mean CIs at all sites except the spleen were below 1. Statistical analysis indicated that the output ratios at two of the enteric sites, the ileum and the colon, were significantly lower than the input ratio in the inocula ($P \leq 0.01$). Although the level of attenuation was modest, these results confirm that BaeS contributes to intestinal colonization of calves by *S. Dublin*. The disparity between phenotypes of the

Fig. 2. BaeS contributes to the virulence of *S. Dublin* in calves. Calves were co-inoculated with SD3246 Nal^R and the $\Delta baeS::mini-Tn5Km2$ mutant by the oral route ($n=6$) or intravenously (i.v.) ($n=3$). CIs were determined in samples at 3 days post-infection. The CI was calculated as output ratio (mutant/wild-type) divided by input ratio (mutant/wild-type). The mean and SEM at each site are shown (bars). Output and input ratios were compared using a Mann–Whitney non-parametric test. $P \leq 0.05$ was taken as significant (indicated by asterisks).

baeS mutant when screened by the oral route among 42 other mutants (Table 3) or in a 1:1 ratio with the parent (Fig. 2) likely reflects differences in competition dynamics. The role of BaeS following intravenous inoculation was also assessed by co-infection of three calves. The CIs were determined in the liver and spleen (Fig. 2), but were not statistically significant, although there was a tendency towards significance in the liver ($P=0.076$).

Conclusions

We present the first complete survey of the role of all two-component sensory systems during typhoidal salmonellosis in a highly relevant model of infection. Inactivation of eight SK genes (*barA*, *phoQ*, *envZ*, *ssrA*, *baeS*, *dpiB*, *qseC* and *citA*) impaired the ability of *S. Dublin* to translocate from the bovine intestines to visceral organs via the lymphatic system. Only mutants lacking *barA* and *phoQ* proved to be significantly negatively selected in the liver and spleen after intravenous dosing of calves, implying that some of the SKs that play a role after oral infection may mediate responses to niche-specific extrinsic signals. We confirmed a novel role for BaeS in intestinal colonization of calves by *S. Dublin*, and further studies are required to define the BaeSR regulon and its activating signal(s). Signature-tagged mutagenesis relies on screening complex pools of mutants followed by amplification and hybridization of associated tags. Though a powerful screening tool, we cannot preclude the possibility that subtle

phenotypes would be evident if mutants were screened in isolation and viable bacteria were enumerated directly. However, it is clear that a large number of the TCSs are not essential for enteric or systemic virulence in the models used. There are a number of possible reasons for this. First, functional redundancy may mask the role of TCSs to ensure that key virulence factors are expressed. For example, T3SS-1 and -2 are regulated by a number of TCSs and other regulators, so the absence of a single regulator does not necessarily abolish their expression. For example, SsrB can replace SlyA, and in some cases, SlyA can replace SsrB for expression of SPI-2-encoded virulence factors (Yoon *et al.*, 2009). Similarly, the existence of multiple regulators of anaerobic respiration (NarX, NarQ, DcuS, TtrS and TorS) and utilization of carbon sources (PgtAB, TctDE and UhpAB) may afford sufficient flexibility to allow the use of alternative pathways in the absence of a single TCS. A second explanation for the absence of SK phenotypes may be that some RRs are phosphorylated *in trans* by non-cognate SKs to ensure that genes under the control of the RR are expressed. For example, in *E. coli*, the BarA, BaeS and EnvZ SKs can phosphorylate non-cognate RRs (Yamamoto *et al.*, 2005). Analysis of such 'cross-talk' will be required to understand how signals downstream of the eight SKs implicated here in *S. Dublin* pathogenesis are transduced. A further layer of complexity exists, as TCSs often act in regulatory networks, where one TCS controls the expression of another or its regulator(s) (Oshima *et al.*, 2002).

Phenotypes of SK mutants may also have been missed owing to the use of a single time interval post-inoculation. For example, mutants lacking *copS*, *dcuR* and *pmrB* are negatively selected in mice at 28 days after intraperitoneal inoculation (Lawley *et al.*, 2006), but are not attenuated in comparable screens of a shorter duration (Santiviago *et al.*, 2009; Yoon *et al.*, 2009). Interestingly, screening of *S. Dublin* SK mutants revealed a number that were positively selected *in vivo* relative to input pools (Table 3). Evidence of enhanced fitness requires studies with strains in isolation or involving co-infection with the parent strain, and may be a consequence of derepression of genes with a positive effect on virulence, or of the absence of induction of those with a suppressive effect. For example, it has been reported that mutation of *rcsS* in *S. Typhimurium* increases expression of SiiE (García-Calderón *et al.*, 2007), an adhesin that influences intestinal colonization in calves (Morgan *et al.*, 2004). Such events may partially explain the over-representation of RcsC/YojN mutants in output pools following oral inoculation of calves.

Although the role of TCSs has been studied in murine models using defined or random mutants, most research has involved intravenous or intraperitoneal delivery, and the role of TCSs in intestinal colonization is less well understood. The present study represents a significant advance, as the role of all predicted SKs in both intestinal colonization and systemic translocation has been evaluated in a model of naturally occurring systemic salmonellosis. Reassuringly, many of the

phenotypes assigned to *S. Dublin* SKs in calves mirror those seen with *S. Typhimurium* in atypically susceptible inbred mice; for example, the roles played by BarA, EnvZ, PhoP and SsrA in calves are in agreement with the results of several screens in murine models (Chaudhuri *et al.*, 2009; Santiviago *et al.*, 2009; Yoon *et al.*, 2009). The tagged mutations created herein may be useful for studies in other *Salmonella*-host combinations to evaluate the extent to which TCSs play host- and niche-specific roles.

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