Full Title:

Combining *Shigella* Tn-seq data with Gold-standard *E. coli* Gene Deletion Data
Suggests Rare Transitions between Essential and Non-essential Gene Functionality

Short Title:

Transitions between Essential and Non-essential Functionality

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Abstract

Gene essentiality - whether or not a gene is necessary for cell growth - is a fundamental

component of gene function. It is not well established how quickly gene essentiality can

change, as few studies have compared empirical measures of essentiality between closely

related organisms. Here we present the results of a Tn-seq experiment designed to detect

essential protein coding genes in the bacterial pathogen *Shigella flexneri* 2a 2457T on a

genome-wide scale. Superficial analysis of this data suggested that 451 protein-coding genes

in this *Shigella* strain are critical for robust cellular growth on rich media. Comparison of this

set of genes with a gold-standard data set of essential genes in the closely related *Escherichia*

coli K12 BW25113 suggested that an excessive number of genes appeared essential in

Shigella but non-essential in *E. coli.* Importantly, and in converse to this comparison, we

found no genes that were essential in *E. coli* and non-essential in *Shigella*, suggesting that

many genes were artefactually inferred as essential in *Shigella.* Controlling for such artefacts

resulted in a much smaller set of discrepant genes. Among these, we identified three sets of

functionally related genes; two of which have previously been implicated as critical for

Shigella growth, but which are dispensable for *E. coli* growth. The data presented here

highlight the small number of protein coding genes for which we have strong evidence that

their essentiality status differs between the closely related bacterial taxa *E. coli* and *Shigella.*

A set of genes involved in acetate utilization provides a canonical example. These results

leave open the possibility of developing strain-specific antibiotic treatments targeting such

differentially essential genes, but suggest that such opportunities may be rare in closely

related bacteria.

Author Summary

Essential genes are those that encode proteins required for growth and survival in a particular

environment. We performed experiments using transposons, genetic elements that disrupt

gene function, to determine the set of essential genes in the pathogenic bacteria *Shigella*

flexneri. We then compared our results to the well-characterized set of essential genes in the

closely related, yet non-pathogenic, bacteria *Escherichia coli*. We found only a small number

of genes that are important for growth in *Shigella flexneri*, yet not in *Escherichia* coli. We

believe these findings are interesting for several reasons; they help us better understand how

quickly the functions of proteins change over time; they suggest possible targets for

developing strain-specific antibiotic treatments; and they expand our basic understanding of

this pathogen's metabolic processes.

Introduction

One general functional characteristic of a gene is essentiality - whether that gene is required for cellular viability and growth. In haploid (e.g. bacterial) genomes, this characteristic can be assessed by attempting to delete a specific gene from a genome. When such a deletion is not possible, this gene is frequently termed "essential" [1], implying that the gene is necessary for cell growth and viability. Gene disruption, although less precise, is more commonly used to infer essentiality using a similar criterion. For example, genes that cannot be disrupted by transposon insertion have been inferred as being essential (e.g. [2]).

One important question is how quickly essential functions change over evolutionary time. If orthologous protein coding genes in two bacterial strains differ in their essentiality classification, this suggests that either the biochemical nature of the protein has changed, or that the cellular context in which the protein acts has changed [3]. It has been experimentally established that such transitions can occur [4-6]. Here we examine how frequently proteins go from being essential to non-essential and vice versa in nature.

A recent study quantified changes in the essentiality classifications of protein coding genes between three alpha-proteobacteria: *Caulobacter crescentus*, *Brevundimonas subvibrioides*, and *Agrobacterium tumefaciens* [3]. The analysis showed that although orthologous cell components are well conserved, the essentiality of such components (e.g. those involved in the cell cycle) had changed considerably, with only 106 orthologous genes being essential in all three organisms, despite their relatively close evolutionary relationship (89%-93% identity in 16S RNA genes).

In this study we combine dense transposon mutagenesis with high-throughput sequencing (Tn-seq [7]) to quantify gene essentiality in *Shigella flexneri* 2a 2457T (hereafter referred to as *Shigella*). We compare the essentiality classifications of protein coding genes in *Shigella* with a gold-standard assessment of essentiality in the closely related strain *Escherichia coli* K12 BW25113 (hereafter referred to as *E. coli*) [1]. These two stains are 99.5% identical in their 16S RNA genes and share approximately 70% of their genomic content.

This proximity in evolutionary distance, and the use of a gold-standard data set, brings two unique advantages that have not been available in other studies that have used Tn-Seq or similar methods to quantify gene essentiality [3, 7-19]. First, by relying on the null hypothesis that protein coding genes do maintain their essentiality characteristics, we can objectively assess which quantitative features in the *Shigella* Tn-seq data best predict essentiality or non-essentiality of their orthologous counterparts in *E. coli*; such a comparison to a gold-standard has not yet been used to assess the quality and sensitivity of Tn-Seq data [20], although several studies have validated a small number of Tn-seq-inferred growth defects using clean

deletion methods (e.g. [10]). Second, the use of very closely related taxa allows us to quantify

on a much shorter time scale the fraction of the essential gene complement that has changed,

providing a fine scale window into the rate with which orthologues change in their essential

functions.

The data presented here suggest that the essential gene complement of *Shigella* and *E. coli*

overlap considerably. Indeed, we find no strong evidence that there are any protein-coding

genes that are essential in *E. coli* but not *Shigella*. Conversely, we do find a small number of

genes that play critical roles for *Shigella* growth, but which have dispensable roles in *E. coli*,

or which are absent entirely from *E. coli.* This implies that the functional correspondence, in

terms of essentiality, has changed for only a small number of protein-coding genes.

However, our analysis also suggests that some protein-coding genes that we observe as

undisrupted by transposon insertions are in fact not essential for cell growth. Instead, they are

either essential for transposon insertion to occur successfully, or their disruption (but not

clean deletion) is detrimental to cell growth. This result emphasizes that in high throughput

transposon mutagenesis studies, false positive inferences of essentiality may be common, and

that simply increasing the resolution or precision of a dataset cannot necessarily solve this

problem.

Taken together, our data suggests that the essential gene complement is relatively static over

short time scales. However, when protein-coding genes do change from being non-essential to

being essential, this appears more likely to occur in pathogenic organisms, perhaps because

host environments absolve the organism from manufacturing its own nutrients, or because

such organisms have smaller population sizes and are prone to the accumulation of

deleterious mutations. It would be interesting to see if this pattern is observed when

comparing other pathogens to their free-living sister taxa. If antibiotics can be directed against

the function or expression of such differentially essential genes, this may allow targeting such

treatments toward specific bacterial strains.

Results and Discussion

A Transposon Mutagenesis Library Provides Fine-scale Resolution of Gene
Essentiality

We generated a transposon insertion library by transforming a *Shigella icsA* mutant [21] with

a plasmid containing a mini-Tn10 transposase with decreased hotspot activity ([22]; **Fig. 1A**,

inset) inducible by isopropyl-β-D-thiogalactopyranoside (IPTG) [23, 24]. After overnight

growth on Tryptic Soy Broth (TSB) agar plates containing IPTG, we harvested

approximately 10^6 colonies carrying transposon insertions. We pooled and then split this

library of clones into six replicates. Three replicates were subject to additional growth step

inside the cytoplasm of HeLa cells for four hours. The resulting cells were then harvested, the

replicates were bar coded and libraries were prepared. We sequenced all six of these pools on

a single Illumina HiSeq lane. For all the analyses presented in this study, we have pooled the

data from all replicates and from both of these treatments, as we are focusing on *Shigella*

genes that are essential across any permissive growth conditions.

From this pool, we mapped insertions at 131,670 unique positions on the *Shigella*

chromosome (with many insertions occurring on both the forward and reverse strands but at

the same position), and 12,552 unique positions on the large *Shigella* virulence plasmid (see

Methods). The median distance between inserts on the chromosome was 17 base pairs (bp);

on the plasmid this distance was 9 bp. 95% of all inter-insert distances on the chromosome

were less than 107 bp; the corresponding figure for the plasmid was 59 bp (**Figs. 1A and B**).

Although the distribution of transposon inserts was relatively even across both the

chromosome (**S1 Fig.**), at smaller scales we found many regions in which few or no insertions

occurred. Quantitative analyses showed that regions containing no transposon insertions for

100bp or more were considerably enriched (see **Methods**; **Figs. 1C and D**). It is likely that

many of these regions are critical for cellular growth in *Shigella*. Indeed, we found that for

many of the protein-coding genes in these regions, the orthologous *E. coli* genes are known to

be essential (**Figs. 2** and **3**).

In contrast to the *Shigella* chromosome, we found that few open reading frames on the

virulence plasmid were devoid of insertions. Only six out of 263 plasmid ORFs had no inserts.

Two of these were replication proteins (CP0258 and CP0259), and two (CP0217 and CP0218)

were located within the plasmid stabilisation region. The remaining two, *mxiH* and *acp*, are

both less than 250 bp in length (the cut-off used here to classify ORFs as essential; see below),

and thus have a lower likelihood of being hit due simply to their smaller target size. The third

replication protein of the plasmid, CP0260, contained a single insert in its 858 bp length. The

absence of inserts in the plasmid replication or stabilisation regions is explained by the fact

that if such insertions did occur, the plasmid would be lost; such insertions would thus never

be sequenced. Thus, this data is consistent with the fact that the *Shigella* plasmid contains no

essential genes [25], and suggested that our transposon library provided a fine-scale

assessment of which *Shigella* chromosomal ORFs provide critical cellular functions.

Average Distance Between Inserts Clearly Delineates Essential and Non-essential

ORFs

We next quantified which transposon insertion patterns in the chromosome were good

predictors of the essentiality of open reading frames. To do so, we first identified 3,027

orthologous open reading frames present in both *E. coli* and *Shigella* for which we also had

data on essentiality from both the Keio [1, 26] and the Profiling of the Escherichia coli

Chromosome (PEC) [27] studies (**S1 Table**). We considered this combined gene set as a gold-

standard of essentiality, for two reasons: it is not subject to artefacts that might exist in Tn-seq

dataset, such as insertion biases or biases arising during sequencing library preparation (e.g.

[28]); and combining both the Keio and PEC datasets should result in few false positive or

false negative essentiality characterizations. This set consisted of 277 orthologues considered

essential by both studies, 2,717 genes considered non-essential by both studies, and 33 genes

for which the two studies disagree.

We next quantified several characteristics for each protein-coding gene in our Tn-seq data set,

including the total number of inserts per ORF, the mean distance between inserts, the length

of the 5' fraction of the ORF upstream of the first insertion, the largest uninterrupted region in

the ORF, and others (**S2 Fig.**). We took as a null hypothesis that generally, genes have

maintained their essentiality characteristics since the divergence of *E. coli* and *Shigella*. We

then tested which of these characteristics best predicted the essentiality status of their

orthologous counterparts in the gold-standard dataset of open reading frame essentiality in *E.*

coli (the Keio and PEC datasets).

We found that the best predictor of essentiality status in *E. coli* was the mean distance

between transposon insertions in their *Shigella* orthologues (Materials and Methods; **S2 Fig.**).

For the *Shigella* orthologues of the 277 *E. coli* essential genes, only four had a mean distance

between inserts of less than 150bp. 17 (6%) had a mean inter-insert distance less than 250bp.

In contrast, only 6% of the orthologues of non-essential *E. coli* genes had a mean distance

between inserts of greater than 250bp (**Fig. 3A**). We selected this mean inter-insert distance

of 250bp as a cut-off for classifying *Shigella* ORFs as essential, as it provided a balance

between protein coding genes classified as essential in *E. coli* but non-essential in *Shigella* (a

6% false negative rate) versus non-essential in *E. coli* and essential in *Shigella* (a 6% false

positive rate). By extension, genes that are less than 250bp in length and in which we do not

observe insertions were inferred as essential (26 ORFs in total, of which 12 were ribosomal

proteins and five were leader peptides). We note, importantly, that almost all of the predictors

we tested performed extremely well (**S2 Fig.**).

We next investigated in greater detail the disagreements in essentiality classification between

E. coli and *Shigella* (**Fig. 3B**). Of the17 *E. coli*-essential genes that this metric identified as

non-essential in *Shigella*, all are likely to be false negatives (i.e. in fact essential in *Shigella,*

but not classified as such by our criterion). All 17 have a mean distance between inserts of

greater than 100 bp (**Fig. 3A**), and nine are uninterrupted for more than 90% of their reading

frame. This suggests, surprisingly, that there are no genes that are essential in *E. coli* but

whose *Shigella* orthologues are non-essential. This similarity in essentiality is not due to the

fact that we use a characteristic that most closely predicts essentiality in the gold standard

dataset – this result is robustly corroborated by any meaningful metric that we used (e.g. using

other mean distances between inserts as cut-offs for essentiality, using the total number of

inserts, the longest uninterrupted gene fraction, or others (**S2 Fig.**)). Overall, this data gives us

a very strong prior that genes have maintained their essentiality status (or near-lethal effects

on growth) since the divergence of *E. coli* and *Shigella*.

Many Non-essential *E. coli* orthologues of Essential *Shigella* Genes Exhibit

Impaired Growth

As a result of this strong prior, we thus expect that many of the discrepancies in essentiality

between *E. coli* and *Shigella* are false positives due simply to the *Shigella* mutants being non-

essential, but having significantly impaired growth. Indeed, of the 160 discrepant genes

classified as non-essential in *E. coli* but essential in *Shigella*, 34% of the orthologous *E. coli*

deletion genotypes exhibit low growth yields (less than 0.5 OD600 after 22 hours of growth

in LB [1]). This contrasts strongly with the 2557 ORFs we classified as non-essential in

Shigella: only 3.7% of the orthologous *E. coli* deletion genotypes had low growth yields (**Fig.**

4). Similar but less striking patterns were observed for growth in glucose minimal MOPS

media (**S3 Fig**.).

It is important to note that Tn-seq assays have only limited power to differentiate essential

genes from those whose deletion results in severe growth deficiencies. During the course of

preparing the library for sequencing, we estimate that at least 20 generations of growth

occurred. If a mutant has a growth rate even 60% that of the wild type, we would expect it to

undergo only 12 doublings in contrast to the 20 of the wild type. This would result in a greater

than 200-fold underrepresentation of such a mutant ($2^{12}/2^{20}$). In addition, this calculation does

not take into account any effects that the mutations have on the length of the lag time, which

might also have significant effects on the relative frequency of some mutants.

In light of this limited resolution power; given that our prior expectation is that essentiality

status changes only rarely; and because we are specifically interested in genes that may have

changed in essentiality status, from this point on we focus our analysis on essential *Shigella*

genes whose orthologous *E. coli* deletion genotype exhibits robust growth yields (OD600

greater than 0.75 after 22 hours growth in LB (**Fig. 4**)). For these genes, we have relatively

high confidence that while their deletion in *E. coli* has few effects on growth, their disruption

in *Shigella* is lethal or results in a severe growth deficiency.

Artefacts of the Transposon Screen Explain Some False Positive Discrepancies

35 essential *Shigella* genes have orthologous *E. coli* deletion genotypes with growth yields higher than 0.75 OD600 after 22 hours growth in LB [1] (**Fig. 4**). Careful inspection suggested that some genes were present in this set due to differences in the growth conditions between *E. coli* Keio and PEC collections and our own. For example, *fhuACD*, and *tonB* all appeared in this set of genes (**Table 1**). All four are involved in iron acquisition, and it is likely that iron was limiting in the solid agar media [29] used during the preparation of the Tn-seq library, as compared to the liquid LB used to measure growth in the Keio study.

Table 1. Genes artefactually inferred as essential in *Shigella*.

Gene(s)	Evidence for Artefactual Inference of *Shigella* Essentiality
acrAB, tolC, ybaB, ksgA, yebC, smpB (0.73)[1]	Affect kanamycin resistance [30-32]
dnaQ, holD, recD, xseA, ruvA (0.58), *ruvB* (0.60), *ruvC* (0.61), *recB* (0.58), *recC* (0.65)	Likely to affect the transposition process; *dnaQ, holD, ruvA,* and *ruvB* inferred as essential using Tn-seq in *Salmonella* [13]
priB	Deficient in plasmid maintenance [33, 34]
fhuACD, tonB	Involved in iron acquisition which is critical for growth in the iron limited media used in this study [29]
rpsT	S20 ribosomal subunit; new data indicates mutants have poor growth [35] (in conflict with Keio data)
miaA	tRNA dimethyl transferase; previous data indicates *E. coli* mutants have poor growth [36] (in conflict with Keio data)
ompA	Outer membrane porin; clean knockouts appear viable [37]; mutant forms are frequently lethal [38]
pitA	Metal phosphate transporter with ten transmembrane segments; transposon disruption of substrate transporters is three-fold more likely to be inferred as essential compared to clean deletion (see main text; **Fig**)
potB	Type I ABC transporter (Putrescine / spermidine transporter)
cysU	Type I ABC transporter (Sulfate / thiosulfate transporter)
sapB	Type I ABC transporter (unknown substrate)
ptsH	Short 306 bp reading frame
ydhR	Short 258 bp reading frame

[1] Gene deletions of orthologous *E. coli* genes with growth levels less than OD600 0.75 have these levels in parentheses

For a second set of genes, the discrepancies are likely due to the differences in methodology between the *E. coli* (precise gene deletions) and *Shigella* (transposon inactivation) studies (**Table 1**). We inferred *acrAB* and *tolC* as essential. These genes act together as an efflux pump, and mutations in these genes result in hypersensitivity to antibiotics [39]. Thus, clones with transposon insertions in these genes are unlikely to survive during library growth under kanamycin selection. A similar explanation likely underlies the fact that we inferred *ybaB*, *ksgA, yebC,* and *smpB* as essential: these four play role in aminoglycoside resistance [30-32].

We also inferred *priB*, *dnaQ*, *holD*, *xseA*, and *recD* as being essential in *Shigella*, although the *E. coli* deletion genotypes exhibit robust growth. All of these are involved in DNA replication, recombination, and double strand break repair, all of which are essential processes in the completion of the transposition process [40]. The related genes *recBC* and *ruvABC* contained a single insert between the five of them, while the *E. coli* deletion genotypes all exhibit only slightly impaired growth of 0.6 OD600 or more (**Table 1**). Certain *recBC* mutants can have considerable effects on the rate of Tn10 excision [41, 42] and we speculate that this may be one reason why we rarely observed insertions in these loci. It has also been speculated that *ruv* mutants inhibit transposition [43]. We propose that after transposition occurs, in order for the event to be successfully resolved, transcription of these genes is often required, and the transposition itself precludes the formation of a proper transcript.

Thus, the dispensability of these ten genes in *E. coli*, and the similarity in their function, suggests that they all affect successful transposon insertion rather than having critical effects on growth. Notably, *priB*, *dnaQ*, *holD, ruvA,* and *ruvB* were also inferred as essential in the closely related bacterium *Salmonella typhimurium* via a high-throughput transposon assay. In the same study *ruvC, recB, recC,* and *ybaB,* were inferred as extremely important for growth while *ksgA* and *yebC* were inferred as significantly impairing growth [13]. Again, the majority of these knockouts in *E. coli* exhibit very robust growth (greater than 0.75 OD600 after 22h growth in LB). Given the roles that these genes are known to play in transposition and antibiotic resistance, this suggests that the inference of essentiality may be due to artefacts of the transposon screen.

For a third set of genes, the literature presents conflicting information on the growth phenotypes, with studies that have individually assessed growth rates suggesting poor growth. These include *rpsT* [35], *miaA* [36], and *ompA* [38] (**Table 1**).

There were also two open reading frames that we inferred as differentially essential as they were completely uninterrupted in our data. However, these two open reading frames, *ydhR* and *ptsH*, are very small and less likely to be disrupted, being 306 bp and 258 bp long,

respectively. It is probable, then, that this discrepancy is not driven by different physiological
roles that they play in *E. coli* as compared to *Shigella*.

Finally, we tested for other possible artefactual patterns in the data based on gene function.
We asked whether there were specific functional categories in which genes were more likely
to be inferred as essential using the transposon mutagenesis screen in *Shigella* as opposed to
clean deletions in *E. coli*. We found two functional categories of genes that showed clear
enrichment: genes involved in substrate transport and / or active transport, which were 3- and
2.1-fold enriched, respectively (**Fig**. **5**). We hypothesize that one reason for this enrichment is
that truncated versions of these proteins disturb the operation of the *sec* machinery, thereby
decreasing or stopping growth. Thus, we propose that the four active transporters we infer as
essential in *Shigella* but not *E. coli* (**Table 1**) are artefacts due to the transposition process
resulting in truncated proteins.

Genes uniquely essential in *Shigella flexneri*

While many differences in essentiality classification between *Shigella* and *E. coli* are likely
due to (1) severe growth defects present in both *E. coli* and *Shigella* rather than strict
essentiality; and (2) differences in environmental conditions (e.g. iron) between the *E. coli*
and *Shigella* assays; and (3) artefacts of the *Shigella* transposon screen that do not occur in
the *E. coli* knockout screen, we do find a number of genes which we infer to be uniquely
essential to Shigella. We expect that the physiological differences between *E. coli* and
Shigella are driving these differences in gene essentiality (**Table 2**).

Among the set of genes essential in *Shigella* but dispensable in *E. coli* is *lysS*: this ORF has a
functional homologue in *E. coli* (*lysU* [45]), while in *Shigella flexneri* there is no homologue.
Also in this set of genes are *proA, proB, and proC*. These genes act in proline biosynthesis.
Given the rich media the cells were grown in, it is surprising that they would be essential. In
addition, as *proB* is involved in the first committed step of proline synthesis, its disruption
should not cause accumulation of toxic intermediates. However, the data provide strong
evidence that the disruption of any these three genes is either lethal or causes severe growth
defects (**Fig. 6**). Interestingly, the active proline transporter *putP* is absent from *Shigella* [46].
It is also known that in *Salmonella,* the cryptic proline transporter *proY* is silent [47], and we
hypothesize that this may also be true of this transporter in *Shigella.* Thus, inefficient proline
transport from the media might necessitate biosynthesis.

A suite of genes involved in acetate utilization (*aceE*, *aceF*, *ackA*, *pta*, and *pykF*) were all
inferred as essential in *Shigella* but dispensable in *E. coli.* The significantly detrimental effect
on growth that such mutants have has been noted previously using a completely different
approach [21]. The difference in essentiality between these two organisms is most likely due

to the absence of acetyl CoA synthetase from *Shigella,* and confirms the sensitivity and

relevance of our transposon mutagenesis assay for assaying differences between *E. coli* and

Shigella biology.

Table 2. Genes inferred as uniquely essential in *Shigella*. All gene deletions in
homologous *E. coli* genes show robust growth in rich media after 22 hours (greater than 0.75
OD600), suggesting that these genes are uniquely essential in *Shigella* as compared to *E. coli*.

Gene(s)	Function [44]	Evidence for Different Physiological Roles in E. coli and Shigella
lysS	Aminoacyl tRNA synthetase, tRNA modification	The *lysU* functional homologue is absent in Shigella [45]
proABC	Proline biosynthesis	The active proline transporter *putP* is absent from Shigella [46]. The cryptic transporter *proY* may be silent, as observed in Salmonella [47], possibly necessitating proline biosynthesis
ackA	acetate kinase	All affect acetate accumulation [48] and utilization [21], which is required for robust growth (Shigella lacks the acetyl CoA synthetase present in *E. coli* K12 [49])
pta	phosphotransacetylase	
aceEF	pyruvate dehydrogenase	
pykF	pyruvate kinase	
rfbF, rfbG, rfc, and *rfbI*	sugar nucleotide biosynthesis for LPS	No *E. coli* K12 orthologues, as this locus has been replaced by the laterally transferred *wbb* locus [50]
spr	Murein DD-endopeptidase	None known
tufB	Elongation factor EF-Tu	None known

For only two other orthologous gene pairs is there strong evidence of discrepant essentiality

status: *tufB* (two insert locations; **Fig. 7**) and *spr* (one insert at base pair 543 across 567 bp).

For neither of these genes do we have a hypothetical causal explanation. Interestingly, we

also found very few transposon insertions in the *tufB* paralogue *tufA* (three insert locations;

Fig. 7), suggesting that this gene, too, is important for *Shigella* growth despite its relative

dispensability in *E. coli* (0.72 OD600 after 22h in LB). We note that these two genes are

nearly identical in their sequence, which creates ambiguities in mapping some reads.

However, this does not explain the absence of reads mapping to either of them.

Understanding the molecular mechanisms driving these apparent disparities in growth

phenotypes between *Shigella* and *E. coli* is an important topic for future research.

Finally, the transposon insertion data indicated that a within single large operon, containing

the ORFs *rfbACEFGI / rfc,* four genes completely lacked insertions (*rfbF, rfbG, rfc,* and *rfbI*)

(**Fig. 8**). Only *rfbA* and *rfbC* in this operon have *E. coli* orthologues. The remaining genes lie

within a commonly laterally transferred region of the *E. coli* chromosome containing

wbbHIJKL, wzxB (*rfbX*), and *glf*. These were all laterally transferred into the K12 lineage [50],

replacing the *Shigella*-like *rfb* operon. The genes in this operon all play a role in sugar nucleotide biosynthesis necessary for O-antigen synthesis and production of the lipopolysaccharide component of the outer membrane [44]. This provides some evidence that specific aspects of this process have become essential in *Shigella*, despite these genes having been replaced by a laterally transferred set in *E. coli* K12.

Conclusions

By exploiting the extremely close evolutionary relationship of *Shigella flexneri* with *E. coli* K12, the bacterial strain that has been the most extensively and carefully characterized for its essential gene complement [1, 27], we were able to develop an objective metric to precisely quantify how the results of the Tn-seq data relate to essentiality.

A superficial analysis of our Tn-seq data suggested that a total of 451 ORFs in *Shigella* were essential for cellular growth in rich media. This is very much in line with what other Tn-Seq studies have found, with numbers ranging from 480 in *Caulobacter crescentus* [8] to 447 in *B. subvibrioides* to 372 in *Agrobacterium tumefaciens* [3]. However, it is considerably more than the number that had been found in *E. coli* using in-frame gene knockouts, which is on the order of 300 essential genes. In addition, we found that close to 100% of the reading frames that were classified as essential in *E. coli* K12 were also essential in *S. flexneri*, giving us a strong prior expectation that the essentiality classifications should match between these two taxa.

A more nuanced analysis suggested four explanations for artefactual discrepancies in essentiality between *E. coli* and *Shigella*: (1) many *Shigella* genes were not strictly essential but instead gene disruption caused severe growth impairment; (2) differences in experimental conditions (i.e. iron availability); (3) many of the genes we inferred as essential were important for antibiotic resistance or successful transposition, and are in fact dispensable for growth; and (4) transposon disruption of specific functional classes of genes may result in systematically different effects as compared to gene deletions, for example due to the production of truncated protein products. By carefully dissecting the functions of discrepant genes that do not appear to be artefactual, we were able to pinpoint several genes for which there is some evidence of differential physiological roles in *E. coli* and *Shigella*. Among others, these included *lysS,* three genes involved in proline biosynthesis, and a suite of genes involved in acetate utilization (**Table 2**). In addition to these, we found one large operon which appears to have an essential role in *Shigella* growth but which is missing completely in *E. coli.* Surprisingly, we found only two additional genes that are differentially essential (*tufB* and *spr*) (**Table 2**).

Even after attempting to decrease false positive inferences of gene essentiality in *Shigella*, it
appears to be considerably more common for genes to be dispensable for growth in *E. coli*,
but critical for growth in *Shigella*. We suggests that one reason *Shigella* more may have a
larger complement of essential genes than *E. coli* is that it frequently lives as an intracellular
pathogen, and may have lost some of the functional redundancy that is present in *E. coli*. This
may occur because host environments provide an abundance of nutrients, or because
pathogens requiring a small infectious dose, such as *Shigella* [51], have inherently smaller
population sizes and are more subject to genetic drift. A third possibility is that changes in
gene function or redundancy may have occurred through selection for increased virulence,
which has resulted in the inactivation of certain genes being selectively advantageous. Finally,
we note that the discrepancies in essentiality between these two bacteria may be exploited to
develop antibiotics that have strain-specific effects [21].

Methods

Strains

For all experiments, *Shigella flexneri* 2457T Δ*icsA* was provided by M. B. Goldberg was used
as the parental strain. This strain is unable to exploit the host actin cytoskeleton for motility
and spreading [52]. Bacterial cells were grown in Tryptic Soy Broth (TSB) media. For
experiments using eukaryotic cells, HeLa cells were cultured in DMEM supplemented with
10 mM Hepes, 25 mM glucose, and 4 mM glutamine. *Shigella* were grown to exponential
phase in tryptic soy broth, coated with poly-L-lysine, and added at a multiplicity of infection
of 25, resulting in an infection rate of around 60%. *Shigella* was centrifuged onto HeLa cells
($600 \times g$ for 5 min). At 30 min postinfection, we added gentamicin (100 μg/mL) to kill
extracellular bacteria. Bacterial cells were allowed to grow within HeLa cells for a total of 4
hours.

Transposon library

Using a Tn10 transposon with a T7 promoter [23, 24] we created a library consisting of
approximately 10^6 clones. This library was created by mating *E. coli* strain BW20767
containing the pJA1 transposon plasmid with a spontaneous nalidixic acid resistant clone of
Shigella flexneri 2457T Δ icsA for 5 hours. Transposase expression was induced by plating
onto TSB plates containing 0.2 mM isopropyl-β-D-thiogalactoside (IPTG). Colonies were
allowed to grow at 37°C for 18 hours on TSB agar plates. All colonies from these plates were
then pooled and 100μl aliquots of the transposon library were stored at -80°C.

Three replicate experiments were carried out on different days in which an aliquot of the
transposon library was grown for 18 hours in TSB to stationary phase, diluted 1:100 and

grown to exponential phase (0.7 OD600). This exponential phase culture was split into two:

part of the bacterial culture was pelleted and saved and other was used for infecting HeLa

cells (as described in [21]). After 3.5 hours, HeLa cells infected with the *Shigella* transposon

library were trypsinized and pelleted. Uninfected HeLa cells were also collected and used to

spike the original bacterial culture not used for HeLa infection in order to account for HeLa

DNA. All resulting DNA was extracted using the Bacterial Genomic Miniprep Kit (Sigma).

Sequence library construction and sequencing

To amplify the transposon region from these pools, we used one top strand primer annealing

to the transposon and a pool of three bottom strand primers each of which consisted of 10

random nucleotides followed by a pentamer of common nucleotides in *E. coli* [53]:

N_{10}GGTGC, N_{10}GATAT, and N_{10}AGTAC, using Phusion pfu (**S4 Fig**). A nested PCR was

then performed to add the P7 and P5 Illumina adapters, as well as a barcode. The products

from this second PCR were then size selected for inserts between 200bp and 300bp,

quantified using a Qubit, and sequenced on an Illumina HiSeq2000 at the D-BSSE

Quantitative Genomics Facility resulting in 49bp single end reads. We used a custom

sequencing primer on the P5 end of the molecule such that on both ends of the molecule,

reads started directly on the chromosome.

Read mapping

In total, we obtained 198,682,954 reads. We found that the number of reads at each location

in the genome varied by up to four orders of magnitude. For this reason, we considered only

whether an insertion had occurred at a specific location, and not on the number of reads we

obtained at a specific location, which is likely to be highly biased due to PCR artefacts. We

thus first deduplicated the reads using *tally* [54], and then used bowtie2 [55] to align the reads

to the *Shigella flexneri* 2a 2457T genome and the *Shigella flexneri* 2a str. 301 plasmid

pCP301. The sequence of the *S. flexneri* 2457T plasmid is not available. However, the *S.

flexneri* 2457T and 2a str. 301 plasmids are nearly identical in sequence (differing by 30

SNPs; see below). Sequence reads were not trimmed for quality as read quality is taken into

account in bowtie2. We used the --sensitive-local option to allow soft clipping on the 3' end

of the reads (so that reads that contained adapter sequences at the 3' end could map

successfully), and required at least 22bp of matching sequence at the 5' end of the read.

We checked for single nucleotide polymorphisms (SNPs) on both the chromosome and the

plasmid using the samtools mpileup and bcftools utilities [56, 57]. We retained as possible

SNPs only those sites that fulfilled the following three criteria: (1) the SNP was inferred as

homozygous (necessarily true, as *Shigella* is haploid); (2) the quality score was above 20; and

(3) at least three reads on both the reverse and forward strands confirmed the SNP. We found

99 SNPs on the chromosome (as compared to the reference *Shigella flexneri* 2457T in NCBI)

and 30 SNPs on the plasmid (as compared to the *Shigella flexneri* 2a str. 301 plasmid in

NCBI (in addition to 12 and 2 small indels, respectively). These are detailed in **S2 Table** and

S3 Table, respectively.

Within chromosomal protein coding regions, 44% of all SNPs were synonymous, while 32%

fell outside of genic regions (i.e. protein coding or RNA genes). These fractions are greater

than one would expect if such SNPs were randomly located on the genome. Only 24% of all

mutations in chromosomal coding regions are expected to be synonymous (not accounting for

mutational biases), and only 28% of the chromosome is annotated as nongenic (including

repeat regions, although for many of these regions, the absence of an annotation may be

erroneous). Additionally, only 2 of the 12 (17%) small chromosomal indels fell in coding

regions. This suggests that there was some selection against nonsynonymous substitutions

that occurred during the culturing and derivation of the *Shigella flexneri* 2a 2457T *virG*

mutant. More importantly, the small number of SNPs that we found suggests that few, if any,

reads remained unmapped due to sequence differences between the strain used in our

experiments and the sequenced GenBank strain.

In total, the reads mapped to 89,028 unique locations on the forward strand and 83,074 on the

reverse strand of the chromosome, for a total of 172,102 insertions. Some of these insertions

occurred at identical positions but on opposite strands, so in total, insertions occurred at

131,670 unique sites in the chromosome. Correspondingly, the reads mapped to 8,208 unique

locations on the forward strand and 8,585 unique locations on the reverse strand of the

plasmid, for a total of 12,552 unique sites. During the insertion of the Tn10 transposon, a 9 bp

target DNA sequence is duplicated [58]. We accounted for this duplication in calculating the

distances between insertions (by moving the inferred site of insertion for one direction (we

arbitrarily selected the antisense direction) backward by 9 bp). Similarly, this duplication was

accounted for in calculating various statistics of insertions within genes: sense insertions that

were inferred as occurring in the last 9 bp of a gene were ignored in calculating the mean

number of insertions per gene (as these bp are duplicated upstream of the insertion).

Antisense insertions occurring in the first 9 bp of a gene were ignored, as these bp are

duplicated downstream of the insertion.

Using the read frequencies at all unique insert locations, we found that the transposon

insertions occurred in a biased manner, integrating more often at sites similar to the known

9bp consensus NGCTNAGC [58], although this bias was relatively weak (**Figs. 1A** and **B**,

insets). This low level of bias is likely due to our using a transposon with reduced hotspot

activity [22]. In addition, we found that insertion frequency was slightly influenced by

nucleotides further downstream of this 9bp consensus (**Figs. 1A** and **B**, insets). Sequence

logos for this analysis were visualized using the R package seqLogo [59].

The median distance between inserts was 17 bp in the chromosome and 9 bp in the plasmid

(**Fig. 1B**), suggesting that the transposon libraries yielded a relatively fine-grained map of the

essential genomic complement for both the chromosome and the plasmid.

As expected given the variation in insertion densities across the chromosome, we found high

variance in the distribution of inter-insert distances. The total length of the *S. flexneri* genome

is 4,599,354 bp in total. Given that we observed 131,670 inserts, under a model of random

insertion, we would expect a median distance between inserts of 35bp, with 95% of all inter-

insert distances being less than 107 bp (under the assumption that these distances are

distributed in a geometric manner (i.e. a negative binomial with the number of successes set

to one). For the plasmid, we observed 12,552 inserts over 221,618 bp, such that we expect a

median distance of 18bp between inserts, and that 95% of all inter-insert distances are less

than 59 bp. However, as noted above, we found that on average transposons insertions were

separated by a median of 17bp on the chromosome and 9bp on the plasmid. Fitting a

geometric distribution to the observed data over 99% of the range of the inter-insert distances

(i.e. from 1 to 237 bp for the chromosome and from 1 to 78 bp for the plasmid) more exactly

quantified this over-dispersion, and showed that uninterrupted regions in the chromosome

greater than 100 bp were considerably enriched (**Fig. 1C**).

Paired end read mapping and inference of IS element dynamics

We used 100 bp paired end Illumina sequencing data from this same library to look for

structural rearrangements due to IS elements in the genome. However, this analysis was

complicated by the fact that many IS elements share close to 100% identity with others

around the genome. During these analyses we thus restricted our searches to regions of the

genome for which we had *a priori* expectations that they harboured a rearrangement (i.e. if

there were no inserts and the orthologous *E. coli* locus was non-essential or absent).

Specifically, we followed the following procedure: we extracted a 50 kilobase pair (Kbp)

region from the genome surrounding each hypothesized rearrangement (in all cases, this was

a deletion). We then used bowtie2 with the paired end option, allowing up to 10 Kbp inserts

to map all reads from our 100 bp PE dataset. From these mapped reads, we retained only read

pairs that had (1) mapping quality scores greater than 20; (2) at least one read that matched

perfectly (i.e. at all 101 bases of the read) to the genome; and (3) were unique in their length

at any specific location (thereby excluding artefacts such as PCR doublets). From these paired

reads we then inferred the insert size, which is plotted in **S5 Fig**. The vast majority of insert

sizes ranged between 100 and 400bp. However, some were much larger (e.g. up to 9,000 bp

in **S5B Fig.**). We inferred that these surrounded regions of the genome that must have been

deleted.

Such deletions would result in the set of genes contained within as being inferred as essential

because of their lack of transposon insertions. However, in the vast majority of cases, we

found that when large operons lacked insertions but had non-essential orthologous operons in

E. coli, or were missing entirely from *E. coli*, these operons were in fact missing from the

Shigella clone that we used, most likely due to the rapid dynamics of IS elements in this

bacterium [60]. For example, no sequence reads we obtained mapped to the *yeaKLMNOP*

operon, which spans a total of 9,240 bp. Upon further analysis using a paired end genomic

data set, we found that this region was clearly missing from our *Shigella* clone (**S6B Fig**.).

This was similarly true for several other operons, as well as for single genes. We did not

consider any region in which we identified a deletion in our downstream analyses.

Essential open reading frames

We identified 3,027 unambiguously ORFs that were present in both *E. coli* and *Shigella*

flexneri 2457T [61], and for which we had essentiality data. We used reciprocal shortest

distance [62] to find orthologues, with the requirement that the alignment of the two

hypothetical orthologues extend over at least 60% of the longer ORF. To establish a gold-

standard set of essential genes we combined the data from two studies of the effects of gene

deletion on growth in *E. coli* K12: the Keio collection [1] and the PEC study [27]. We

retained only those ORFs which we had data on essentiality from both studies. We then

quantified which transposon insertion patterns that most closely corresponded with the

essentiality delineations in theses studies. Specifically, we selected the feature that maximized

the number of true positive essential genes (maximizing the sensitivity) while minimizing the

number of FP (maximizing specificity) (this metric is a receiver operator characteristic for

which we quantified the area under the curve (AUC; **S2 Fig.**)). We selected from eleven non-

independent features: (1) the total number of insertions; (2) the mean number of bp between

insertions; (3) the median number of bp between insertions; (4) the number of bp in the 5' end

preceding the first insertion; (5) the number of bp in the 5' end preceding the first insertion

relative to the total bp in the gene; (6) the number of bp in the 5' end preceding the second

insertion; (7) the number of bp in the 5' end preceding the second insertion relative to the

total bp in the gene; (8) the number of bp in the longest uninterrupted stretch of the gene; (9)

the number of bp in the longest uninterrupted stretch of the gene relative to the total length of

the gene, and (10) the number of bp in the longest stretch of the gene interrupted by at most

one insertion; (11) the number of bp in the longest stretch of the gene interrupted by at most

one insertion, relative to the total length of the gene.

We found that for both the PEC dataset and the Keio dataset, the two best predictors of
essentiality were the mean distance between inserts (AUC = 0.972 for the PEC dataset, 0.952
for the Keio dataset, and 0.973 for the genes on which both datasets agreed on the essentiality
classifications); and the fraction of the gene that lay in the longest uninterrupted region (AUC
= 0.969 for the PEC dataset, 0.955 for the Keio dataset, and 0.971 for the genes on which both
datasets agreed on the essentiality classifications) (**S2 Fig.**). We selected mean distance as on
average, it marginally outperformed the other statistic on the gold standard data set.

We note that for eight of the 14 genes classified as essential solely in the Keio dataset, the
orthologous *Shigella* ORFs have mean distances less than 30 bp, suggesting that these genes
may be falsely annotated as essential in the Keio study. In contrast, nine of the ten genes
inferred as essential solely in the PEC dataset have mean distances greater than 200 bp; the
tenth has a mean distance of 189 bp.

tRNA disruptions
We found insertions in 27 out of 99 tRNAs, with tRNAs for certain amino acids being
considerably overrepresented (**S2 Table**).

Additional analyses of differentially classified essential genes
We also tested for the enrichment of certain functional categories in the set of genes that were
classified as being essential in *Shigella* but not *E. coli*. This differs from the analysis present
in **Fig. 5** in that we are asking whether across a broad set of functions, are specific categories
enriched for *Shigella*-essential genes. In **Fig. 5** we ask whether *within* a single functional
category, is there a much higher fraction of *Shigella* essential genes than we would expect,
given the fraction of genes in that functional category that are essential in *E. coli.*

Thus, for this analysis, we separated genes by primary functional category and secondary
subcategory using the MultiFun designations (e.g. the primary functional category cell
processes divided into the secondary subcategories of cell division, SOS, stress, protection,
and motility). We then calculated the fraction of *Shigella*-essential genes within each
secondary subcategory and compared this to the total fraction of *Shigella*-essential genes
within the primary category (e.g. we calculated the fraction of essential genes in Ribosomal
Function (the secondary category) and the fraction of *Shigella*-essential genes in all other
categories in Cell Structure (the primary category) (**S6 Fig**.). We tested for enrichment
(depletion) using a Fisher exact test.

We also examined gene conservation. Highly conserved genes were considered to be those
present in more than 50% of all gamma-proteobacteria [61]. We found that genes classified as
uniquely essential in *Shigella* were much more conserved across gamma-proteobacteria (79%

highly conserved) compared to genes that were found non-essential in both *E. coli* and

Shigella (36% highly conserved; p=1.0e-33, Wilcox rank sum test).

Availability of supporting data

All read data are in the SRA with accession numbers XXX.

List of abbreviations used

bp - base pairs; *Shigella – Shigella flexneri* 2a 2457T; *E. coli – Escherichia coli* BW25113; ORF – open reading frame; PEC - Profiling the E coli Chromosome database

Competing interests

The authors declare no competing interests.

Authors' contributions

NEF, DB, and OKS conceived and designed the transposon mutagenesis. NEF performed the mutagenesis and sequencing. OKS analysed the data with input from DB and NEF. NEF and OKS wrote the paper.

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Endnotes

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Table and Figure legends

Table 1. Genes artefactually inferred as essential in *Shigella*. Listed here are those genes that were likely inferred as essential largely due to the gene disruptions having direct effects on (1) antibiotic resistance, (2) successful transposition events, (3) differences between the growth conditions of the *E. coli* and *Shigella* essentiality studies, or (4) systematically different effects of gene disruption versus gene deletion.

Table 2. Genes inferred as uniquely essential in *Shigella*. Listed here are genes inferred as essential in *Shigella*, but which have orthologous *E. coli* deletion genotypes that exhibit robust growth (greater than 0.75 OD600 after 22h growth in LB). The genes in the *rfb* operon have no orthologues in *E. coli* K12 (see main text).

Fig 1. **Histograms of distances between inserts on the chromosome for (A) the *Shigella* chromosome and (B) the *Shigella* virulence plasmid**. The median distance between inserts is indicated by the dotted line. The insets in (A) and (B) indicate the slight but detectable biases in transposon insert location using a weight matrix motif. The reverse cumulative plots show the observed fractions of distances between inserts for the chromosome (C) and the plasmid (D). In blue, the observed frequencies are plotted. In black, the expected frequencies are plotted, given a geometric distribution (negative binomial with the number of successes set to one) of inter-insert distances (see main text). For both the chromosome and the plasmid, there are considerably more large regions uninterrupted by transposons than one would expect given the geometric null model, observed as a shift of the curve to the right.

Fig 2. **Orthologous genes known to be essential in *E. coli* are also essential in *Shigella*.** A region of the *Shigella* chromosome is shown, with genes whose orthologues are known to be essential for growth in *E. coli* (coloured in white) [1, 27], or non-essential (coloured in grey). The unique locations of transposon insertions are plotted as vertical black segments. In the genome region shown here, none of the genes essential in *E. coli* have orthologues that are interrupted in *Shigella*.

Fig 3. Differences in essentiality classification between *E. coli* K12 and *Shigella*. (A) Cumulative distributions showing the mean distances between inserts for ORFs depending on whether their orthologues are known to be non-essential (black curve) or essential (blue curve) in *E. coli*. All ORFs that are completely uninterrupted by transposons have been plotted at the very right end of the x-axis. The dotted vertical line indicates the cut-off that we used to delineate essentiality in *Shigella* (a mean distance between transposons of 250 bp or more). The 17 blue points to the left of the dotted vertical line indicate ORFs that are essential in *E. coli* but not *Shigella* by our metric. These are likely to be false positives (i.e. non-essential in both *Shigella* and *E. coli*), as all have inter-insert distances greater than 100 bp

(see main text). Black points to the right of the dotted vertical line indicate ORFS that we

classify as essential in *Shigella* but not in *E. coli*. Many of these ORFs have *E. coli*

orthologues whose deletion genotypes exhibit robust growth, suggesting that their essentiality

status has changed. (B) A Venn diagram showing the overlap between essential orthologous

ORFs in *E. coli* and *Shigella*.

Fig 4. **Orthologous gene pairs that are non-essential in *E. coli* but inferred as essential in**

***Shigella* (blue) tend to exhibit low growth yields in *E. coli*.** ORFs that we infer to be

uniquely essential in *Shigella* consistently have *E. coli* orthologues with low growth

phenotypes in LB media after 22 hours (apparent as a strong leftward shift in the cumulative

curve). For genes inferred as uniquely essential in *Shigella,* 34% of the orthologous *E. coli*

deletion genotypes exhibit low growth yields (less than 0.5 OD600 after 22 hours of growth

in LB). For genes we classified as non-essential in *Shigella* and *E. coli* only 3.7% exhibit low

growth yields. Thus, some genes we infer as essential in *Shigella* may not be strictly essential,

but instead be required for robust growth. Despite this enrichment for low-growth phenotypes,

there are many genes which we infer as essential in *Shigella*, but which have *E. coli*

orthologues whose deletion genotypes exhibit robust growth (OD600 greater than 0.75 after

22 hours growth in LB).

Fig 5. **Transposon disruption of *Shigella* genes with transport-related functions are**

more likely to be inferred as essential compared to clean deletions of similarly

functioning genes in *E. coli*. We classified genes according to function using the MultiFun

functional classification system [63]. For any category containing more than ten essential *E.*

coli genes, we also calculated the number of *Shigella*-essential genes. As expected, most

categories show a relative excess of *Shigella*-essential genes, as we inferred approximately

50% more genes as being essential in *Shigella* versus *E. coli* (**Fig. 3B**). However, two

functional categories show a clear excess above this level: substrate transport and active

transport, showing a 3- and 2.1-fold increased probability of inferring a gene as being

essential in *Shigella* as opposed to *E. coli*. This provides evidence that genes in these

functional categories may be more likely to be inferred as artefactually essential. For each

functional category (y-axis), we show the number of genes in that category (to the right of

each bar); the number of genes found to be essential in *E. coli* (within each bar); and the level

of enrichment of essential genes in *Shigella* (x-axis).

Fig 6. Three genes involved in proline biosynthesis (*proABC*) appear uniquely essential

in *Shigella*. The orthologous *E. coli* deletion strains exhibit robust growth (OD600 greater

than 0.75 after 22 hours growth in LB), but are essential by our criteria**.** *proA* and *proC*

completely lack transposon insertions*,* while *proB* contains only two insertions near the 3'end,

which leaves approximately 70% of the gene intact, including the entire kinase and substrate-
binding domain.

Fig 7. Both elongation factor paralogues *tufA* and *tufB* appear differentially essential in
***Shigella* as compared to *E. coli*.** The orthologous *E. coli* deletion strains of *tufA* and *tufB*
exhibit robust growth (OD600 of 0.72 and 0.78 after 22 hours in LB), but are essential by our
criteria**.** Both genes contain insertions only at the 5' or 3' ends of the genes. Genes that are
essential in both *E. coli* and *Shigella* are coloured in white. Those inferred as being essential
in *Shigella* but for which the orthologous deletion genotypes exhibit robust growth in *E. coli*
are indicated in blue. Genes inferred as essential in *Shigella* and which do not exhibit robust
growth in *E. coli* are coloured in light blue. tRNA genes are indicated in dark grey.

Fig 8. The region of the genome containing the *rfb* operon is largely uninterrupted by
transposon insertions. *rfbI*, *rfc*, *rfbG*, and *rfbF* are completely uninterrupted by transposon
insertions; *rfbE* is uninterrupted over 90% of its length. None of these genes have orthologous
counterparts in *E. coli* K12 due to a lateral transfer event that occurred at this locus (see main
text). This operon encodes genes active in O-antigen biosynthesis.

S1 Table. Full table of gene characteristics and orthologue relationships used in the analyses.

S2 Table. List of chromosomal SNPs and indels observed in the *Shigella* strain used here that
differ from the GenBank sequence NC004741.

S3 Table. List of plasmid SNPs and indels observed in the *Shigella* strain used here that are
different from the GenBank sequence of the *Shigella flexneri* 2a strain 301 virulence plasmid
pCP301 (NC_004851).

S4 Table. Table listing tRNA genes and the number of insertions in each.

S1 Fig. Distribution of transposon insertions across the genome. We observed little bias
on the chromosomal level of insert locations.

S2 Fig. ROC curves showing the predictive power of various features. To select a feature
that was the best predictor of essentiality in *E. coli* orthologues*,* used only ORFs that we had
data on essentiality from both the Keio and PEC studies. We then selected transposon
insertion patterns that most closely match the essentiality delineations in theses studies.
Specifically, we selected the feature that maximized the number of true positive "essential"
genes (maximizing the sensitivity) while minimizing the number of FP (maximizing
specificity). We selected from eleven (non-independent) features shown here: (1) the total
number of insertions; (2) the mean number of bp between insertions; (3) the median number
of bp between insertions; (4) the number of bp in the 5' end preceding the first insertion; (5)
the number of bp in the 5' end preceding the first insertion relative to the total bp in the gene;

(6) the number of bp in the 5' end preceding the second insertion; (7) the number of bp in the

5' end preceding the second insertion relative to the total bp in the gene; (8) the number of bp

in the longest uninterrupted stretch of the gene; (9) the number of bp in the longest

uninterrupted stretch of the gene relative to the total length of the gene, and (10) the number

of bp in the longest stretch of the gene interrupted by at most one insertion; (11) the number

of bp in the longest stretch of the gene interrupted by at most one insertion, relative to the

total length of the gene. See the **Methods** section for more details of this analysis.

S3 Fig. **Analogous plots to that shown in Fig. 3, for growth in minimal glucose MOPS**

media after (A) 24 and (B) 48 hours. In both cases, we find that the shift is less pronounced

than that observed for LB.

S4 Fig. **Schematic of the primer positions used for Illumina sequencing of transposon**

insertions.

S5 Fig. Inferred fragment lengths of perfectly mapped reads across several genomic

regions. For each plot, the inferred fragment lengths are arranged by increasing length

(ranked on the y-axis). Thus, very long fragments are present at the top of the y-axis. Most

fragments have lengths between 100bp and 400bp; a small number have lengths over 1000bp

or more. It is very likely that these are not the true insert sizes, but appear that way because of

large scale deletions in our *Shigella* clone compared to the clone present in the NCBI genome

database; see **Methods** for more details. (A) A region of the chromosome in which a

complicated series of rearrangements has occurred, leading to paired end reads perfectly

matching to different locations in this region. 45 mapped read pairs span more than 1.5 Kbp, a

size that is not concordant with the majority of insert sizes. (B) A genomic region where an

approximately 10Kbp deletion occurred, removing a region containing the *yeaKLMNOP*

operon. 92 mapped read pairs span more than 8.5Kbp. This region is flanked by two IS

elements. (C) A region where an approximately 4Kbp deletion occurred, removing two genes

with no *E. coli* K12 orthologues. 68 mapped read pairs span more than 4 Kbp, and again this

region is flanked by two IS elements. (D) A genomic region where an approximately 2Kbp

deletion occurred, removing *yhdW*. 244 mapped read pairs span more than 2Kbp, and the

region is flanked by two IS elements. (E) A deletion in the region of the chromosome

containing *S4145 (yiaN)*. 232 mapped read pairs spanned more than 1.8 Kbp, and this region

is also flanked by two IS elements. (F) A region of the chromosome containing the *rfb* operon.

Most of the genes within this operon are uninterrupted by transposons. However, we find no

evidence that this is due to a deletion of this region in our *Shigella* clone, as we find no reads

mapping across the region; a small number of reads map within the region; and the closest IS

elements are 15 Kb upstream of *rfbJ* and 20 Kb downstream of *rfbA*. The genes in this operon

have no orthologues in *E. coli* K12.

S6 Fig. **Functional categories of non-essential *E. coli* genes that are enriched (or**
depleted) for essential *Shigella* genes. We classified genes according to function using the
MultiFun classification system [63]. For this analysis we considered only genes that are non-
essential in *E. coli*. We find that genes uniquely essential in *Shigella* are enriched in some
functional categories. For example, of the 27 ribosomal proteins identified as non-essential in
E. coli, we identify approximately 45% as being essential in *Shigella.* In contrast, of the 565
membrane proteins identified as non-essential in *E. coli*, we find that less than 10% are
essential in *Shigella.* Thus, ORFs uniquely essential in *Shigella* are far more likely to function
in the ribosome than one would expect. The number of non-essential *E. coli* genes is indicated
above each bar; the probability of finding the level of enrichment (or depletion) that we
observe in each secondary category is indicated for cases in which this probability is less than
0.025, using a Fisher exact test. (A) All non-essential genes in *E. coli*. (B) An identical
analysis excluding all non-essential genes in *E. coli* that exhibit very low growth yields
(OD600 less than 0.5 after 22 hours of growth LB). In both cases, the only subcategory
notably enriched for essential genes is that containing ribosomal proteins. The only categories
appreciably depleted for genes with essential function are genes with function in RNA
processing and to some extent, energy production.

S7 Fig. Transposon insertion locations across the entire *Shigella* chromosome. Each
insertion site is indicated by a vertical red line. ORFs are indicated in light green; rRNAs
in dark green; and tRNAs in dark blue. The annotation is taken from the GenBank sequence
of *Shigella flexneri* 2a 2457T.

Fold−enrichment of Shigella−essential genes

1: Arbitrary PCR
using phusion PCR enzyme, so that no A's added at 3' end

```
1_transp_deep_seq_F1:  TCTATCGCCTTCTTGACGAG
```

```
1_transp_deep_seq_R2: GTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT NNNNNNNNNNggtgc
1_transp_deep_seq_R3: GTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT NNNNNNNNNNgatat
1_transp_deep_seq_R4: GTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT NNNNNNNNNNagtac
```

2: Nested PCR (addition of P7 and P5)
using phusion PCR enzyme, so that no A's added at 3' end

```
2_transp_deep_seq_ FP5:  AATGATACGGCGACCACCGAGATCT actggtcggcg CATTAGGGGATTCATCAG
```

```
Rev_P7 6_02
CAAGCAGAAGACGGCATACGAGAT agtctt GTGACTGGAGTTCAGACGTGT
CAAGCAGAAGACGGCATACGAGAT gtatct GTGACTGGAGTTCAGACGTGT
CAAGCAGAAGACGGCATACGAGAT tcatgg GTGACTGGAGTTCAGACGTGT
CAAGCAGAAGACGGCATACGAGAT cgcgac GTGACTGGAGTTCAGACGTGT
CAAGCAGAAGACGGCATACGAGAT acgata GTGACTGGAGTTCAGACGTGT
CAAGCAGAAGACGGCATACGAGAT tataga GTGACTGGAGTTCAGACGTGT
```

double stranded PCR product,
gel purify band/smear at (300-400bp)
(insert at 200-300bp)

3: Sequencing on Illlumina chip

```
transp_deep_seq_Custom seq 1:  actggtcggcgCATTAGGGGATTCATCAG
```

4: Barcode is read on Illlumina chip

```
transp_deep_seq_Index seq:  GATCGGAAGAGCACACGTCTGAACTCCAGTCAC
```

51 – 101 Kb

151 – 201 Kb

201 – 251 Kb

ldcC yaeR tilS yaeO yaeQ yaeJ cutF yaeF proS yaeB rcsF metQ yaeE metN yaeD rrsH ileV alaV rrlH metU asaU dkgB S0201 yafC yafD yafE mltD gloB yafS rnhA dnaQ aspV safA S0212 safB safC S0215 sat1 sat2 S0219 S0220 S0221 S0223 S0224 S0229 S0230 S0231 S0232 S0233 S0234 S0235 S0236 S0237 S0238 S0239 S0240 S0241 S0242 S0243 S0244 S0245

|205 kb |210 kb |215 kb |220 kb |225 kb |230 kb |235 kb |240 kb |245 kb |250 kb

5 kb

301 – 351 Kb

351 – 401 Kb

malZ queA tgt yajC secD secF yajD tsx yajI nrdR ribD ribH nusB thiL pgpA dxs ispA xseB yajK thiJ apbA yajQ S4809 S4810 S0373 S4811 S4812 S0373 cyoE cyoD cyoC S0379 S0380 S0383 ampG yajG bolA tig clpP clpX lon hupB ybaU ybaV ybaW ybaX

355 kb   360 kb   365 kb   370 kb   375 kb   380 kb   385 kb   390 kb   395 kb   400 kb

5 kb

401 − 451 Kb

451 – 501 Kb

5 kb

501 – 551 Kb

551 – 601 Kb

5 kb

601 – 651 Kb

5 kb

651 – 701 Kb

701 – 751 Kb

751 – 801 Kb

801 – 851 Kb

5 kb

851 – 901 Kb

901 – 951 Kb

951 – 1001 Kb

5 kb

1001 – 1051 Kb

1051 – 1101 Kb

5 kb

1101 – 1151 Kb

5 kb

1151 – 1201 Kb

1201 – 1251 Kb

5 kb

1251 – 1301 Kb

S1289 ychF pth ychH ychM prsA ipk lolB hemA prfA hemK S1300 ychA kdsA chaA chaB chaC ychN ychP narL narX narK narG narH narJ narI tyrT purU ychJ ychK hnr galU hns tdk S1325 adhE ychE S1328 S329 oppA oppB oppC oppD

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S3115 S3116 S3117 S3118 yggD yggF S3122 S3124 S3126 tktA yggG speB S3131 S3132 speA yggC yggD metK galP sprT endA yggJ gshB yggE yggF yggR yggS yggT yggU yggV yggW S3151 S3152 S3153 S3154 S3155 yggM ansB yggN yggL trmB mutY yggX mltC nupG speC yggA pheV S4822 S3169

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4201 – 4251 Kb

ftsE ftsX rpoH livJ yhhK livK livH livM livG livF ugpB ugpA ugpE ugpQ yhhA ggt yhhY yhhX yhhW gntR gntK gntU yhgN asd glgB glgX glgC glgA glgP S4313 S4314 glpD glpE glpG glpR rtcR rtcB yhgJ malP

|4.205 Mb |4.21 Mb |4.215 Mb |4.22 Mb |4.225 Mb |4.23 Mb |4.235 Mb |4.24 Mb |4.245 Mb |4.25 Mb

5 kb

4251 – 4301 Kb

5 kb

4301 – 4351 Kb

5 kb

4351 – 4401 Kb

5 kb

4401 – 4451 Kb

5 kb

4451 – 4501 Kb

5 kb

4501 – 4551 Kb

5 kb

4551 – 4601 Kb

5 kb