

Reoccurring *Escherichia coli* O157:H7 Strain Linked to Leafy Greens–Associated Outbreaks, 2016–2019

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Genomic characterization of an *Escherichia coli* O157:H7 strain linked to leafy greens–associated outbreaks dates its emergence to late 2015. One clade has notable accessory genomic content and a previously described mutation putatively associated with increased arsenic tolerance. This strain is a reoccurring, emerging, or persistent strain causing illness over an extended period.

Escherichia coli O157:H7 is estimated to cause ≈63,000 domestically acquired foodborne illnesses and 20 deaths in the United States each year (1). *E. coli* O157:H7 infections are typically associated with abdominal cramps, bloody diarrhea, and vomiting; however, a rare but serious condition called hemolytic uremic syndrome can develop, resulting in anemia and acute renal failure (2). Healthy cattle serve as the main reservoir for *E. coli* O157:H7, and contaminated food, water, and environmental sources, as well as contact with animals, have been the source of outbreaks of *E. coli* O157:H7 infections (3,4). More recently, contaminated leafy greens have been recognized as a major source of *E. coli* O157:H7 illnesses and outbreaks. In foodborne illness attribution estimates for 2020 based on outbreak data, 58.1% of *E. coli* O157:H7 illnesses were attributed to vegetable row crops, a category that includes leafy greens (<https://www.cdc.gov/foodsafety/ifsac/>

<https://www.cdc.gov/foodsafety/ifsac/> annual-reports.html). During 2009–2018, a total of 32 confirmed or suspected outbreaks of *E. coli* O157:H7 infections linked to contaminated leafy greens occurred in the United States and Canada (5).

A large *E. coli* outbreak in late 2019, hereafter referred to as outbreak A, caused 167 cases, hospitalized 85 persons from 27 states, and was associated with the consumption of romaine lettuce from Salinas Valley, California, USA (<https://www.cdc.gov/ecoli/2019/o157h7-11-19/index.html>). We characterized isolates from outbreak A and highly related isolates by using a variety of molecular methods.

The Study

A query of the PulseNet database revealed 356 isolates related to the outbreak strain that had <15 core-genome multilocus sequence typing (MLST; cgMLST) allele differences (Table 1; Appendix 1 Table 1, <https://wwwnc.cdc.gov/EID/article/29/9/23-0069-App1.xlsx>) (6). Of those, 302 isolates corresponded to human cases associated with 6 outbreaks spanning 3 years; dates of isolation ranged from September 27, 2016, to January 3, 2020. An additional 54 isolates were either clinical isolates not associated with a recognized outbreak (n = 14) or from environmental (n = 20), food (n = 8), or animal (n = 12) samples. Seven-gene MLST and Manning clade typing revealed all isolates were sequence type (ST) 11 and belonged to Manning clade 2 (Appendix 2, <https://wwwnc.cdc.gov/EID/article/29/9/23-0069-App2.pdf>). In silico PCR of the Shiga toxin (stx) genes revealed that all

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Table 1. Summary of outbreaks caused by reoccurring *Escherichia coli* O157:H7 strain REPEXH02 linked to leafy greens–associated outbreaks, 2016–2019*

Outbreak	No. sequences	Timeframe	No. sequences, subset	Median subset allele differences (min–max)	Median hqSNP subset differences (min–max)	Outbreak source	Growing region
D	20	Sep 27–Dec 5, 2016	20	2 (0–5)	3 (0–17)	Unknown	NA
C	23	Nov 10–Dec 14, 2017	23	0 (0–3)	1 (0–5)	Leafy greens	Likely SW USA, Mexico
B3	7	Jul 31–Aug 15, 2018	7	0 (0–3)	1 (0–10)	Unknown	NA
B2	71	Oct 8–Dec 7, 2018	69	1 (0–4)	2 (0–10)	Romaine lettuce	Santa Maria, CA
B1	19	Nov 1–Dec 18, 2018	18	2 (0–5)	4 (0–10)	Leafy greens	NA
A	179	Sep 27, 2019–Jan 3, 2020	84	1 (0–5)	2 (0–12)	Romaine lettuce	Salinas Valley, CA
Nonhuman samples collected in Santa Maria	23	Nov 14, 2019	12	0 (0–1)	1 (0–5)	NA	Santa Maria, CA
Not associated with known outbreak	14	Oct 18, 2016–Aug 4, 2019	12	1 (0–3)	8 (0–18)	NA	NA
All	356	Sep 27, 2016–Jan 3, 2020	245	2 (0–8)	10 (0–39)	NA	NA

*hqSNP, high-quality single nucleotide polymorphism; NA, not applicable.

but 2 isolates contained *stx2a*, whereas 2 remaining isolates had no detectable *stx* genes. We generated a closed-reference genome, 2019C-3201 (Strain: PNUSAE020169; BioSample: SAMN10432148), using PacBio Sequel technology (<https://www.pacb.com>) and assembled with Flye version 2.6 (7). The sequence data assembled into a single complete chromosomal contig and 3 plasmids (Table 2).

We selected a subset of 245 isolates for further genomic analysis to more evenly sample across outbreaks and to reduce computational demands. Isolates were characterized by core genome MLST implemented in BioNumerics 7.6 (6) and high-quality single-nucleotide polymorphism (SNP; hqSNP) methods using Lyve-SET version 1.1.4f (9), using the chromosomal sequence of 2019C-3201 as a reference and the Lyve-SET presets for *E. coli*. Overall, hqSNP was more discriminatory, differentiating isolates by a median of 10 pairwise hqSNPs (0–39 SNPs), whereas cgMLST differentiated isolates by a median of 2 allele differences (0–8 alleles) (Table 1). This finding was foreseeable because hqSNP does not depend on a predefined scheme; therefore, intergenic SNPs between

loci, multiple SNP differences within a given locus, or SNPs in loci not included in the cgMLST schema can result (9).

Time-tree analysis using BEAST version 2.6.3 (10) revealed the divergence of this strain into 2 clades that last shared a common ancestor around late 2015 (median December 19, 2015; 95% highest posterior density interval December 7, 2014–July 10, 2016) (Figure 1). After outbreak D in 2016, sequences corresponding to a given outbreak belonged to 1 of 2 clades; outbreaks B2 and C were associated with clade 1, and outbreaks A, B1, and B3 were associated with clade 2. Of note, outbreak A was traced to romaine lettuce from Salinas Valley, whereas traceback and sampling in outbreak B2 linked some illnesses to romaine lettuce from Santa Maria, California (<https://www.fda.gov/food/outbreaks-foodborne-illness/investigation-summary-factors-potentially-contributing-contamination-romaine-lettuce-implicated-fall>; <https://www.fda.gov/food/outbreaks-foodborne-illness/outbreak-investigation-e-coli-romaine-salinas-california-november-2019>). Lettuce from Salinas was not considered a source of any illnesses in outbreak B2.

Table 2. Genomic attributes of the 2019C-3201 reference genome of reoccurring *Escherichia coli* O157:H7 strain REPEXH02 linked to leafy greens–associated outbreaks, 2016–2019*

Contig name	GenBank accession no.	Length, bp	Sequence coverage	Replicon	PTU (8)
2019C-3201 chromosome	CP090856	5,488,442	866	None	NA
p2019C-3201_1	CP090857	87,920	732	Incl1-l(gamma)	PTU-I1
p2019C-3201_2	CP090858	61,933	560	IncFII(pHN7A8), IncFII(pSFO)	PTU-F _E
p2019C-3201_3	CP090859	92,724	623	IncFIB, IncFII	PTU-E5

*PTU, plasmid taxonomic unit.

Environmental sampling in Santa Maria in 2019 yielded isolates clustering closely with outbreak B2 in the time tree.

We analyzed the closed reference sequence of 2019C-3201 using Prokka version 1.8 to enable SNP annotation (11). We examined output from Lyve-SET to determine the SNPs differentiating the 2 clades in our phylogenetic analysis (Appendix 1 Table 2). This work confirms a previous study reporting a nonsense mu-

tation in the *arsR* gene, an arsenical resistance operon repressor (12). All clade 1 isolates in this study possess a G→A mutation resulting in a premature stop codon. This mutation could decrease the activity of this repressor and lead to constitutive expression of this operon. Agricultural soils and water sources can contain increased arsenic levels because of natural processes, industrial sources, or agricultural uses of arsenic, such as application of arsenic-containing herbicides,

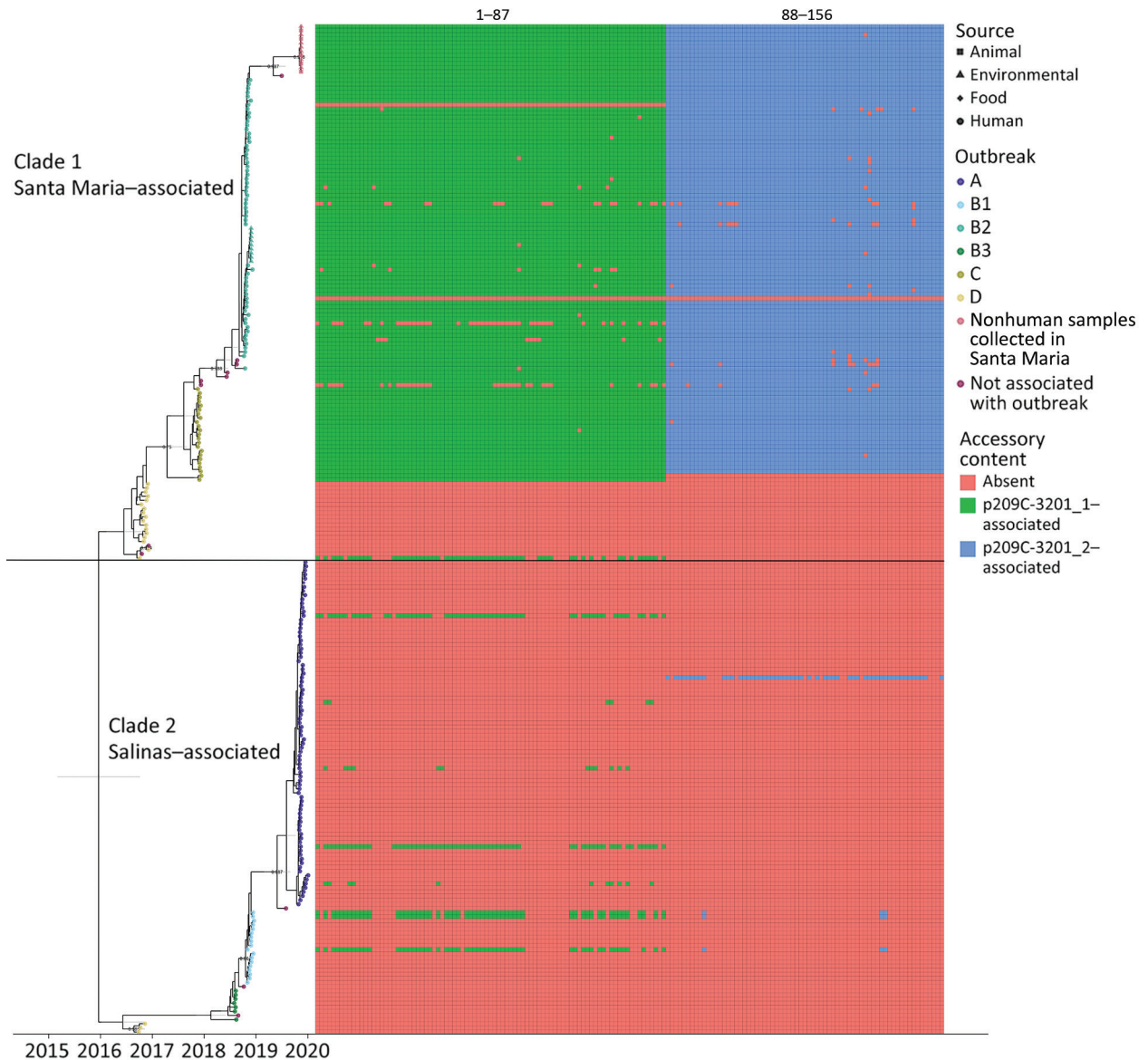


Figure 1. Tip-dated maximum clade credibility tree of 245 isolates of reoccurring *Escherichia coli* O157:H7 strain REPEXH02 linked to leafy greens-associated outbreaks, 2016–2019, generated in BEAST2 (<https://www.beast2.org>). Tips are aligned with the date of collection; calendar year is shown on the x-axis. Tips are colored according to the outbreak to which each isolate belonged; the shape corresponds to sample type (e.g., human, animal, environmental, or food). A horizontal black line segregates the two identified clades. Clade 1 contains outbreak B2 where some illness was traced back to Santa Maria, California, USA, as well as environmental samples collected in that region. Clade 2 contains outbreak A, which was traced back to the Salinas Valley, California. The presence/absence matrix to the right of the tree displays accessory genome content identified using Roary/scoary with 90% sensitivity and specificity to a subset of clade 1 isolates. A legend for accessory genome feature labels is included in Appendix 1 Table 5 (<https://wwwnc.cdc.gov/EID/article/29/9/23-0069-App1.pdf>).

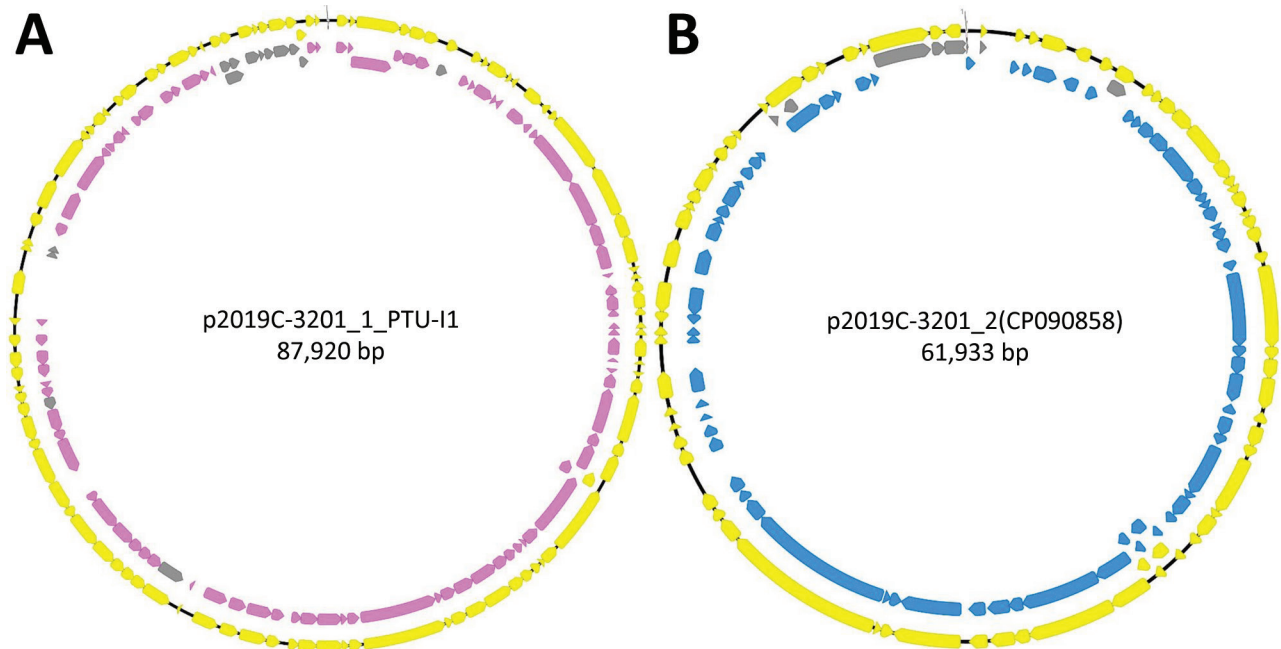


Figure 2. Annotated plasmids of reference genome 2019C-3201 of *Escherichia coli* O157:H7 containing clade-specific genomic features. A) p2019C-3201_1 annotated with prokka version 1.14.5 (yellow annotations) (11). Mapped list of Roary features (pan_genome_references.fa) onto plasmid (95% nucleotide identity; gray annotations) (14). Features highlighted had ≥ 90 sensitivity and ≥ 90 specificity to a subset of clade 1 isolates (pink annotations). The region with specific/sensitive features covers a large portion of the plasmid and predominately contains genes encoding hypothetical proteins with unknown functions and common plasmid-associated genes. Three features did not map in Geneious because they were either below 95% identity (2 features) or were identified as partial copy (1 feature). B) p2019C-3201_2 annotated with prokka v1.14.5 (yellow annotations). Mapped list of Roary features (pan_genome_references.fa) onto plasmid (100% nucleotide identity; gray annotations). Features highlighted had ≥ 90 sensitivity and ≥ 97 specificity (blue annotations) to a subset of clade 1 isolates. The region with specific/sensitive features covers a large portion of the plasmid and is associated with conjugation. Image was generated using Geneious version 2021.2 (<https://www.geneious.com>).

pesticides, or animal drugs (13). This mutation could provide an ecologic advantage in environments containing high levels of arsenic. This finding underscores the potential need to routinely screen enteric bacterial strains for heavy metal resistance determinants, as well as to consider heavy metal levels in soil as part of traceback investigations.

We further characterized isolates through assembly and annotation using Shovill-SPAdes version 1.0.9 and Prokka version 1.14.5 (11) and subsequent analysis in Roary version 3.11.2 (14) and scoary version 1.6.16 (15) to identify differences in the pangenome among isolates. We compared differentially distributed genes with the reference genome using BLASTn (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to identify feature location (chromosome/plasmid). Roary/scoary analysis revealed a subset of clade 1 isolates with additional genomic content. A total of 156 genomic features had ≥ 90 sensitivity and ≥ 90 specificity to this subset of clade 1. Of those, 87 (56%) are on plasmid p2019C-3201_1, and 69 (44%) are on p2019C-3201_2 (Figure 2; Appendix 1 Tables 3, 4). Prokka-annotated features associated with p2019C-3201_1 (Figure 2; Appendix 1 Table

3) were predominantly genes encoding hypothetical proteins with unknown functions and common plasmid-associated genes. Annotated features associated with p2019C-3201_2 (Figure 2; Appendix 1 Table 4) were predominantly associated with conjugation and span a large portion of that plasmid. Additional work is necessary to characterize the role of these plasmids in clade 1. When visualizing the distribution of these clade 1-specific features alongside the maximum-clade credibility tree (Figure 1; Appendix 1 Table 5), it appears those features were acquired after clade 1 and clade 2 diverged. Given the geographic distribution of isolates, these features might be a result of adaptation to a particular niche or environment.

Conclusions

In summary, a specific strain of *E. coli* O157:H7 associated with leafy greens has been the source of ongoing enteric illness since late 2016. This strain is estimated to have emerged in late 2015 and consists of 2 clades with different geographic distributions, 1 of which has notable genomic features. After this analysis, an additional outbreak associated with this strain was

detected in late 2020 in which a reported 40 infections occurred in 19 states; 20 persons were hospitalized, and 4 developed hemolytic uremic syndrome (<https://www.cdc.gov/ecoli/2020/o157h7-10-20b/index.html>). After that outbreak, no further outbreaks have been detected, and only a single clinical isolate associated with this strain has been identified by PulseNet. The Centers for Disease Control and Prevention has classified this strain as a reoccurring, emerging, or persistent (REP) strain (<https://www.cdc.gov/nceid/dfwed/outbreak-response/rep-strains.html>) with the designation REPEXH02. REP strains represent a new paradigm in enteric molecular surveillance, distinct from discrete outbreaks where numerous cases occur in a relatively short time frame. Detailed genomic characterization of additional REP strains, using the types of approaches outlined in this study, is necessary to elucidate factors contributing to their emergence and persistence in specific environments.

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Appendix 2

Supplemental Methods

Reference genome generation and characterization

We generated a closed reference genome, 2019C-3201 (WGSID: PNUSAE020169), using PacBio Sequel technology and assembled with Flye v.2.6 with the `–plasmids` option (1). The final assembly was then polished with Illumina reads using `unicycler_polish` scripts from Unicycler suite version v0.4.8 (2). Plasmids were characterized using a modified PlasmidFinder database (<https://github.com/StaPH-B/resistanceDetectionCDC/blob/master/plasmidDatabase.fasta>) (3) and COPLA (4) to identify plasmid replicons and plasmid taxonomic units respectively.

Genomic subtyping

MLST was determined using *mlst* (<https://github.com/tseemann/mlst>) with the Achtman seven-gene scheme. Stx gene subtype(s) were determined using in-silico PCR (<https://github.com/ucscGenomeBrowser/kent/tree/master/src/isPcr>) with the primers described by Flemming, et al. (5). The O157 Manning Clade was determined by identifying the four informative SNPs at loci ECs2357, ECs2521, ECs3881, and ECs4130. The matching clade was assigned based on these SNPs as described by Riordan, et al (6).

Phylogenetic analysis

Isolates were characterized by high-quality single nucleotide polymorphism (hqSNP) methods using Lyve-SET v.1.1.4f (7), using the chromosomal sequence of 2019C-3201 as a

reference and the Lyve-SET presets for *Escherichia coli*. The closed reference sequence of 2019C-3201 was analyzed using Prokka v1.8 to facilitate SNP annotation (8). To generate a time tree in BEAST v.2.6.3 (9) the alignment from Lyve-SET was processed using gubbins 3.0.0 (10) to generate a recombination-free SNP alignment. The resulting tree was first analyzed in TempEST v.1.5.3 (11) to assess suitability for molecular clock analysis. The resulting alignment was analyzed in BEAST2 while accounting for constant sites by modifying the xml file from Beauti to include `<data id = "filt" spec = "FilteredAlignment" filter = "-" data = "@filtOriginal" constantSiteWeights = "470006 589755 590021 468034"/>`. bModelTest v1.2.1 (12) was employed to average across appropriate substitution models. All coalescent tree priors under both a strict and relaxed clock and were evaluated with an initial clock rate of 5.0×10^{-7} SNPs/site/year, and analyses were run for 500,000,000 iterations with sampling every 50,000 iterations. A maximum clade credibility tree was generated using TreeAnnotator and the median options for height. The resulting time tree was visualized using the ggtree package (13) for R v.4.1.1.

Pangenome analysis

Isolates were assembled and annotation using Shovill-spades v.1.0.9 (<https://github.com/tseemann/shovill>; with the options `-trim,-depth 100,-mincov` set to 10% average genome coverage) and Prokka v.1.14.5 (8). Subsequent analysis in Roary v.3.11.2 (14) and scoary v.1.6.16 (15) using default parameters was performed to identify differences in the pangenome among isolates.

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Appendix 2 Figure. Map illustrating growing regions in California, USA, of which some REPEHX02-linked illnesses are associated with. More specifically, outbreak A in this study was traced to romaine lettuce from Salinas Valley, California, while traceback and sampling in outbreak B2 linked some illnesses to romaine lettuce from Santa Maria, California, both labeled and shaded in grey on the map. Image adapted with permission from the Food and Drug Administration (Original source: <https://www.fda.gov/media/147349/download#:~:text=As%20a%20result%20of%20this,and%202020%20leafy%20greens%20outbreaks>).

