

Potential confounding mutations in Keio knockout strains: implications for research accuracy

Oishi Sen,^{1,2} Xianghui Liu,¹ Staffan Kjelleberg,^{1,2} Scott A. Rice,^{1,3,4} Thomas Seviour^{1,5}

AUTHOR AFFILIATIONS See affiliation list on p. 8.

ABSTRACT The Keio library of single-gene knock-out mutants of *Escherichia coli* is useful for the research community. It has been used to analyze the role of various *E. coli* genes in alcohol tolerance, multi-drug resistance, and biofilm formation. The current study provides a general overview of potential single nucleotide polymorphisms (SNPs), insertion–deletion of bases (≤ 50 nucleotides, INDELs) in the genome of a set of 21 knock-out mutants of the Keio collection in comparison to the parent strain. A small number of SNPs and INDELs were predicted in the coding and intergenic regions of the knock-out mutants in comparison to the parental strain through sequencing and bioinformatic analysis. Mutations in the coding regions of genes (different from the actual gene knocked out in the mutants) led to different types of mutations in the affected genes, ranging from nonsense mutations to frameshift mutations, which could affect the functionality of the resulting gene products. These mutations in the intergenic and coding regions could lead to phenotypic differences in the single-gene knock-out mutant strains in comparison to the parent strain, independent of the desired gene deletion. This, in turn, could be misinterpreted by researchers using these strains as differences caused by the missing gene. While this is a preliminary study based on only 21 strains of the Keio collection, the deleted genes in the mutants used in this study were approximately evenly distributed across the entire genome. This study likely indicates the possibility of such mutations in other Keio strains, although a larger sample size of knock-out mutants would be required to understand the likelihood of such mutations across the library.

IMPORTANCE The Keio library of single-gene knock-out mutants of *Escherichia coli* has been widely used for a variety of studies. However, mutations might appear in the genome of these strains over time, leading to differences in the characteristics of the mutant and parent strains that are independent of the gene deletions of interest. This study predicts the presence of a few SNPs and INDELs in some of the knock-out mutants from the Keio collection, which could potentially alter the phenotypic attributes of the knock-out mutants with no role of the deleted gene towards this change. Therefore, this study highlights the possibility of the presence of such mutations in other strains of the library and the importance of conducting additional steps, such as complementation assays, to confirm the outcomes of studies comparing specific attributes of the knock-out mutants with the parental strain.

KEYWORDS *Escherichia coli*, gene knockout, whole genome sequencing, genomic mutations

Knocking out non-essential genes from the parent bacterial strain offers a valuable way of studying the role of individual genes in bacterial fitness under various environmental conditions. However, the process of knocking out genes can be time-consuming. Thus, a comprehensive library of almost all non-essential, single-gene

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Address correspondence to Thomas Seviour, twseviour@bce.au.dk.

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knock-out mutants from *Escherichia coli* BW25113 was developed and made available to the scientific community (i.e., the Keio collection) (1). This has subsequently been widely used for many biological studies (2–16), especially given that many fundamental biological processes are often first studied in *E. coli* as a model bacterium before extending understandings to other non-model bacteria or eukaryotes. For example, 84 genes were screened using knock-out mutants from the Keio collection to study their role in the butanol tolerance of *E. coli* (9). In another study, the Keio library was used to identify 1,564 chemical-genetic interactions for 15 antibiotics in *E. coli*, which revealed common genes possibly involved in multidrug resistance (10). The knock-out strains from the Keio collection have also been used to investigate the involvement of specific genes in biofilm formation (11, 12), ethanol tolerance (13), zinc resistance (14), colistin sensitivity (15) and boric acid resistance (16).

However, it has been more than 17 years since this library was first made. Sub-culturing of these strains may result in the introduction of spontaneous mutations in the genome of the single-gene knock-out mutants, which might differ from the parental strain. Such mutations could, in turn, confound attributing phenotypes to the gene deletions of interest. Therefore, in this study, we subjected 21 single-gene knock-out mutants from the Keio collection to whole genome sequencing and compared the results with the parental strain, which was also re-sequenced here, to describe the presence and distribution of single nucleotide polymorphisms (SNPs) and insertions and deletions that are ≤ 50 nucleotides (INDELs) using breseq software (17).

RESULTS AND DISCUSSION

The genomes of the single-gene knock-out mutants confirmed the absence of the respective genes of interest

For the development of the single-gene knock-out mutants, the coding regions of the genes of interest were replaced by a kanamycin cassette, leading to their inactivation (1). The locations of the deleted genes in the knock-out mutants used in this study were approximately evenly distributed across the entire genome (Fig. S1), with the exception of a few genes occupying the same operons (such as $\Delta rcsB/C/D$, $\Delta acrA/B$, $\Delta envZ/OmpR$, and $\Delta cpxA/P/R$). These were assessed to determine whether gene deletions belonging to the same operon or similar genomic locations lead to similar genomic mutations. Knock-out mutants $\Delta ompC$ and $\Delta rcsD$ with deleted genes belonging to different operons but very close in their genomic locations were also analysed. The deleted genes in the knock-out mutants used in this study encoded different types of proteins, such as cytoplasmic, periplasmic, inner membrane, and outer membrane proteins (Table S1), and the genomic mutations were compared between knock-out mutants lacking proteins from the same cellular locations (such as the periplasm, cytoplasm, inner membrane, or outer membrane). All 21 knock-out mutant strains of the Keio collection used in the present study (Table S1) were verified by whole genome sequencing, which confirmed that the genes of interest were indeed deleted. When the rest of the genome was compared with the parental sequence (NCBI accession: [CP009273.1](https://.ncbi.nlm.nih.gov/nucl/CP009273.1)), the mutations predicted by breseq in the single-gene knock-out mutant strains are described in Table 1.

A small number of SNPs/INDELs were predicted in some knock-out mutants

There were no SNPs or INDELs detected in the resequenced parental strain, $\Delta degP$, $\Delta lamB$, $\Delta ompC$, $\Delta fadL$, and $\Delta tolC$ mutants in comparison to the reference parental genome in the mutation predictions by breseq (Table 1). Overall, the number of SNPs or INDELs found in the mutant strains compared with the parental strain was quite low. The breseq results indicated the presence of one SNP in one of the coding regions of $\Delta acrA$, $\Delta acrB$, $\Delta ompR$, $\Delta ompT$, $\Delta rcsB$, $\Delta soxS$, and Δspy mutants, and the presence of two SNPs in the coding regions of the $\Delta cpxA$ mutant (Table 1). Out of these SNPs, the SNP in the *gshB* gene of the Δspy mutant is an example of a silent mutation (Fig. 1A) since the codon resulting from the mutation (CAA) codes for the same amino acid as the codon before the mutation

TABLE 1 Mutation predictions by breseq (one-letter abbreviations are used for the amino acids)

Strain	Mutation	Genomic position	Annotation ^a	Gene
Δ <i>acrA</i>	C→G	434,596	M346I (ATG→ATC), missense mutation	<i>dxs</i>
	Insertion of 1,199 bp	2,400,257	Intergenic (-137/-780), IS5 mediated	<i>lrhA/alaA</i>
Δ <i>acrB</i>	C→T	3,941,103	W75* (TGG→TGA), nonsense mutation	<i>hdfR</i>
Δ <i>cpxA</i>	A→T	435,152	F161Y (TTT→TAT), missense mutation	<i>dxs</i>
	G→T	1,293,170	F136L (TTC→TTA), missense mutation	<i>adhE</i>
	T→C	2,719,426	Intergenic (-321/+2)	<i>kgtP/rrfG</i>
Δ <i>cpxP</i>	Insertion of 1,199 bp	1,972,818	Intergenic (-364/-413), IS5 mediated	<i>flhD/uspC</i>
Δ <i>cpxR</i>	Deletion of 1 bp	446,710	Coding (357/948 nt), frameshift mutation	<i>cyoA</i>
	Insertion of 1,199 bp	1,972,967	Intergenic (-513/-264), IS5 mediated	<i>flhD/uspC</i>
	T→C	2,719,426	Intergenic (-321/+2)	<i>kgtP/rrfG</i>
Δ <i>envZ</i>	T→C	2,719,426	Intergenic (-321/+2)	<i>kgtP/rrfG</i>
	Deletion of 6 bp	3,941,538	Coding (93-98/339 nt), non-frameshift mutation	<i>yifE</i>
Δ <i>ompA</i>	Insertion of 1,199 bp	1,972,967	Intergenic (-513/-264), IS5 mediated	<i>flhD/uspC</i>
Δ <i>ompF</i>	Insertion of 6 bp	3,046,837	Coding (41/1179 nt), non-frameshift mutation	<i>ubiH</i>
Δ <i>ompR</i>	G→A	2,400,090	L11F (CTC→TTC), missense mutation	<i>lrhA</i>
	T→C	2,719,426	Intergenic (-321/+2)	<i>kgtP/rrfG</i>
Δ <i>ompT</i>	G→A	4,036,889	A38T (GCG→ACG), missense mutation	<i>dsbA</i>
Δ <i>ompX</i>	T→C	2,719,426	Intergenic (-321/+2)	<i>kgtP/rrfG</i>
	G→T	4,259,218	Intergenic (-278/-124)	<i>yjbS/aphA</i>
Δ <i>rscB</i>	C→T	170,655	V239M (GTG→ATG), missense mutation	<i>hemL</i>
	T→C	2,719,426	Intergenic (-321/+2)	<i>kgtP/rrfG</i>
Δ <i>rscC</i>	T→C	2,719,426	Intergenic (-321/+2)	<i>kgtP/rrfG</i>
	Insertion of 1,199 bp	3,797,089	Coding (406-409/1080 nt), frameshift mutation, IS5 mediated	<i>waaB</i>
Δ <i>rscD</i>	T→C	2,719,426	Intergenic (-321/+2)	<i>kgtP/rrfG</i>
Δ <i>soxS</i>	T→C	2,719,426	Intergenic (-321/+2)	<i>kgtP/rrfG</i>
	T→G	3,940,652	T226P (ACC→CCC), missense mutation	<i>hdfR</i>
Δ <i>spy</i>	T→C	2,719,426	Intergenic (-321/+2)	<i>kgtP/rrfG</i>
	G→A	3,085,323	Q29Q (CAG→CAA), silent mutation	<i>gshB</i>

^a / Intergenic region in between two genes, → Substitution of one base with another, - Number of bp upstream of the gene, + Number of bp downstream of the gene.

(CAG), i.e., glutamine (Q). A nonsense mutation was observed in the *hdfR* gene of the Δ *acrB* mutant (Fig. 1B), which codes for the *flhDC* operon transcriptional repressor HdfR (18). In the current study, the SNP found in the gene *hdfR* of the Δ *acrB* mutant would result in the replacement of the codon (TGG) for tryptophan (W) in the 75th amino acid position of the protein with a stop codon (TGA). This would generate a truncated protein with only 74 amino acids (aa) instead of 279 aa for the native gene. It is reported that a loss of function mutation in the *hdfR* gene increases the availability of NADPH in the parent strain *E. coli* BW25113, probably because HdfR facilitates the expression of enzymes involved in NADPH-dependent glutamate synthesis (19).

Interestingly, an SNP (T→C) in the same genomic position was found in the Δ *cpxA*, Δ *cpxR*, Δ *envZ*, Δ *ompR*, Δ *ompX*, Δ *rscB*, Δ *rscC*, Δ *rscD*, Δ *soxS*, and Δ *spy* mutants (Fig. 1C). However, this nucleotide falls in the intergenic region in between genes *kgtP* and *rrfG*. Therefore, the SNP does not alter the proteins encoded by the flanking genes. Similarly, another SNP was found in the intergenic region in between genes *yjbS* and *aphA* in the Δ *ompX* mutant (Table 1). While these are unlikely to alter the encoded proteins, mutations in the intergenic regions might affect the expression of nearby genes or non-coding regulatory RNA molecules (20). It is reported that intergenic mutations in bacteria may alter the transcription level of genes and may contribute to the evolution of bacterial traits, such as antibiotic sensitivity (21) and adaptation to the gut environment (22).

Sequence analysis indicated the deletion of one nucleotide from the coding region of the *cyoA* gene in the Δ *cpxR* mutant. This would lead to a frameshift mutation in the gene (Fig. 1D). In the Δ *envZ* mutant, a deletion of six bp was observed in the *yifE*

mutants, except the one found in the $\Delta rcsC$ mutant. The transposon-mediated insertion in the $\Delta rcsC$ mutant occurs in the coding region of the *waaB* gene, which codes for lipopolysaccharide 1,6-galactosyltransferase (18). The insertion, which is 1,195 (IS5) + 4

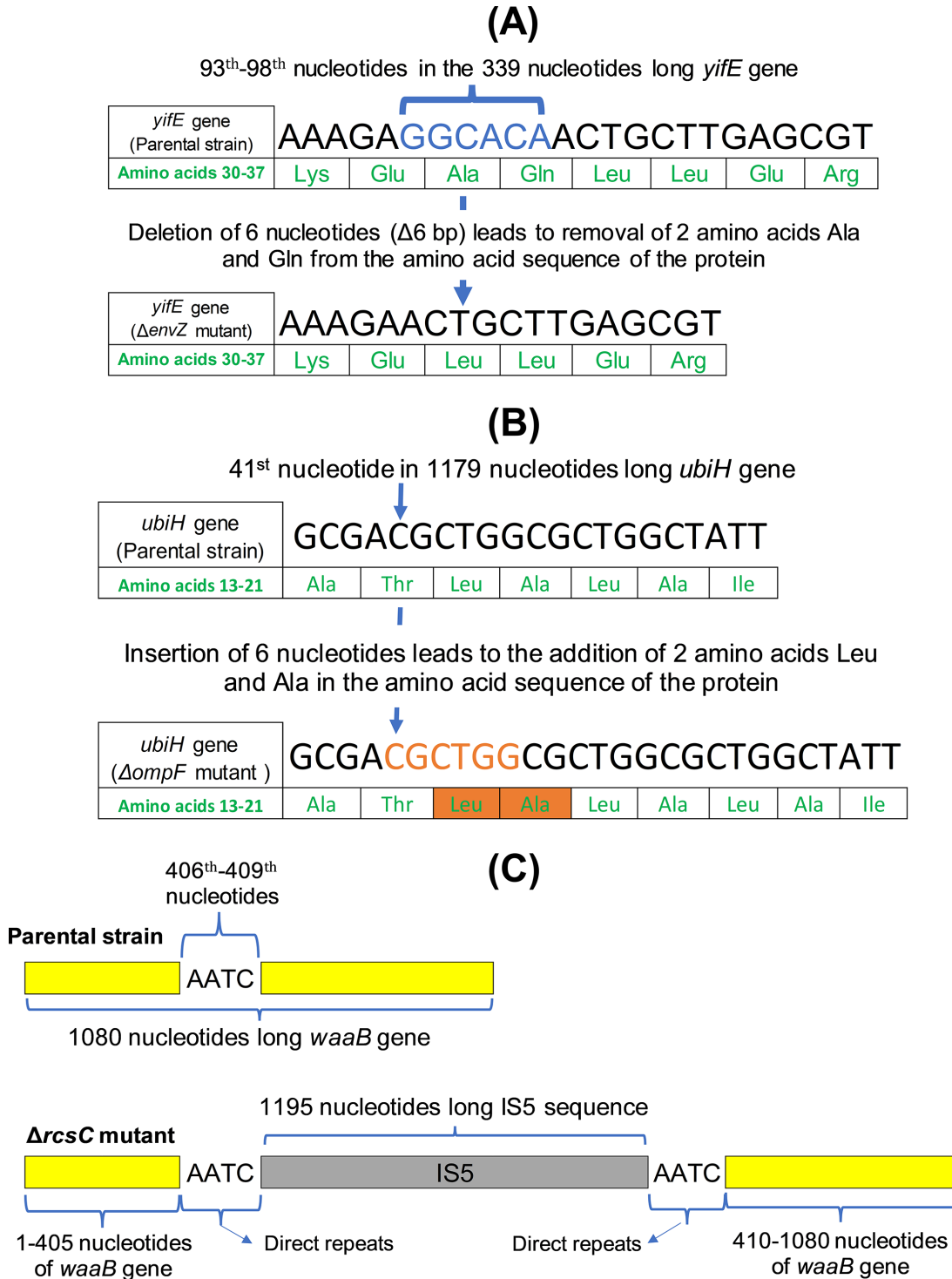


FIG 2 (A) Deletion of six nucleotides (93rd–98th nucleotides) from the *yifE* gene of $\Delta envZ$ mutant would result in the loss of two amino acids (32nd and 33rd amino acids) from the protein. The deleted bases in the $\Delta envZ$ mutant are shown in blue in the parental strain. (B) Insertion of six nucleotides in the coding region of the *ubiH* gene in the $\Delta ompF$ mutant would result in the addition of two amino acids to the protein. The inserted sequence and the two newly incorporated amino acids in the $\Delta ompF$ mutant are colored in orange. (C) Insertion of IS5 (1,195 bp) + direct repeat (4 bp) after the 409th nucleotide in the *waaB* gene (colored in yellow) of $\Delta rcsC$ mutant would likely lead to frameshift mutation and gene inactivation.

nucleotides (direct repeat) long, occurs after the 409th nucleotide of the 1,080 nt long *waaB* gene and, most likely, would lead to frameshift mutation and inactivation of the gene (Fig. 2C).

No similarity was observed in the pattern of SNPs in the mutant strains with deleted genes belonging to the same operon, such as $\Delta rcsB/C/D$, $\Delta acrA/B$, $\Delta EnvZ/OmpR$, and $\Delta cpxA/P/R$ (Table 1). Likewise, no similarity in mutation patterns was observed between $\Delta ompC$ and $\Delta rcsD$ mutants (i.e., different operons but similar genomic location). The same SNP in the intergenic region between *kgtP* and *rrfG* genes (Fig. 1C) was found in different mutant strains, with gene deletions from different operons and genomic locations. The IS5-mediated insertion was predicted in the same intergenic region between genes *flhD* and *uspC* (Table 1) in $\Delta cpxP$ and $\Delta cpxR$ mutants (with deleted genes from the same operon). However, an IS5-mediated insertion in the same intergenic region was also predicted in the $\Delta ompA$ mutant. Therefore, the mutations predicted in this study were likely not influenced by the genomic locations/operon of the genes deleted. No notable similarity in mutation patterns was observed between knock-out mutants lacking proteins belonging to the same cellular locations.

The development of mutations in *E. coli* over time in a laboratory environment is not uncommon. In a long-term evolution experiment where *E. coli* was maintained in a simple lab environment, *E. coli* continued to improve their fitness despite being in the same environment for >35 years (23). Whole genome sequencing suggested the appearance of several SNPs and INDELS in the *E. coli* strains after 20 years of cultivation in a laboratory environment (24). However, all the mutations predicted in this study should be confirmed through phenotypic studies to understand if they have any impact on gene function or expression. Future studies might consider testing the phenotypic effect or functional impacts of such mutations via genetic manipulation and phenotypic assays. Nonetheless, the results in the current study highlight the importance of carrying out additional experiments, such as complementation experiments, to validate phenotypic observations gleaned from Keio strains. While this should be considered standard, conclusions are sometimes drawn from Keio mutant strains without such additional validation studies. Therefore, our observations will serve as a timely reminder to researchers to consider SNPs and INDELS when assigning phenotypic changes to knocked out genes. This study will likely raise awareness in the scientific community about this issue.

The single gene knock-out mutant library of *E. coli* is an invaluable resource, enabling rapid screening of the effect of individual gene deletions across a range of applications. It is cheap, convenient, and time-saving for the research community. Here, we provide preliminary studies indicating a small number of SNPs and INDELS in a subset of knock-out strains of the Keio collection in comparison to the parent strain. These mutations in the intergenic and coding regions could potentially lead to phenotypic differences in the mutant strains compared with the parent strain that are independent of the desired gene deletion and therefore could mislead studies comparing specific phenotypes between the knock-out strains and the parent strain. This problem has been overcome in various studies by expressing the deleted gene in the knock-out mutants (complementation experiments) and cross-checking if the desired or missing phenotype is recovered when the gene is expressed (14–16, 25, 26). However, this might not always work since single-gene deletions also affect the expression levels of downstream genes belonging to the same operon known as the polar effects (27). In such cases, complementation cannot restore the indirect effects of gene deletion. For instance, it is reported that the UV sensitivity of the $\Delta ybaB$ mutant is not due to the absence of YbaB but due to lower expression of adjacent gene *recR* as an indirect effect of *ybaB* gene deletion (27). Therefore, in such cases, the genes downstream to the deleted gene and belonging to the same operon also need to be analyzed through gene knock-out and complementation studies.

While the current study involving 21 knock-out mutant strains from a library of 3,985 mutants (1) is not sufficient to draw a definitive conclusion about the entire Keio library,

the location of the knocked out genes in the mutants used in this study are approximately evenly distributed across the entire genome representing the absence of genes from different genomic locations across the library. Moreover, these knocked out genes encode proteins belonging to the cytoplasm, periplasm, and inner or outer membrane, representing the absence of proteins from different locations of the cell. Mutation predictions by breseq indicated some form of mutation (SNPs/INDELS/ISS-mediated mutations) in 16 out of 21 mutants tested. Therefore, our findings on this subset of the Keio collection suggest the possibility of such mutations in other knock-out strains of the library. While this study suggests potential risks, this study should be expanded to a larger subset of the Keio collection to obtain a more robust numerical or statistical assessment of the likelihood of such mutations across the library.

MATERIALS AND METHODS

Strains and culture medium used in this study

All the strains listed in Table S1 were obtained from NBRP (NIG, Japan): *E. coli*. All the strains were grown in BD Difco Luria–Bertani Broth (Lennox) with the following composition: 10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl. The knock-out mutants were grown in the presence of 30 mg/mL kanamycin.

Genomic DNA isolation

The genomic DNA of the knock-out mutants and their parent strain *E. coli* BW25113 (listed in Table S1) was isolated using the Invitrogen PureLink genomic DNA extraction kit according to the manufacturer's instructions. The quality of the isolated genomic DNA was analyzed on 0.8% agarose gel to ensure the genomic DNA was intact and without any RNA contamination. The concentration of the genomic DNA was measured by Qubit dsDNA High Sensitivity (HS) Assay Kit.

Whole genome sequencing

The genomic DNA samples were sequenced on Illumina MiSeq V3 Run with 301 bp paired-end sequences, which was carried out by the SCELSE sequencing facility. One sequencing library was produced from each sample, and the sequencing was performed in paired-end mode, yielding two reads (R1: read 1 and R2: read 2) for each sample. The sequences (fastq files) can be found at the NCBI sequence-read archive (<https://www.ncbi.nlm.nih.gov/sra/PRJNA1095198>).

QC and reads trimming

The quality of the Illumina pair-ended reads was first assessed using FastQC version (28). Illumina reads were processed to cut the adaptor and quality-trimmed using Trimmomatic version 0.38 (29).

Variant calling

Software breseq version 0.35.5 (17) was used, with default parameters, to map merged trimmed reads and identify mutations. *E. coli* BW25113 genome (NCBI accession: CP009273.1) was used as the reference for all the samples, and the mutations were then determined by comparing the knock-out strains with the parent strain.

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AUTHOR AFFILIATIONS

¹Singapore Centre for Environmental Life Sciences Engineering, Nanyang Technological University, Singapore, Singapore

²School of Biological Sciences, Nanyang Technological University, Singapore, Singapore

³The Australian Institute for Microbiology and Infection, University of Technology Sydney, Ultimo, Australia

⁴CSIRO, Agriculture and Food, Westmead and Microbiomes for One Systems Health, Canberra, Australia

⁵WATEC Aarhus University Centre for Water Technology, Universitetsbyen, Aarhus, Denmark

AUTHOR ORCIDs

Oishi Sen  <http://orcid.org/0000-0001-7282-0997>

Scott A. Rice  <http://orcid.org/0000-0002-9486-2343>

Thomas Seviour  <http://orcid.org/0000-0002-8816-9796>

AUTHOR CONTRIBUTIONS

Oishi Sen, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review and editing | Xianghui Liu, Data curation, Formal analysis, Investigation, Methodology, Writing – review and editing | Staffan Kjelleberg, Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review and editing | Scott A. Rice, Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review and editing | Thomas Seviour, Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review and editing

DATA AVAILABILITY

The sequences (fastq files) can be found at the NCBI sequence-read archive (<https://www.ncbi.nlm.nih.gov/sra/PRJNA1095198>).

ETHICS APPROVAL

Not applicable.

ADDITIONAL FILES

The following material is available [online](#).

Supplemental Material

Supplemental material (Spectrum02036-24-S0001.docx). Fig. S1; Table S1.

REFERENCES

- Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, Mori H. 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol* 2:2006–0008. <https://doi.org/10.1038/msb4100050>
- Price MN, Wetmore KM, Waters RJ, Callaghan M, Ray J, Liu H, Kuehl JV, Melnyk RA, Lamson JS, Suh Y, Carlson HK, Esquivel Z, Sadeeshkumar H, Chakraborty R, Zane GM, Rubin BE, Wall JD, Visel A, Bristow J, Blow MJ, Arkin AP, Deutschbauer AM. 2018. Mutant phenotypes for thousands of bacterial genes of unknown function. *Nature* 557:503–509. <https://doi.org/10.1038/s41586-018-0124-0>
- Schmidt A, Kochanowski K, Vedelaar S, Ahrné E, Volkmer B, Callipo L, Knoop K, Bauer M, Aebersold R, Heinemann M. 2016. The quantitative and condition-dependent *Escherichia coli* proteome. *Nat Biotechnol* 34:104–110. <https://doi.org/10.1038/nbt.3418>
- Hirayama S, Nakao R. 2020. Glycine significantly enhances bacterial membrane vesicle production: a powerful approach for isolation of LPS-reduced membrane vesicles of probiotic *Escherichia coli*. *Microb Biotechnol* 13:1162–1178. <https://doi.org/10.1111/1751-7915.13572>
- Guo C, Nolan EM. 2022. Heavy-metal trojan horse: enterobactin-directed delivery of platinum(IV) prodrugs to *Escherichia coli*. *J Am Chem Soc* 144:12756–12768. <https://doi.org/10.1021/jacs.2c03324>
- Wong F, Stokes JM, Cervantes B, Penkov S, Friedrichs J, Renner LD, Collins JJ. 2021. Cytoplasmic condensation induced by membrane damage is associated with antibiotic lethality. *Nat Commun* 12:2321. <https://doi.org/10.1038/s41467-021-22485-6>
- Sen O, Hinks J, Lin Q, Lin Q, Kjelleberg S, Rice SA, Seviour T. 2023. *Escherichia coli* displays a conserved membrane proteomic response to a

- range of alcohols. *Biotechnol Biofuels Bioprod* 16:147. <https://doi.org/10.1186/s13068-023-02401-4>
8. Rutherford BJ, Dahl RH, Price RE, Szmidski HL, Benke PI, Mukhopadhyay A, Keasling JD. 2010. Functional genomic study of exogenous n-butanol stress in *Escherichia coli*. *Appl Environ Microbiol* 76:1935–1945. <https://doi.org/10.1128/AEM.02323-09>
 9. Reyes LH, Almario MP, Kao KC. 2011. Genomic library screens for genes involved in n-butanol tolerance in *Escherichia coli*. *PLoS ONE* 6:e17678. <https://doi.org/10.1371/journal.pone.0017678>
 10. French S, Mangat C, Bharat A, Côté JP, Mori H, Brown ED. 2016. A robust platform for chemical genomics in bacterial systems. *Mol Biol Cell* 27:1015–1025. <https://doi.org/10.1091/mbc.E15-08-0573>
 11. Zhang XS, García-Contreras R, Wood TK. 2007. YcfR (BhsA) influences *Escherichia coli* biofilm formation through stress response and surface hydrophobicity. *J Bacteriol* 189:3051–3062. <https://doi.org/10.1128/JB.01832-06>
 12. Niba ETE, Naka Y, Nagase M, Mori H, Kitakawa M. 2007. A genome-wide approach to identify the genes involved in biofilm formation in *E. coli*. *DNA Res* 14:237–246. <https://doi.org/10.1093/dnares/dsm024>
 13. Zhang DF, Ye JZ, Dai HH, Lin XM, Li H, Peng XX. 2018. Identification of ethanol tolerant outer membrane proteome reveals OmpC-dependent mechanism in a manner of EnvZ/OmpR regulation in *Escherichia coli*. *J Proteomics* 179:92–99. <https://doi.org/10.1016/j.jprot.2018.03.005>
 14. Shirakawa R, Ishikawa K, Furuta K, Kaito C. 2023. Knockout of ribosomal protein RpmJ leads to zinc resistance in *Escherichia coli*. *PLoS ONE* 18:e0277162. <https://doi.org/10.1371/journal.pone.0277162>
 15. Kano T, Ishikawa K, Furuta K, Kaito C. 2024. Knockout of adenylosuccinate synthase purA increases susceptibility to colistin in *Escherichia coli*. *FEMS Microbiol Lett* 371:fnae007. <https://doi.org/10.1093/femsle/fnae007>
 16. Çöl B, Kürkcü MS, Di Bek E. 2024. Genome-wide screens identify genes responsible for intrinsic boric acid resistance in *Escherichia coli*. *Biol Trace Elem Res* 202:5771–5793. <https://doi.org/10.1007/s12011-024-04129-0>
 17. Deatherage DE, Barrick JE. 2014. Identification of mutations in laboratory-evolved microbes from next-generation sequencing data using breseq. *Methods Mol Biol* 1151:165–188. https://doi.org/10.1007/978-1-4939-0554-6_12
 18. Bateman A, Martin MJ, Orchard S, Magrane M, Ahmad S, Alpi E, Bowler-Barnett EH, Britto R, Bye-A-Jee H, Cukura A. 2023. UniProt: the universal protein knowledgebase in 2023. *Nucleic Acids Res* 51:D523–D531. <https://doi.org/10.1093/nar/gkac1052>
 19. Reynolds TS, Courtney CM, Erickson KE, Wolfe LM, Chatterjee A, Nagpal P, Gill RT. 2017. ROS mediated selection for increased NADPH availability in *Escherichia coli*. *Biotechnol Bioeng* 114:2685–2689. <https://doi.org/10.1002/bit.26385>
 20. Morales-Laverde L, Trobos M, Echeverez M, Solano C, Lasa I. 2022. Functional analysis of intergenic regulatory regions of genes encoding surface adhesins in *Staphylococcus aureus* isolates from periprosthetic joint infections. *Biofilm* 4:100093. <https://doi.org/10.1016/j.bioflm.2022.100093>
 21. Khademi SMH, Sazinas P, Jelsbak L. 2019. Within-host adaptation mediated by intergenic evolution in *Pseudomonas aeruginosa*. *Genome Biol Evol* 11:1385–1397. <https://doi.org/10.1093/gbe/evz083>
 22. Frazão N, Konrad A, Amicone M, Seixas E, Güleresi D, Lässig M, Gordo I. 2022. Two modes of evolution shape bacterial strain diversity in the mammalian gut for thousands of generations. *Nat Commun* 13:5604. <https://doi.org/10.1038/s41467-022-33412-8>
 23. Barrick JE, Blount ZD, Lake DM, Dwenger JH, Chavarria-Palma JE, Izutsu M, Wiser MJ. 2023. Daily transfers, archiving populations, and measuring fitness in the long-term evolution experiment with *Escherichia coli*. *J Vis Exp* 198. <https://doi.org/10.3791/65342>
 24. Barrick JE, Yu DS, Yoon SH, Jeong H, Oh TK, Schneider D, Lenski RE, Kim JF. 2009. Genome evolution and adaptation in a long-term experiment with *Escherichia coli*. *Nature* 461:1243–1247. <https://doi.org/10.1038/nature08480>
 25. Velur Selvamani RS, Telaar M, Friehs K, Flaschel E. 2014. Antibiotic-free segregational plasmid stabilization in *Escherichia coli* owing to the knockout of triosephosphate isomerase (tpiA). *Microb Cell Fact* 13:1–13. <https://doi.org/10.1186/1475-2859-13-58>
 26. Kruszewska-Naczka B, Grinholc M, Waleron K, Bandow JE, Rapacka-Zdończyk A. 2024. Can antimicrobial blue light contribute to resistance development? Genome-wide analysis revealed aBL-protective genes in *Escherichia coli*. *Microbiol Spectr* 12:e0249023. <https://doi.org/10.1128/spectrum.02490-23>
 27. Mateus A, Shah M, Hevler J, Kurzawa N, Bobonis J, Typas A, Savitski MM. 2021. Transcriptional and post-transcriptional polar effects in bacterial gene deletion libraries. *mSystems* 6:e0081321. <https://doi.org/10.1128/mSystems.00813-21>
 28. Andrews S. 2010. FastQC. A quality control tool for high throughput sequence data. <https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>.
 29. Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30:2114–2120. <https://doi.org/10.1093/bioinformatics/btu170>