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Title

## Abstract

### 1 Introduction

Generating genomic variants that carry genes responsible for essential cellular processes can open up new research directions. Adding genes or metabolic pathways to cells provides new variants with specialised phenotypes. Modified cells have different potential applications in biotechnology [Juhas et al., 2014], fuel production [Seo et al., 2013], healthcare, and food production [Juhas et al., 2013]. Another important application for studying essential genes is in drug discovery [Juhas et al., 2012]. Infectious diseases are among the top major causes of mortality worldwide. Even though antibiotic resistance is growing among bacteria, the antibiotic discovery and development rate is diminishing [Fischbach and Walsh, 2009, Nathan, 2004]. Therefore, it is urgent to find new drugs for infectious diseases. New antibiotics target genes that are essential for the survival of pathogenic bacteria in order to control disease. Some well-known antibiotics that target essential functions are tetracyclines that bind to small ribosomal subunit and interfere with protein translation [Brodersen et al., 2000], penicillins that target peptidoglycan and inhibit the cell wall synthesis [Chung et al., 2009], and quinolones that target DNA Gyrase [Marcusson et al., 2009].

In the earliest attempt for the identification of essential genes, Mushegian and Koonin compared the genomes of *Haemophilus influenzae* and *Mycoplasma genitalium*, assuming that the genes that are shared in these two phylogenetically distant bacteria are indispensable and reported 256 genes fulfilling this requirement [Mushegian and Koonin, 1996]. With the advent of sequencing technologies and availability of more genome sequences,

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the number of core genes in different prokaryotic genomes declined to less than 50 which is not enough for performing all essential functions in a cell [Charlebois and Doolittle, 2004]. Therefore, the use of experimental methods for the identification of essential genes is vital. Researchers have now studied the essential genes in organisms from all three domains of life [Luo et al., 2014] using a number of different methods. Baba et al. [Baba et al., 2006] made a library of single gene deletions for *Escherichia coli* K-12. The 303 genes where viable *Escherichia coli* colonies failed to grow are the candidate essential genes. Another group used an antisense RNA knockdown approach to study gene essentiality in *Staphylococcus aureus* [Forsyth et al., 2002]. In this method, if the expression of an antisense RNA hinders the growth of the cell, its cognate gene is known as essential. Both of these methods are labour intensive and are dependent on the accuracy of genome annotations. Another widely used procedure is transposon mutagenesis combined with high-throughput sequencing [Chao et al., 2016, van Opijnen and Camilli, 2013, Barquist et al., 2013a] which includes different approaches namely, Tn-Seq [van Opijnen et al., 2009], INSeq [Goodman et al., 2009], HITS [Gawronski et al., 2009] and TraDIS [Langridge et al., 2009]. These procedures differ in the type of transposon, sample preparation methods, and data analysis [van Opijnen and Camilli, 2013]. Nonetheless, all share the same workflow: pools of single insertion mutants are constructed using transposon mutagenesis. After a growth phase, mutants that are fitter outnumber the less fit ones. Using high-throughput sequencing and tallying transposon junctions gives an indication of whether a genomic region is essential or not. A high number of transposon insertions in a gene indicates that the gene is not essential in its growth medium and conversely, a low number of transposon insertions indicates that a gene is essential in the medium.

There are two groups of essential genes: core essential genes are indispensable for all cells, and accessory essential genes that are required for some organisms. Core essential genes can shed light on the genome structure of the last universal common ancestor and the evolution of living cells [Koonin, 2003] and have been used for the synthesis of minimal cells [Hutchison et al., 2016]. On the other hand, accessory essential genes are helpful in the study of specific lineages. Accessory essential genes may be useful in species-specific antibiotic discovery. Antibiotics that target core essential genes in pathogens may not be

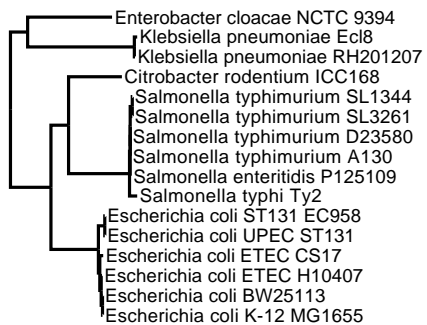
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ideal, as they may attack homologous genes in their hosts or commensal bacteria.

Freed et al. [Freed et al., 2016] have investigated the difference between essential genes in *Shigella flexneri* 2a 2457T and *Escherichia coli* K12 BW25113 and shown that there are no genes that are essential in *Escherichia coli* and not essential in *Shigella flexneri*, while some genes are only essential in *Shigella flexneri*. These include a group of genes involved in cysteine, proline and sugar nucleotide biosynthesis, acetate utilisation, translation elongation, aminoacyl tRNA synthetase, murein DD-endopeptidase, and soxR-reducing complex, many of which are essential in *Shigella flexneri* due to the absence of paralogs or other alternative systems that exist in *Escherichia coli*.

Canals et al. [Canals et al., 2012] have compared the essentiality of genes in *Salmonella typhimurium* ATCC 14028, two isolates of *Salmonella typhi* Ty2 (varying in *htrA*, *aroC* and *aroD* genes), and *Escherichia coli* K12 BW25113 and found that these cells share 268 essential genes. Nine genes are essential in *Escherichia coli* and not essential in *Salmonellas*, three of which can tolerate insertions only in some parts and one has distant paralogs in *Salmonellas* that is missing in *Escherichia coli*. For other genes, there are a few insertions in *Salmonellas*, but the number is not small enough to call those genes essential. Moreover, 159 genes were almost essential in all *Salmonellas* but not in *Escherichia coli*. These include genes involved in replication and genes related to ribosome and its accessory proteins. The authors also found 26 genes that are under greater selection in *Salmonella Typhimurium* compared to *S. typhi* and 10 genes vice versa. Barquist et al. [Barquist et al., 2013b] have used transposon-directed insertion-site sequencing to compare the essentiality of genes in *Salmonella Typhi* Ty2, *Salmonella Typhimurium* SL1344, and *Escherichia coli* K12 BW25113. These genomes share 228 essential genes which are mostly involved in cell division, transcription, translation, and fatty acid and peptidoglycan biosynthesis. Additionally, many of the serovar-specific essential genes in *Salmonellas* are phage repressors. Another key difference between these two serovars is that a set of genes that are putatively involved in cell wall biosynthesis are essential in *Salmonella Typhimurium* and not essential in *salmonella Typhi* which gives an indication of the adaptation of these two *Salmonella* serovars to their niches.

Although there are some studies on differentiation of essentiality in different organisms,



**Figure 1.** The species tree containing the 16 genomes used in this study. We have generated the tree by running RAXML [Stamatakis, 2014] on Phylosift [Darling et al., 2014] amino acid markers.

these studies usually include a few genomes. We are going to extend these studies to a larger scale in Enterobacteriaceae family. Our aim is to study the essentiality of genes in in 16 different organisms. These include *Enterobacter cloacae* NCTC 9394, *Klebsiella pneumoniae* Ecl8, *Klebsiella pneumoniae* RH201207, *Citrobacter rodentium* ICC168, *Salmonella* Typhimurium SL1344, *Salmonella* Typhimurium SL3261, *Salmonella* Typhimurium D23580, *Salmonella* Typhimurium A130, *Salmonella* Enteritidis P125109, *Salmonella* Typhi Ty2, *Escherichia coli* ST131 EC958, *Escherichia coli* UPEC ST131, *Escherichia coli* ETEC CS17, *Escherichia coli* ETEC H10407, *Escherichia coli* BW25113, and *Escherichia coli* K-12 MG1655. The phylogenetic tree containing all these species is depicted in Fig. 1.

## 2 Results and Discussion

### 2.1 Incorporating multiple measures of gene essentiality improves prediction

In this section we will compare a number of methods of quantifying the essentiality of genes based on transposon insertion data and introduce our own method.

#### 2.1.1 Comparison of old methods

A number of methods have been used for evaluating the essentiality of genes using transposon insertion data. Freed et al. [Freed et al., 2016] have compared eleven features that can quantify the essentiality of genes. These include: the number of insertion sites

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within genes; the mean distance between insertion sites, the median distance between 99  
insertion sites, the number of base pairs before the first insertion in the 5' end, the ratio 100  
between the number of base pairs before the first insertion in the 5' end and gene length, the 101  
number of base pairs before the second insertion in the 5' end, the ratio between the number 102  
of base pairs before the second insertion in the 5' end and gene length, the length of the 103  
longest uninterrupted region in the gene, the ratio between the length of the longest 104  
uninterrupted region in the gene and gene length, the length of the longest region in the gene 105  
containing at most one insertion divided by gene length, and the number of insertion sites 106  
divided by gene length. Among these, the average distance between insertion sites and the 107  
length of the longest uninterrupted region were the best predictors. 108

Barquist et al. [Barquist et al., 2016] have used insertion indices which are calculated by 109  
dividing the number of insertion sites by gene length for defining the essentiality of genes. 110  
Plotting all insertion indices for all genes in a genome gives a bimodal plot, each mode 111  
representing a group of genes (essential or non-essential). They have fitted two gamma 112  
distributions to these modes and calculated the log odds value for each gene to test which 113  
mode it belongs to. 114

Turner et al. [Turner et al., 2015] have randomly sampled insertions in a genome and 115  
using DESeq package [Anders and Huber, 2010] calculated log fold changes and P-values for 116  
the actual number of insertions compared to the expected number of insertions obtained 117  
from the sampling method. 118

We have compared the predictive power of the average distance between insertion sites in 119  
a gene, the length of the longest uninterrupted region, insertion index, log odds value 120  
obtained from insertion index value, and P-value and log fold change calculated using DESeq 121  
package after sampling. To evaluate the accuracy of these methods, we have compared the 122  
predicted essential genes to the essential genes in *Escherichia coli* K-12 MG1655 in EcoGene 123  
database [Zhou and Rudd, 2013]. The results are depicted in Figure 2a. Among all methods, 124  
those that try to fit a distribution to data and predict essentiality based on that, namely log 125  
odds value from insertion indices and P-value from DESeq after sampling, are the least 126  
accurate methods. Sampling and then calculating log fold changes using DESeq package is 127  
the most accurate predictor and the other three methods are also good predictors despite 128

their simplicity.

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### 2.1.2 PCA based method

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We selected four of the predictors in previous section: insertion index, log fold change using  
sampling and DESeq, the average distance between insertion sites in a gene, and the length  
of the longest uninterrupted region. These predictors were selected as they were more  
accurate and less dependent on each other. Afterwards, we ran principal component analysis  
on these predictors using *prcomp* function in R and selected the first principal component.  
As Figure 2a shows, PCA results gave us the most powerful tool for quantifying the  
essentiality of genes compared to other methods. We have plotted the results for one sample  
genome and the results for other genomes are shown in **SUPPLEMENTARY**. Figure 2b  
shows the distribution of PCA results after zero mean unit variance normalisation. We call  
this value NPEQ (Normalised Pca based Essentiality Quantifier) in this paper. The genes  
whose NPEQ values are less than -1.644854 after normalisation are beneficial losses and the  
genes with NPEQ greater than 1.644854 are essential. The rest are non-essential genes. The  
cutoff 1.644854 has been selected because if we assume that NPEQ distribution is normal,  
this cutoff shows the P-value 0.95. Moreover, by trying to define a cutoff that maximises  
Matthews correlation coefficient for each genome using NPEQ, we get 1.650449 as the  
average cutoff which is very close to this value.

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## 2.2 TraDIS data is biased

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In Section 2.1.2 we proposed NPEQ method that quantifies the level of essentiality of a gene.  
However, if the transposon insertion is biased to specific regions in the genome, it can  
increase/decrease the values predicted by our method for genes and put them in a different  
level of essentiality. Different articles have reported biases in transposon insertion using  
Tn5 [Barquist et al., 2013b, Green et al., 2012, Rubin et al., 2015, Canals et al.,  
2012, Langridge et al., 2009]. We performed a detailed study of these biases. The biases that  
we studied include: origin of replication bias, preferred insertion motif bias, and positional  
bias within genes.

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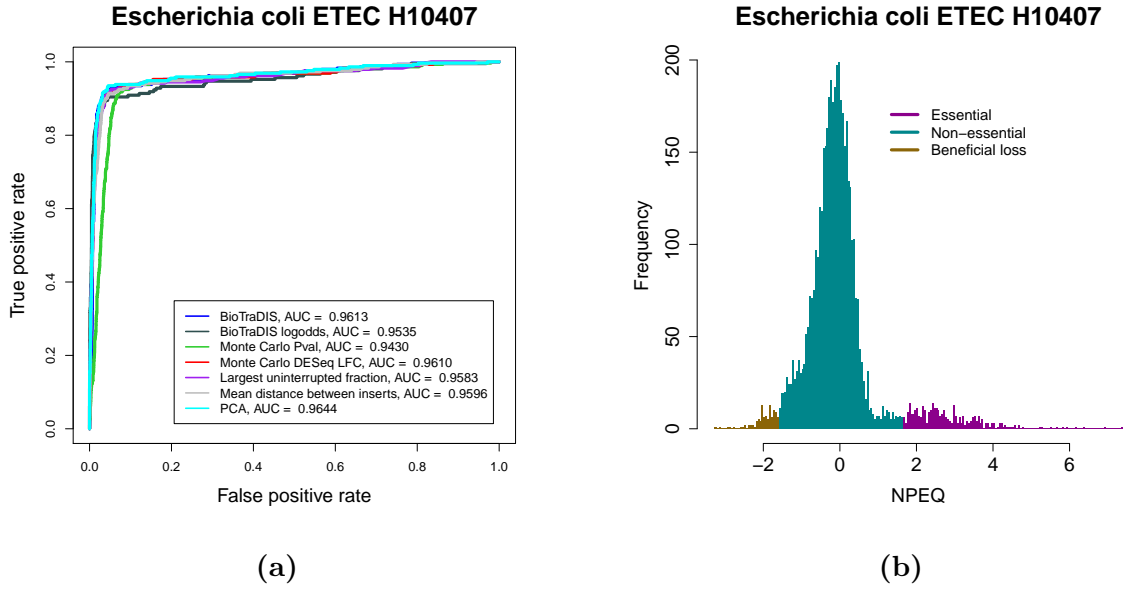
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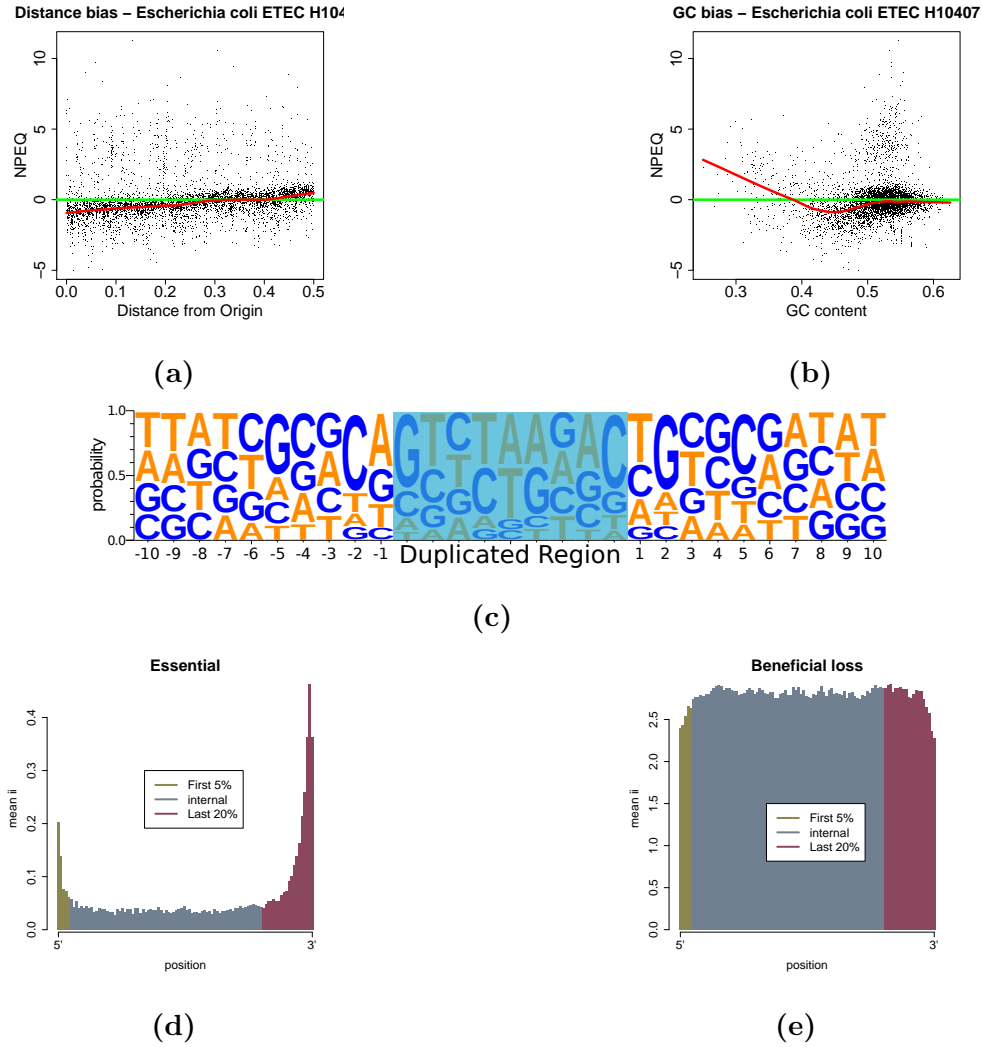
**Figure 2.** (a) The accuracy of 7 different prediction methods for quantifying the essentiality of genes. The higher the area under the curve, the more accurate the method is. (b) The distribution for our proposed method (NPEQ)

### 2.2.1 Distance from the origin of replication bias

While a study has reported Tn5 insertion bias near the origin of replication [Barquist et al., 2013b], another study has reported no bias [Rubin et al., 2015]. To study the bias towards the position of gene within genome, we plotted NPEQ for each gene versus the distance of the gene from the origin of replication normalised by the length of the genome in Fig. 3a. The figure indicates that NPEQ increases when the genes are located further from the origin of replication. When the bacteria are under replication during the transposon insertion process, there are more copies of the genes close to the origin of replication than the genes further away due to the initiation of different replication forks. This results in more insertions in the genes near the origin of replication which can influence the accuracy of our predictions.

### 2.2.2 Preferred nucleotide composition bias

Another concern while inferring essentiality from transposon insertion data is that transposon insertion is biased to certain compositions of nucleotides and high number of insertions in genes reflects the enrichment of the motifs that transposon insertion is biased



**Figure 3.** (a) The distance of the genes from DnaA gene normalised by the lengths of the genomes versus NPEQ. The distance from DnaA gene has been calculated in both directions and then the minimum value has been used for distance. The red curves show the loess curve when the smoothness parameter is 0.2 and the green line shows no bias. (b) G-C content of genes against their NPEQ values. The red curves show the loess curve when the smoothness parameter is 0.2 and the green line shows no bias. (c) Sequence logo plots generated using sequences from 10 nucleotides flanking the 100 top most frequent insertion sites from each genome. The height of each character shows the relative frequency of that character. (d) and (e) The plots show the average insertion index in percentiles of all essential genes (d) and beneficial losses (e). The genes are divided into 3 segments: 5% of the genes on the 5' end, 20% of the genes on the 3' end, and the rest in the middle. These are shown by khaki, slate gray, and violet red respectively.



towards, instead of their essentiality level. During Tn5 transposition, a sequence of 9 nucleotides is duplicated. Lodge et al. [Lodge et al., 1988] showed that these duplicated regions have G-C pairs at two ends and replacing these G-C pairs with A-T pairs reduces the number of transposon insertions by more than fivefold. Goryshin et al. [Goryshin et al., 1998] reported the palindromic sequence A-GNTYWRANC-T as the consensus target site for Tn5 transposition where the 9 letters in middle show the consensus sequence in the duplicated region. In some other research a similar consensus motif has been found for Tn5 [Canals et al., 2012] which is CGCGCA-GTTYWRAAC-TGCGCG. Others [Green et al., 2012, Rubin et al., 2015] have not found such a sequence but shown that the duplicated regions are G-C rich. We used Weblogo [Crooks et al., 2004] to generate a logo from duplicated regions and 10 nucleotides flanking the 100 top most frequent insertion sites in each genome. The results in Figure 3c show the consensus motif that we have found is very similar to [Green et al., 2012] and the only difference is in positions 3 and 7 within the duplicated region.

The other possible source of bias is if transpositions are more inclined to G-C or A-T rich regions. Rubin et al. [Rubin et al., 2015] have reported that the number of Tn5 insertions rises with the increase of G-C content and Green et al. [Green et al., 2012] have shown that the highest number of insertions occur in high G-C content regions. On the other hand, Langridge et al. [Langridge et al., 2009] have seen an increase in the number of Tn5 insertions in 40% G-C content. In Figure 3b, we plotted the G-C content of genes versus their NPEQ values. The red curves are loess curves with smoothness parameter 0.2 and the green line shows no bias. NPEQ decreases gradually (which means an increase in the number of insertions) as G-C content decreases and then somewhere between 40% and 50% G-C content, NPEQ starts to rise again in almost all genomes. In the region where most of the genes are packed, the loess curve is almost flat. On the left side of this flat region, there are genes with different G-C content which are enriched in mobile genetic elements. So, we expect to see more insertions (smaller NPEQ values) in this region. We have low NPEQ values between 40% and 50% G-C content which is expected. However, in most cases when we have less than 40% G-C content, NPEQ value is high. A possible reason for this phenomena is the association of A-T rich sequences and histone-like nucleotide structuring (H-NS) proteins, which reduces the insertions in A-T rich regions. This has been shown for

Tn10 transposon [Kimura et al., 2016], but not for Tn5 transposon, yet. Overall, the results are consistent with Langridge et al. [Langridge et al., 2009]. The G-C content of the most of the genes with large NPEQ values is between 50% and 60% which is inconsistent with Green et al. [Green et al., 2012] as this region contains most of the genes and is not considered as a high G-C content region but rather an average G-C content region.

### 2.2.3 Positional bias within genes

Some research has indicated that himar1 transposons are more probable to get inserted into the two ends of a gene compared to the middle [Griffin et al., 2011]. We have tested this hypothesis using our TraDIS data. We divided every gene into 100 fragments with equal lengths (percentiles) and calculated the mean insertion index for each percentile. Insertion index is calculated using  $\frac{n_p}{l_p} \cdot \frac{l_g}{n_g}$ , where  $n_p$  is the number of insertion sites in a specific percentile,  $l_p$  is the length of that percentile,  $n_g$  is the number of insertion sites in the whole genome and  $l_g$  is genome length. Mean insertion index for each percentile is calculated by averaging over all insertion indices for that specific percentile of genes. We saw almost no bias towards any location when considering all genes together (Fig. ?? SUPPLEMENTARY). We studied the bias in three different groups of genes: essential genes which have no or just a few insertions, non-essential genes which have an intermediate number of insertions, and beneficial losses which have a high number of insertions. The results imply that the number of insertions in the internal region of the essential genes is outnumbered by the number of insertions in the 5' and 3' ends (Fig. 3d) while it is the opposite in beneficial losses (Fig. 3e). High number of insertions at the 3' end of essential genes implies that the functional domains are located before the insertions and the insertions are not interfering with them. On the other hand, high number of insertions at the 5' end of the essential genes indicates there might be alternative start codons in the 5' end or it might be because of annotation errors that have predicted the start codon in an incorrect place before the actual start codon.

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## 2.3 Most essential genes are ubiquitously essential in

### Enterobacteriaceae

Previous studies of gene essentiality in Enterobacteriaceae family have compared essential genes in different genomes in this family and studied the sets of core essential genes and accessory essential genes [Freed et al., 2016, Canals et al., 2012, Barquist et al., 2013b]. Core essential genes are responsible for essential processes such as cell division, DNA replication, transcription and translation and some important pathways like peptidoglycan and fatty acid biosynthesis [Barquist et al., 2013b]. Accessory essential genes differ in genomes due to different reasons such as niche adaptation, gene duplication and the existence of alternative pathways [Freed et al., 2016, Canals et al., 2012, Barquist et al., 2013b]. Another group of accessory essential genes are phage repressors [Barquist et al., 2013b]. Even though these genes are not essential for the growth of a cell, once phages are introduced to a cell, they become essential as long as the phage remains in the cell.

In this study, we have compared the genes in 16 bacteria from Enterobacteriaceae family. For this we needed to study core and accessory sets of genes. Moreover, in the presence of two redundant variations of one gene, if we knock out one copy using TraDIS, the other copy compensates and the organism can still survive [Dean et al., 2008]. This leads to a different essentiality inference using TraDIS. Therefore, in addition to core and accessory genes, we have studied duplicate genes. To study whether each gene in the 16 organisms is core we used Jackhmmer from HMMER package [Eddy, 2011] to iteratively search for homologous proteins in our dataset and cluster them. We divided the clusters of homologous genes into three groups based on their conservation. Genus specific class contain genes that are present only in one genus, the genes in single copy class are present in more than one genus and more than 70% of them are not duplicated (core genes), and the genes in multi-copy class are present in more than one genus and more than 30% of them are duplicated (duplicate genes).

We also evaluated the essentiality of genes and divided the genes into different levels of essentiality using NPEQ values. If NPEQ is less than -1.644854, it means that the gene has tolerated many insertions, so it is beneficial for the organism to lose this gene in the rich medium that we used. However, if NPEQ is greater than 1.644854, the gene can tolerate very

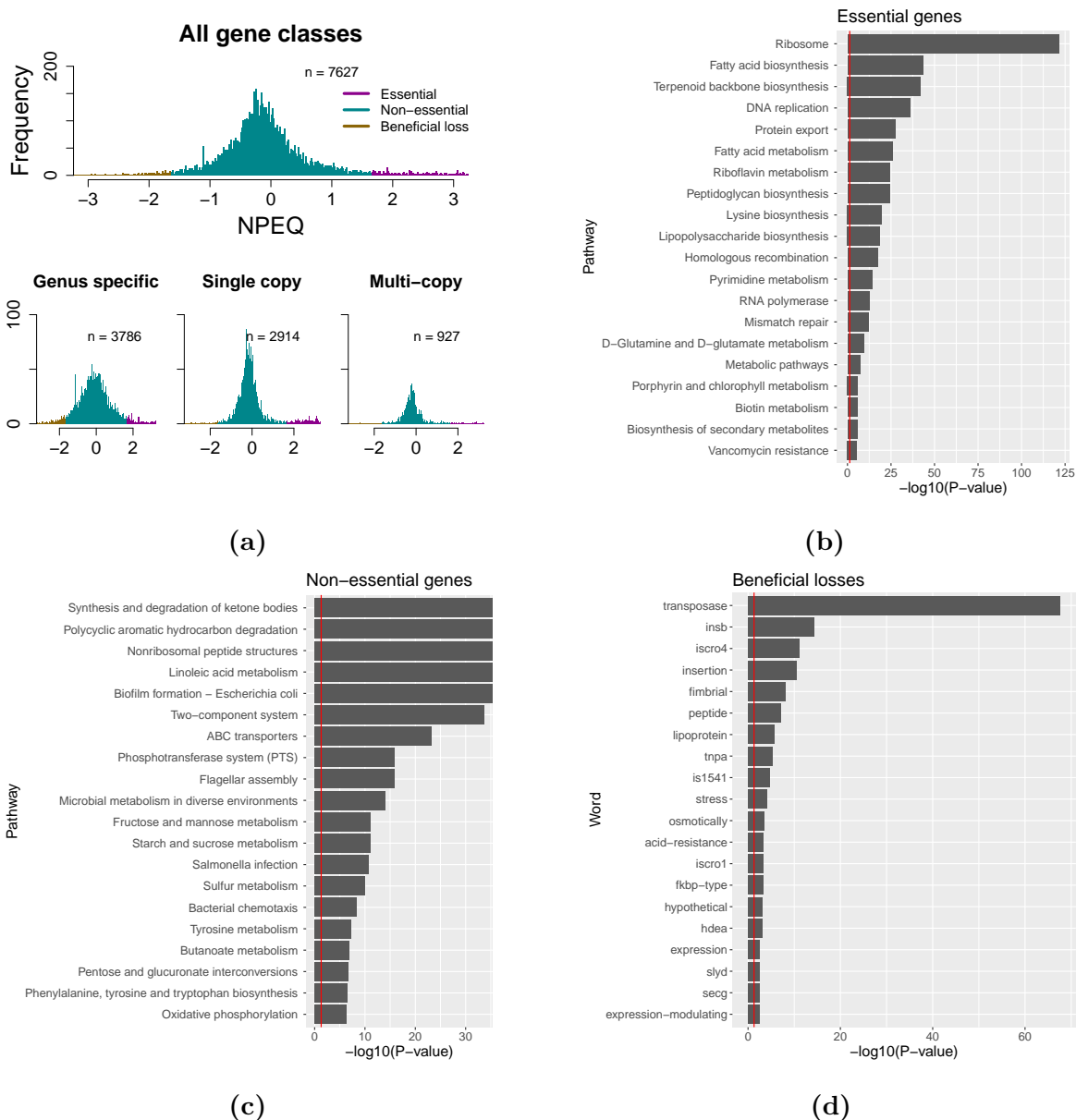
few or no insertions indicating that the gene is essential for cell viability in our test medium. 254  
Any other NPEQ value shows an intermediate number of insertions in genes meaning that it 255  
is not beneficial for the organism to lose these genes, but they are not essential, too. We 256  
have called this group of genes non-essential. 257

The results for comparing three levels of essentiality and three classes of conservation are 258  
depicted in Fig. 4a. The high number of single copy clusters in essential level, indicates that 259  
there is a set of essential genes in Enterobacteriaceae that are conserved and inclined to keep 260  
their essentiality. However, there are also many essential genus specific genes. The figure 261  
also shows that beneficial losses are over represented in genus specific class. Therefore, 262  
beneficial losses are mostly recent genes that the organism tends to lose in the long run. 263  
Besides, most of the multi-copy clusters are non-essential and there are only a few multi-copy 264  
clusters that are essential. This can be explained by the redundancy that duplicate genes 265  
can keep even after  $\sim 100$  million years [Dean et al., 2008]. 266

To study which functions are enriched in each class of essentiality, we used KEGG 267  
pathway enrichment analysis [Kanehisa and Goto, 2000] and compared the genes in each 268  
class with the databases that were available for the genomes in this study. The results show 269  
that essential genes are enriched in pathways related to genetic information processing such 270  
as replication (DNA replication, homologous recombination and mismatch repair), 271  
transcription (RNA polymerase), translation (ribosome), and protein export. These are the 272  
essential functions that every cell needs for its viability. Other enriched pathways are mostly 273  
metabolism related. These include fatty acid biosynthesis that produce cell membrane, 274  
peptidoglycan and lipopolysaccharide biosynthesis that are essential components of cell wall, 275  
terpenoid backbone biosynthesis which feeds peptidoglycan biosynthesis, nucleotide and 276  
amino acid metabolism, and the metabolism of important cofactors and vitamins like 277  
riboflavin, biotin, porphyrin and chlorophyll (Figure 4b). CAN WE HAVE SOMETHING 278  
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Non-essential genes are mostly involved in carbohydrate, energy, and lipid metabolism, 280  
membrane transport, cell motility and cellular community. Even though these functions are 281  
important, they are not essential in vitro and in a rich medium. 282

As most of beneficial losses do not have homologs in other genomes, most of them are not 283



**Figure 4.** (a) The genes have been clustered into homologous groups using Jackhmmer and divided into 3 groups: genus specific, single copy, and multi-copy genes. Then, the essentiality of the clusters has been defined using the insertion indices of the genes in the clusters. The figure shows that most of the essential genes are in single copy group, while most of the beneficial losses are genus-specific. (b) and (c) KEGG Pathways enriched in essential genes (b) and non-essential genes (c). (d) The words enriched in the description of beneficial losses in their embl files compared to other genes. The red line shows  $P\text{-value} = 0.05$ . The  $P\text{-values}$  are calculated using hypergeometric test and then corrected using Benjamini-Hochberg-Yekutieli procedure.

studied and therefore there is no KEGG pathway for them. Because of this reason, we  
studied the description of these genes in their embl files and found the words that were  
enriched. These are shown in Figure 4d. The results show that most of beneficial losses are  
mobile genetic elements like transposases and insertion elements which are not essential in  
their host genomes, genes that are not essential in a rich medium like stress protein and  
acid-resistance protein, and hypothetical proteins.

## 2.4 phylogenetic analysis of gene essentiality identifies ...

## 3 Materials and Methods

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