



Whole-Genome Sequencing and Genetic Analysis Reveal Novel Stress Responses to Individual Constituents of Essential Oils in *Escherichia coli*

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ABSTRACT Food preservation by the use of essential oils (EOs) is being extensively studied because of the antimicrobial properties of their individual constituents (ICs). Three resistant mutants (termed CAR, CIT, and LIM) of *Escherichia coli* MG1655 were selected by subculturing with the ICs carvacrol, citral, and (+)-limonene oxide, respectively. These derivative strains showed increased MIC values of ICs and concomitantly enhanced resistance to various antibiotics (ampicillin, trimethoprim, chloramphenicol, tetracycline, kanamycin, novobiocin, norfloxacin, cephalexin, and nalidixic acid) compared to those for the parental strain (wild type [WT]). Whole-genome sequencing (WGS) of these hyperresistant strains permitted the identification of single nucleotide polymorphisms (SNPs) and deletions in comparison to the WT. In order to analyze the contribution of these mutations to the increased antimicrobial resistance detected in hyperresistant strains, derivative strains were constructed by allelic reversion. A role of the SoxR D137Y missense mutation in CAR was confirmed by growth in the presence of some ICs and antibiotics and by its tolerance to ICs but not to lethal heat treatments. In CIT, increased resistance relied on contributions by several detected SNPs, resulting in a frameshift in MarR and an in-frame GyrB ΔG157 mutation. Finally, both the insertion resulting in an AcrR frameshift and large chromosomal deletions found in LIM were correlated with the hyperresistant phenotype of this strain. The nature of the obtained mutants suggests intriguing links to cellular defense mechanisms previously implicated in antibiotic resistance.

IMPORTANCE The antimicrobial efficacy of ICs has been proven over the years, together with their potential to improve traditional heat treatments by reducing treatment intensity and, consequently, adverse effects on food quality. However, the mechanisms of bacterial inactivation by ICs are still not well understood, in contrast to antibiotics. We performed WGS of three *E. coli* strains that are hyperresistant to ICs. The information provided detailed insight into the mechanisms of bacterial resistance arising from exposure to carvacrol, citral, and (+)-limonene oxide. Future experiments will undoubtedly yield additional insights into genes and pathways contributing to the acquisition of endogenous resistance to ICs.

KEYWORDS whole-genome sequencing, carvacrol, citral, heat treatment, limonene, mechanisms of resistance, pulsed electric fields

Essential oils (EOs) and their individual constituents (ICs) have been proposed as alternatives to control spoiling and pathogenic microorganisms in the food industry (1–4). The antimicrobial activities and mechanisms of inactivation of ICs and EOs have

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been extensively studied (4–8), and they are perceived to be less prone to generating resistant strains, unlike other antimicrobial compounds such as antibiotics (9). Treatment of bacterial cells with these ICs caused the generation of reactive oxygen species (ROS), and bacterial cell death followed the RecA-mediated SOS response pathway (6, 7). Although these pathways could lead to increased mutagenesis (10), we observed a lower mutation rate in *Escherichia coli* cells grown in the presence of sub-MICs of ICs than in cells grown in their absence (11). This protection against mutation was attributed to the potential antioxidant activity of ICs at the low concentrations used (12). Nevertheless, derivative strains showing direct resistance and cross-resistance to multiple antimicrobial treatments (i.e., hyperresistant strains) were isolated after the growth of *Escherichia coli* MG1655 in the presence of sub-MICs of monoterpene ICs [carvacrol, citral, and (+)-limonene oxide] (11). The stability of the resistant phenotype in these derivative strains after their regrowth in the absence of the ICs supported the notion of genotypic modifications from the original strain.

Evolution experiments expose bacterial populations to a drug concentration high enough to inhibit the growth of the wild-type (WT) strain, thus imposing a selective advantage for resistant mutants (13–16). The evolution of resistance through the acquisition of single spontaneous mutations is particularly relevant for certain drugs, such as quinolones and rifampin, for which increased resistance can result from a single point mutation (17, 18). For most antibiotics, however, multiple mutations, or the acquisition of resistance genes by horizontal transfer, are required to develop high levels of resistance (19–22). The application of deep-sequencing methodologies to study antimicrobial-resistant strains is rapidly growing, since they permit the identification of mutations and shed light on the underlying bacterial defense mechanisms. Recent studies include the sequencing of methicillin-resistant *Staphylococcus aureus* isolates from both outbreaks in a regional hospital and intercontinental collections (23–25), whole-genome sequencing (WGS) of multiresistant *Klebsiella* (26) or carbapenem-resistant *Acinetobacter baumannii* (27), and the identification of genes associated with the higher resistance shown by these strains. Therefore, as previously researched in antibiotic-resistant strains (25), WGS of hyperresistant derivative strains previously obtained (11) with ICs would, in principle, permit the identification of single nucleotide polymorphisms (SNPs) and insertions or deletions (indels) in comparison with the WT.

Here, we performed WGS of WT *E. coli* MG1655 and the isogenic strains CAR, CIT, and LIM, isolated and characterized in a previous work (11).

RESULTS AND DISCUSSION

Genomic sequencing of isogenic WT MG1655, CAR, CIT, and LIM strains. Three IC-resistant mutants of *E. coli* MG1655 were selected by subculturing with carvacrol (CAR strain), citral (CIT strain), or (+)-limonene oxide (LIM strain) for 10 days. The MICs of the WT were 200, 1,000, and 750 $\mu\text{l liter}^{-1}$ for carvacrol, citral, and (+)-limonene oxide, respectively, whereas the derivative strains showed increased MICs for the ICs that had been applied in their selection process (Table 1) (11).

In addition, the resistance of derivative strains to antibiotics was compared to that of the WT strain by an agar disk diffusion assay (Table 2). An increase in the resistance of derivative strains to the antibiotics ampicillin, trimethoprim, chloramphenicol, tetracycline, kanamycin, novobiocin, norfloxacin, cephalaxin, and nalidixic acid was detected with regard to the WT ($P < 0.05$). However, no differences in resistance to rifampin were found between derivative strains under our experimental conditions.

We conducted WGS of the WT, CAR, CIT, and LIM strains in order to elucidate the genetic events associated with the IC-resistant phenotype. Since the WT, CAR, CIT, and LIM strains are derivatives of the sequenced MG1655 strain, we used this as a reference to evaluate genome coverage and facilitate contig assembly. Using Illumina-Solexa technology, we obtained 29,978,246-, 36,724,652-, 30,398,902-, and 34,299,888-bp paired-end reads for the WT, CAR, CIT, and LIM strains, respectively, mapping the reference genome. Mapping covered 29,779,959, 35,063,939, 27,666,129, and 30,964,563

TABLE 1 MICs of carvacrol, citral, and (+)-limonene oxide in *Escherichia coli* MG1655 and derivative strains

Strain	MIC ($\mu\text{l liter}^{-1}$) ^a		
	Carvacrol	Citral	(+)-Limonene oxide
MG1655 (WT)	200	1,000	750
CAR	600	>1,750	2,250
BC010	200	>1,750	750
CIT	600	>1,750	2,250
BC020	600	>1,750	2,000
BC022	200	1,000	750
LIM	600	>1,750	1,500
BC030	200	>1,750	2,000
BC032	200	1,000	750

^aIncreases in MICs with regard to the WT strain are shaded.

bases in the WT, CAR, CIT, and LIM strains, resulting in 99.34%, 95.48%, 91.01%, and 90.28% MG1655 genome coverages (4,639,675 bp), respectively. Most of the reference genome was covered sufficiently (minimum coverage of 3 read bases per reference base) to allow SNP detection. The paired-end information was used to detect indels with high confidence. Since we sequenced the MG1655 strain that was the isogenic precursor of the CAR, CIT, and LIM strains, only the differences between our laboratory MG1655 strain, not the reference MG1655 strain, and IC derivatives were extracted.

SNP and indel differences detected between the WT MG1655, CAR, CIT, and LIM strains. Computer analysis of interstrain differences between the assembled WT, CAR, CIT, and LIM genomes first revealed 3 potential SNPs (1 in CAR and 2 in CIT) and 9 potential indels (2 in CAR, 5 in CIT, and 2 in LIM). Each difference was subsequently reexamined by PCR amplification using genomic DNA (gDNA), appropriate primers, and sequencing (Table 3). One potential SNP and 6 potential indels were rejected by this analysis. As shown in Table 4, only 1 SNP difference in each derivative strain apparently differentiated CAR and CIT from their parental strain. Furthermore, the CIT and LIM strains also possessed 2 and 1 indels, respectively, that apparently differentiated them from the WT. The mutations are depicted on circular schematic maps (Fig. 1).

In the CAR derivative strain, we detected a single SNP, changing guanine to thymine (transversion) at genome position 4275900 (Table 4), which corresponds to *soxR* (Fig. 1A). Conceptual translation predicted the SoxR missense mutation D137Y. This open reading frame (ORF) codes for the superoxide response regulator, which controls the transcription of the regulon involved in defense against redox-cycling drugs (28) and in responses to nitric oxide (29), activating genes for resistance to both oxidants

TABLE 2 Zones of growth inhibition for agar disk diffusion assays with antibiotics (disk diameter of 6.0 mm included)^a

Antibiotic	Mean inhibition halo diam (mm) \pm SD for strain					
	WT	CAR	CIT	LIM	BC010	BC020
Ampicillin	19.3 \pm 0.7	16.0 \pm 0.5**	12.3 \pm 0.6**	14.5 \pm 0.5**	18.4 \pm 0.7 NS	11.8 \pm 0.8**
Trimethoprim	25.7 \pm 0.6	17.3 \pm 0.6**	20.7 \pm 1.5**	19.8 \pm 1.0**	24.8 \pm 1.3 NS	18.9 \pm 1.2**
Chloramphenicol	20.2 \pm 0.3	11.3 \pm 0.6**	11.0 \pm 0.2**	15.7 \pm 0.6**	19.6 \pm 0.5 NS	11.1 \pm 0.4**
Tetracycline	23.3 \pm 1.2	20.2 \pm 1.0**	19.7 \pm 0.6**	20.3 \pm 1.2**	6.8 \pm 0.7** ^b	18.6 \pm 0.7**
Rifampin	16.7 \pm 0.6	16.0 \pm 0.2 NS	16.7 \pm 0.6 NS	15.7 \pm 0.6 NS	16.1 \pm 0.3 NS	15.9 \pm 0.3 NS
Kanamycin	22.5 \pm 0.5	19.3 \pm 0.6**	19.7 \pm 0.6**	19.0 \pm 0.2**	22.4 \pm 0.5 NS	6.2 \pm 0.1** ^c
Novobiocin	15.8 \pm 0.8	10.7 \pm 0.6**	10.0 \pm 0.1**	11.0 \pm 0.2**	14.8 \pm 0.7 NS	9.9 \pm 0.4**
Norfloxacin	25.3 \pm 1.2	24.7 \pm 0.6 NS	19.0 \pm 0.1**	20.3 \pm 0.6**	25.6 \pm 0.4 NS	19.8 \pm 0.6**
Cephalexin	19.8 \pm 1.0	18.0 \pm 1.7*	16.3 \pm 0.6**	16.8 \pm 1.0**	20.4 \pm 0.5 NS	16.7 \pm 0.5**
Nalidixic acid	21.3 \pm 0.6	17.8 \pm 0.8**	14.8 \pm 0.8**	16.2 \pm 1.0**	21.2 \pm 0.3 NS	14.4 \pm 0.7**

^aEach value represents the mean diameter of the inhibition halo \pm standard deviation from three independent experiments ($n = 3$). Significant changes with regard to the WT strain are shaded. NS, not significantly different from the WT ($P > 0.05$); *, significantly different from the WT ($P < 0.05$); **, significantly different from the WT ($P < 0.001$).

^bThe presence of the Tet^r marker in the BC010 strain increased tetracycline resistance in relation to the CAR strain, as expected.

^cThe presence of the Kan^r marker in the BC020 strain increased kanamycin resistance in relation to the CIT strain, as expected.

TABLE 3 Primers used for verification

Description of amplified region	Forward sequence (5'→3')	Reverse sequence (5'→3')
<i>acrR</i> SNP in LIM	CTGTAAATTCACGAACATATGGCAC	TCAGATTCAGGGTTATTCGTTAGTG
Deleted 11-kb region in LIM	TCTTATCATGCCTACAAACCACATC	GTTTCTGATTTCAITTTGTGGCAGAC
Deleted 4-kb region in LIM	ATCGATGATCTGAATACGTAGACCT	CTCTGCTTTCTAATCTGAACGGTTA
Flanking regions of 11-kb deletion in LIM	GTAATTCCGGTGATGAAAATGCTG	CCACCACGGTAAGGATGATAATTTTC
Flanking regions of 4-kb deletion in LIM	ATTGATCAACCTTTTCTACCGCTAC	CTTTGACGTGGTGATATGGATGAC
Flanking regions of 95-kb deletion in CAR	ACGAATGATAAGGTATTCAAGGCAG	GATCACAGCTTTCACGACCTTAATA
<i>gyrB</i> deletion in CIT	AGAAGTAGAAGATATTCGGGTGGAT	TATTCGAGGTGGTAGATAACGCTAT
Intergenic region between <i>eptB</i> and <i>yhjX</i> in CIT	AAATGCCTTTCAGACGGTAAAATC	TATGTGACCTTCTACGTGATTTTCG
and deleted 95-kb region in CAR		
<i>marR</i> insertion in CIT	AAGGGGTAAACAAGGATAAAGTGTC	GATCCAGTCCAAAATGCTATGAATG
<i>soxR</i> SNP in CAR	TTCATCTGTTGGGGAGTATAATTCC	ATACTTTTACTGCGGAAGAAACG

and antibiotics (30). It might be possible that this mutation renders the SoxR protein active in the absence of oxidative stress, as observed previously (31–34).

In the CIT derivative strain, we detected an in-frame deletion of 3 nucleotides (GGT) at genome position 3877671 (Table 4) within *gyrB* (Fig. 1B), causing the in-frame removal of glycine 157 (Δ G157). The *gyrB* locus encodes the GyrB subunit of DNA gyrase, which is required for the ATPase activity of the enzyme. A mutation in GyrB could cause an increased resistance of a bacterial strain if citral or other ICs acted as competitive inhibitors of ATPase activity, as novobiocin and other coumarin antibiotics do (35). It might also be possible that the *gyrB* mutation causes pleiotropic effects by modulation of DNA supercoiling leading to increased resistance, consistent with the results shown in Table 2. Also, in the CIT strain, we detected a frameshift mutation caused by an insertion of a single adenine at position 1617171 (Table 4), affecting *marR* (multiple-antibiotic resistance) at glutamic acid 10 (Fig. 1B). This gene participates in controlling several genes involved in resistance to antibiotics, multidrug efflux (36–38), oxidative stress, organic solvents, and heavy metals (39). MarR is part of the *marRAB* operon and negatively autoregulates its own expression (40). Therefore, the inactivation of *marR* results in the increased expression of *marA*, which acts upon several target genes in the cell, leading to the reduced accumulation of antibiotics and phenolic ring-containing compounds (41–43).

In the LIM strain, we confirmed the frameshift mutation caused by a 1-nucleotide insertion of cytosine at position 485340 (Table 4). This change is located at phenylalanine 119 of *acrR* (*acriflavine resistance regulator*) (Fig. 1C). *acrR* has been implicated in the regulation of the expression of genes involved in multidrug transport, such as the *acrAB*-encoded efflux pump (44). The AcrAB-TolC efflux pump has been shown to provide intrinsic tolerance to organic solvents (45) by enhancing the removal of intracellularly accumulated solvents in *E. coli* cells.

TABLE 4 SNPs, indels, and large deletions of *Escherichia coli* MG1655 derivative strains

Mutant strain	Genome position(s) in the WT ^a	WT base	Mutant base(s)	SNP/indel in gene	Locus tag	EcoGene accession no.	Result of mutation
CAR	3623620–3718650 ^b 4275900 (92.15')	G	T	Δ (<i>yhhJ-mokA</i>) <i>soxR</i>	b4063	EG10957	Deletion of 75 genes Predicted SoxR missense mutation D137Y
CIT	1617171 (34.85') 3708613 (79.89')	A	+A C	<i>marR</i> Intergenic <i>eptB-yhjX</i>	b1530 Intergenic b3546-b3547	EG11435 Intergenic EG12267-EG12268	MarR E10 frameshift
	3877671 (83.53')		–GGT	<i>gyrB</i>	b3699	EG10424	GyrB in-frame Δ G157
LIM	485340 (10.45') 1972710–1976520 ^b 2800250–2811150 ^b		+C	<i>acrR</i> Δ (<i>cheA-insA</i>) Δ (<i>nrdE-enrB</i>)	b0464	EG12116	ArcR F119 frameshift Deletion of 3 genes Deletion of 9 genes

^aValues in parentheses represent the position of the mutation in a "100-minute map."

^bEndpoints of large chromosomal deletions.

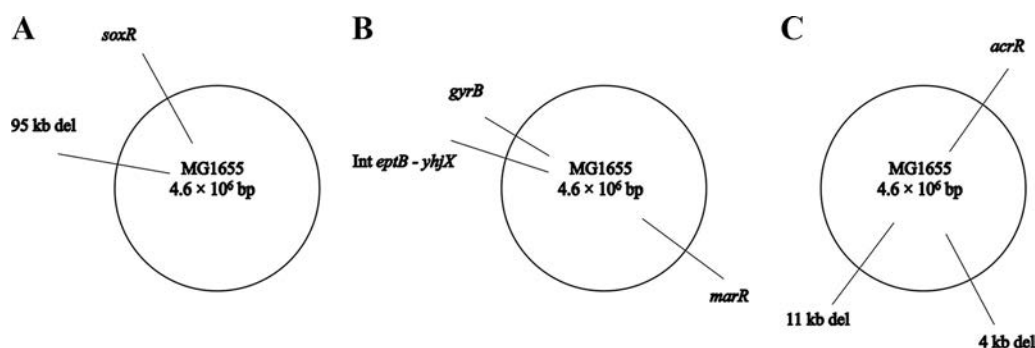


FIG 1 Genomic maps of the chromosomes of the *Escherichia coli* MG1655 derivative strains CAR, CIT, and LIM.

Interestingly, each of the mutated genes detected through WGS in our derivative strains had been previously linked to altered antibiotic resistance. For instance, Webber and Piddock (46) found a number of mutations in the *acrR*, *marR*, and *soxR* repressor genes of fluoroquinolone-resistant *E. coli* strains, correlating with larger amounts of *acrA*, *marA*, and *soxS* mRNAs, respectively. In addition, mutations conferring a multidrug resistance phenotype have been found in the genes *marR*, *soxR*, and *acrR* among both clinical and veterinary *E. coli* isolates (47–51), with *acrAB* and *tolC* being *marA*-*soxS*-*rob* regulon genes (52). Thus, the SNPs and indels found independently in each derivative strain sequenced in this study are remarkably related and could conceivably be causing the increased resistance of the strains through a common mechanism.

Identification of large deletions in CAR, CIT, and LIM compared to WT strain MG1655. In addition to the point mutations described above, we identified one and two large deletions in CAR and LIM, respectively, compared to the WT (Table 4). These deletions were each verified by appropriate PCR and sequence analyses. One 95,030-bp deletion (here called the “95-kb deletion”) was located between bp 3623620 and 3718650 of the CAR strain, leaving a gap just after *yhhI*, a predicted transposase, and immediately before *insJ*, which codes for the IS150 protein InsA. This 95-kb region contained 75 genes in the WT strain (Table 4). As previously shown (11), cells from the CAR derivative strain displayed a filamentous morphology. Interestingly, three of the genes deleted in the CAR strain along with the 95 kb-deletion have been associated with elongation: *rbhA* and *yhjD* double-deletion mutant cells were elongated when grown at 15°C or 32°C and showed impaired translation fidelity (53), and a *bcsQ* mutant exhibited a filamentous morphology, a heterogeneous cell size, and a defect in chromosome partitioning (54). YhiM is necessary for RpoS-, glutamine-, and lysine-dependent acid resistance, but it is not required for arginine-dependent acid resistance (55), which was identified as a final Gene Ontology (GO) term for the ontology tree constructed with the upregulated genes after carvacrol treatment (56).

In the LIM derivative strain, a 3,810-bp deletion (here called the “4-kb deletion”) was located between bp 1972710 and 1976520 upstream of a mobile element. Genes removed by this large deletion include *cheA*, *flhC*, and *insA*. Between bp 2800250 and 2811150, we observed another large deletion in LIM, of 10,900 bp (here called the “11-kb deletion”). Genes affected by these two deletions in the LIM strain are listed in Table 4. Among them, we found *mprA*, also called *emrR* (*E. coli* multidrug resistance regulator), which negatively regulates the transcription of genes coding for multidrug resistance pumps that exclude structurally unrelated antimicrobial agents from the cell (57, 58).

Genetic analysis of each SNP/indel/large deletion and its contribution to MICs of ICs and antibiotics. The evaluation of MICs revealed that the MIC of carvacrol for CAR was three times higher than that for the WT strain, the MIC of citral for CIT was nearly twice the MIC for the WT, and the MIC of (+)-limonene oxide for LIM was twice that for the WT strain. All three derivative strains showed cross-resistance to the other two ICs with which selection had not been performed (Table 1) and to a wide range of

TABLE 5 Strains used in this study^a

Strain	Relevant genotype	Result(s) of mutation	Characteristic(s)	Source or reference
MG1655	F ⁻ λ ⁻ <i>ΔilvG rfb-50 rph-1</i>			ATCC 700926
CAR	<i>soxR</i> (G4275900T); 95-kb deletion (positions 3623620–3718650)	Predicted SoxR missense mutation D137Y; 1 large chromosomal deletion, <i>Δ(yhhJ-mokA)</i>	MG1655 10-day carvacrol selection	11
CIT	<i>gyrB</i> (–GGT3877671); <i>marR</i> (+A1617171); intergenic <i>eptB-yhjX</i> (A3708613C)	GyrB in-frame <i>ΔG157</i> , MarR E10 frameshift	MG1655 10-day citral selection	11
LIM	<i>acrR</i> (+C485340); 4-kb deletion (positions 1972710–1976520); 11-kb deletion (positions 2800250–2811150)	ArcR F119 frameshift; 2 large chromosomal deletions, <i>Δ(cheA-insA)</i> and <i>Δ(nrdE-enrB)</i>	MG1655 10-day (+)-limonene oxide selection	11
CAG12156	MG1655 <i>uvrC279::Tn10</i>			60
CAG12164	MG1655 <i>malF3089::Tn10</i>			60
CAG18592	MG1655 <i>zie-3163::Tn10</i> Kan			60
BC010	<i>soxR</i> rev. WT (MG1655)		CAR, <i>soxR</i> rev. WT, Tet ^r nearby; P1(CAG12164) × CAR	This study
BC020	<i>gyrB</i> rev. WT (MG1655)		CIT, <i>gyrB</i> rev. WT, Kan ^r nearby; P1(CAG18592) × CIT	This study
BC021	<i>gyrB</i> (–GGT3877671)	GyrB in-frame <i>ΔG157</i>	CIT, <i>gyrB</i> Kan ^r nearby; P1(CAG18592) × CIT	This study
BC022	<i>gyrB</i> (–GGT3877671)	GyrB in-frame <i>ΔG157</i>	MG1655, <i>gyrB</i> (–GGT157) Kan ^r nearby; P1(BC021) × MG1655	This study
BC030	4-kb deletion rev. WT (MG1655)		LIM, 4-kb deletion rev. WT, Tet ^r nearby; P1(CAG12156) × LIM	This study
BC031	4-kb deletion (positions 1972710–1976520)	1 large chromosomal deletion, <i>Δ(cheA-insA)</i>	LIM, 4-kb deletion (positions 1972710–1976520) Tet ^r nearby; P1(CAG12156) × LIM	This study
BC032	4-kb deletion (positions 1972710–1976520)	1 large chromosomal deletion, <i>Δ(cheA-insA)</i>	MG1655, 4-kb deletion (positions 1972710–1976520) Tet ^r nearby; P1(BC031) × MG1655	This study

^arev., reverted to.

antibiotics (Table 2). This finding suggests an interrelation between the mechanisms of bacterial inactivation of carvacrol, citral, (+)-limonene oxide, and even of certain classes of antibiotics. Although structures targeted and/or damaged by these antimicrobial compounds might not be the same, these ICs and antibiotics seem to be acting by triggering similar signals or by evoking similar defensive pathways. This scenario would suggest a common mechanism of bacterial death induced by ICs and antibiotics, which could be related to the generation of and/or protection against ROS, as previously demonstrated (6, 7, 59).

In order to analyze the contribution of each mutation to the increased MICs detected in hyperresistant strains, we next constructed allelic-exchange strains of each derivative strain, CAR, CIT, and LIM, in which mutations were reverted to the genotype of the WT strain. To achieve this, we exploited the Singer-Gross collection (60) of marked strains with Tet or Kan resistance cassettes located near the position of our mutations. The proximity of the Kan^r or Tet^r markers to the physical map positions of our detected mutations yielded a >60% cotransduction frequency of the two features (mutation region, i.e., SNP or indel, and resistance marker) with the transducing P1vir phage; allelic reversion was accomplished and generated the following derivative strains (Table 5): BC010, a CAR strain with *soxR* reverted to the WT; BC020, a CIT strain with *gyrB* reverted to the WT; BC022, a WT strain with *gyrB* containing the mutation from the CIT strain; BC030, a LIM strain without the 4-kb deletion, a region which was reinserted from the WT; and, finally, BC032, a WT strain in which the 4-kb region deleted in the LIM strain had been removed. The cotransduction of the mutations with Kan^r or Tet^r was confirmed by sequencing or appropriate PCR assays.

The strategy of allelic reversion with nearby linked resistance markers was not successful in certain cases: (i) the construction of a WT strain with a *soxR* mutant from CAR, (ii) the reversion of mutant *marR* or the intergenic region between *eptB* and *yhjX* from CIT to the WT or the creation of a WT strain with mutant *marR* or the intergenic

region between *eptB* and *yhjX*, and (iii) the reversion of mutant *acrR* or the 11-kb deletion from LIM to the WT or the generation of a WT strain with mutant *acrR* or the 11-kb deletion. In these cases, up to three different strains were used as donors for P1_{vir} infection, and at least five transductants from each reaction were tested by sequencing or PCR assays. The lack of success in constructing the above-mentioned mutants might indicate an incompatibility due to the lethal nature of the presence of the mutations detected by WGS without the specific mutation, which we were trying to revert to the WT; i.e., mutations might be linked and cannot be separated. Uniquely, the size of the 95-kb deletion of the CAR strain could be the reason why P1_{vir} transduction was not successful, since this bacteriophage can package only sequences of around 90 kb (61). Other genetic methods, such as transduction with T4GT7, a derivative of bacteriophage T4 (62), could be used to create these complementation mutants.

In those cases in which allelic exchange was performed, the behavior of the engineered derivative strains in the presence of the ICs carvacrol, citral, and (+)-limonene oxide was analyzed. For BC010 (Table 5), MICs of carvacrol and (+)-limonene oxide (Table 1) and resistance to all antibiotics (Table 2) returned to those of the WT, while the MIC of citral stayed as increased as that for the CAR strain (Table 1). The behavior of BC010 would confirm a role of *soxR* in resistance to carvacrol, (+)-limonene oxide, and antibiotics and an influence of the 95-kb deletion on the increase of the MIC of citral shown by the CAR strain.

As demonstrated by the CIT strain, the BC020 strain (Table 5) also showed higher MICs of ICs and increased resistance to antibiotics compared to the WT (Tables 1 and 2). BC022 (Table 5) behaved as the WT strain with regard to resistance to ICs (Table 1). Thus, *gyrB* is not likely to be involved in the mechanisms leading to the higher MICs of any of the three ICs or increased resistance to practically every tested antibiotic.

For the LIM derivative strain, obtained by selection with (+)-limonene oxide, we were able to assess the contribution of the 4-kb deletion in both WT and LIM strains (Table 5). Whereas the MIC of carvacrol for BC030 was the same as that for the WT strain (Table 1), the MICs of citral and (+)-limonene oxide for both LIM and BC030 were higher than those for the WT. BC032 (Table 5) showed the same MIC of the three ICs as that for the WT (Table 1). As a consequence, it is unlikely that the 4-kb deletion by itself was involved in the increased MICs of any of the ICs tested, and it is most likely that the *acrR* mutation and/or the 11-kb deletion is responsible for the increased MICs of citral and (+)-limonene oxide in the LIM strain.

Further studies should examine the individual contributions of double mutations, especially in the CIT and LIM strains. This information would reveal a potential role of the SNP in the intergenic region between *eptB* and *yhjX* and confirm whether the insertion in *acrR* and the 11-kb deletion act together to increase the resistance of the CIT and LIM strains, respectively. Additionally, the construction of alternative *E. coli* strains (such as MC4100, W3110, or O157:H7) carrying the mutations from CAR, CIT, and LIM is advisable to address whether the phenotype caused by each or several of the mutations detected by WGS is similar in other *E. coli* strains. It would be equally important to distinguish between the dominant or recessive natures of the mutations detected through WGS in *E. coli* MG1655 derivative strains. This could be achieved by the transformation of these strains with plasmids containing the WT form of each mutation or the transformation of the WT strain with plasmids containing each mutation and evaluating resistance to ICs.

Effects of single mutations and their contributions to cell survival against ICs and heat. As described in a previous study characterizing the derivative strains CAR, CIT, and LIM, each strain showed a different behavior when lethal treatments with the ICs carvacrol, citral, and (+)-limonene oxide were applied (11). Analysis of the survival curves shown in Fig. 2 to 5 offers a deeper view into the different tolerances shown by derivative strains with regard to the WT. While the CIT strain was more tolerant to carvacrol treatment than the WT strain (Fig. 2B), it is noteworthy that CAR and LIM displayed different kinetics of inactivation by carvacrol treatments than those of the WT strain (Fig. 2A and C), with these strains showing a higher initial drop of cell counts than

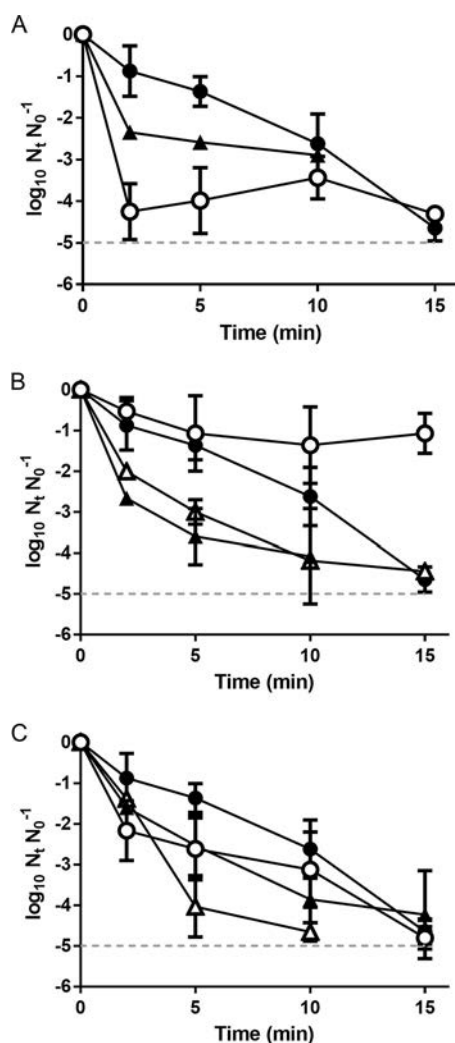


FIG 2 Inactivation of *Escherichia coli* WT strain MG1655 (●) and its derivative strain CAR (A), CIT (B), or LIM (C) (○); a derivative strain from ICs with the reverted genotype, BC010 (A), BC020 (B), or BC030 (C) (▲); and the WT strain with a mutant genotype, BC022 (B) or BC032 (C) (△), after treatment with 200 μ l/liter of carvacrol. Data are means \pm standard deviations (error bars). Horizontal gray dotted lines represent the limit of detection (5 \log_{10} cycles).

WT cells. As a consequence, low rates of survival of CAR and LIM against carvacrol treatment were detected after 2- and 5-min treatments in comparison to the WT strain ($P \leq 0.05$). The inactivation of the CAR and LIM strains after 10 and 20 min of treatment with carvacrol was similar to that of the WT. Contrary to the behavior against lethal treatments with carvacrol, higher tolerances of the three derivative strains to citral or (+)-limonene oxide were observed (Fig. 3 and 4). Finally, since heat inactivation of all the *E. coli* strains tested followed linear kinetics (Fig. 5), decimal reduction times (D_T values) were calculated to compare their survival rates more accurately (Table 6). The D_{55} value was 4.3 min for the WT strain, whereas the three derivative strains had higher D_{55} values (ca. 7 min), indicating a higher thermal tolerance of derivative strains.

The inactivation of the WT by lethal treatment with carvacrol (Fig. 2A), citral (Fig. 3A), or (+)-limonene oxide (Fig. 4A) was compared to those of CAR and BC010 (Table 5). CAR showed higher sensitivity to lethal carvacrol treatment but lower sensitivity to citral and (+)-limonene oxide than the WT, whereas BC010 inactivation resembled that of the WT with all ICs tested (Fig. 2A, 3A, and 4A). These results suggest that *soxR* could be involved in the tolerance of the CAR derivative strain to ICs. However, since BC010 was as thermally sensitive as the CAR strain, the mutation in *soxR* did not detectably play a

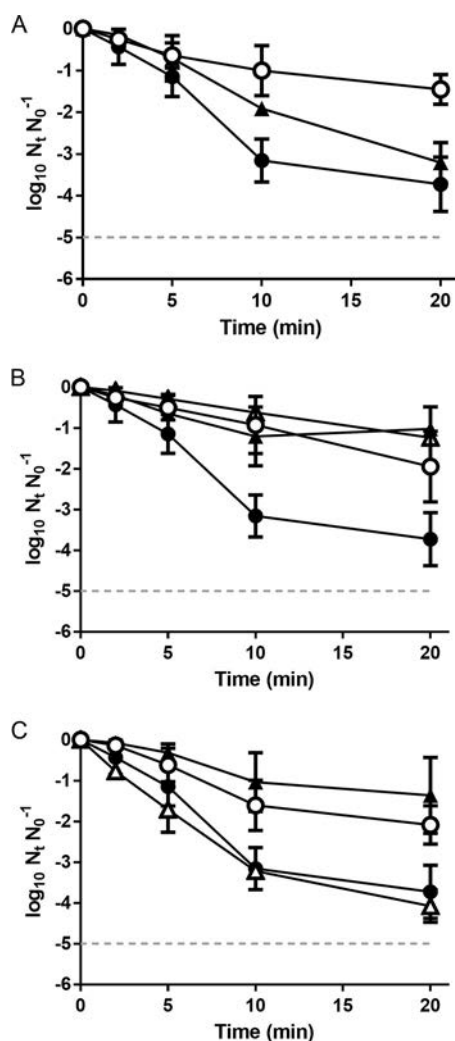


FIG 3 Inactivation of *Escherichia coli* WT strain MG1655 (●) and its derivative strain CAR (A), CIT (B), or LIM (C) (○); a derivative strain from ICs with the reverted genotype, BC010 (A), BC020 (B), or BC030 (C) (▲); and the WT strain with a mutant genotype, BC022 (B) or BC032 (C) (△), after treatment with 300 μ l/liter of citral. Data are means \pm standard deviations (error bars). Horizontal gray dotted lines represent the limit of detection (5 \log_{10} cycles).

role in tolerance to thermal treatments (Table 6). Consequently, the large 95-kb deletion in both CAR and BC010 would be responsible for the higher bacterial tolerance to lethal heat treatments. Further research is needed in order to identify and characterize the role of the gene(s) in the 95-kb region in thermal tolerance. The lower survival rate of CAR cells during lethal carvacrol treatment (Fig. 2A) may indicate different mechanisms for *E. coli* tolerance and resistance to carvacrol.

Regarding CIT, it showed higher rates of survival against lethal treatments with carvacrol (Fig. 2B) and heat (Fig. 5B and Table 6) than the WT. However, both BC020 and BC022 (Table 5) were more rapidly inactivated by lethal carvacrol treatment than the WT strain (Fig. 2B) and were inactivated by heat treatments at the same rate as that of the WT (Fig. 5B and Table 6). In addition, CIT, BC020, and BC022 showed higher rates of survival against lethal treatments with citral and (+)-limonene oxide than did the WT (Fig. 3B and 4B). Therefore, the increased tolerance to these treatments shown by CIT might be related to SNPs in the intergenic region between *eptB* and *yhjX* and/or *marR*, as suggested for the higher resistance of CIT (Table 1).

Although high survival rates of LIM and BC030 were detected after citral treatment (Fig. 3C) in comparison to the WT, the inactivation of BC032 by citral was similar to that of the

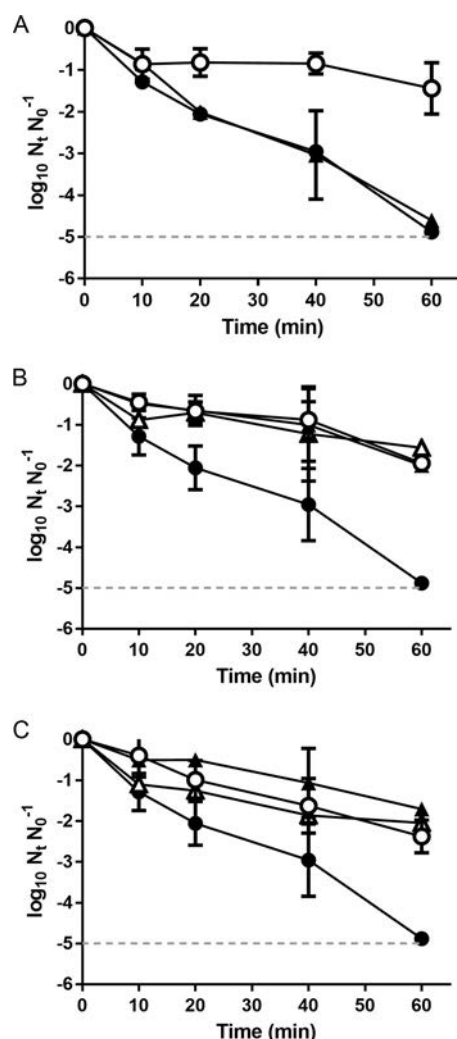


FIG 4 Inactivation of *Escherichia coli* WT strain MG1655 (●) and its derivative strain CAR (A), CIT (B), or LIM (C) (○); a derivative strain from ICs with the reverted genotype, BC010 (A), BC020 (B), or BC030 (C) (▲); and the WT with a mutant genotype, BC022 (B) or BC032 (C) (△), after treatment with 500 $\mu\text{l/liter}$ of (+)-limonene oxide. Data are means \pm standard deviations. Horizontal gray dotted lines represent the limit of detection (5 \log_{10} cycles).

WT, suggesting that the 4-kb deletion by itself could be involved in survival against this IC. Higher rates of survival of LIM, BC030, and BC032 against (+)-limonene oxide challenge than that of the WT (Fig. 4C) indicate a protective role of the *acrR* mutation and/or the 11-kb deletion against this IC, independently of the 4-kb deletion. Moreover, the rates of survival of LIM, BC030, and BC032 against carvacrol treatment were not higher than that of the WT (Fig. 2C), suggesting that the mutation in *acrR* and/or the 11-kb deletion would be a disadvantage for survival in the presence of carvacrol.

Thus, CAR, CIT, and LIM showed higher tolerance and/or resistance to ICs, caused by the point and large mutations uncovered in the present study. In addition, these results suggest different mechanisms for resistance and tolerance to ICs.

Conclusions. The WGS and bioinformatics analyses allowed the identification of mutations in the genomes of hyperresistant *E. coli* MG1655 strains obtained by selection with ICs. The strain obtained by selection with carvacrol (CAR) revealed a large deletion of 95,000 bp and a SNP resulting in a missense mutation (D137Y) in *soxR*, a gene implicated in oxidative stress defense. In a citral-selected strain (CIT), we found a SNP in the intergenic region between *yhjX* and *eptB*; a 3-nucleotide deletion in *gyrB*, encoding DNA-gyrase; and a 1-nucleotide insertion resulting in a frameshift mutation

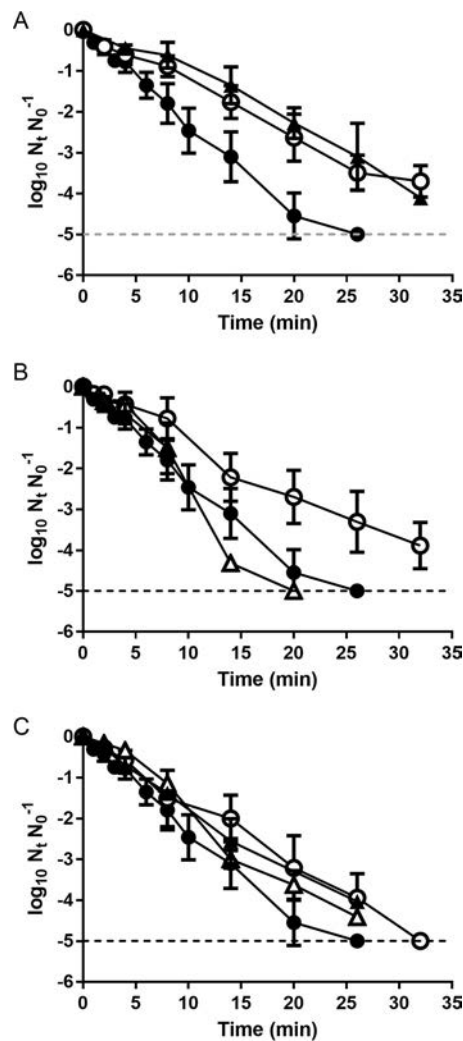


FIG 5 Inactivation of *Escherichia coli* WT strain MG1655 (●) and its derivative strain CAR (A), CIT (B), or LIM (C) (○); a derivative strain from ICs with a reverted genotype, BC010 (A), BC020 (B), or BC030 (C) (▲); and the WT strain with a mutant genotype, BC022 (B) or BC032 (C) (△), after heat treatment at 55°C. Data are means ± standard deviations (error bars). Horizontal gray dotted lines represent the limit of detection (5 log₁₀ cycles).

in *marR*, a gene linked to multiple-antibiotic resistance. Finally, the strain obtained by the selection of (+)-limonene oxide (LIM) was found to possess two large deletions of 4 and 11 kb and a 1-nucleotide insertion leading to a frameshift mutation in *acrR*, a gene involved in multidrug transport gene regulation.

TABLE 6 Decimal reduction values for the *Escherichia coli* MG1655 wild-type strain and its derivative strains CAR, CIT, and LIM heat treated at 55°C^a

Strain	Mean <i>D</i> ₅₅ (min) ± SD
WT	4.43 ± 0.86
CAR	7.72* ± 1.05
BC010	8.79* ± 2.38
CIT	7.03* ± 1.31
BC020	5.50 ± 1.17
BC022	4.00 ± 1.25
LIM	6.27* ± 1.04
BC030	6.36* ± 0.36
BC032	5.43 ± 0.07

^aData are means ± standard deviations. Asterisks indicate significant differences of derivative strains with regard to the WT strain (*P* > 0.05).

The genotypic reversion of hyperresistant strains allowed the study of the contribution of each mutation to the increased antimicrobial resistance and/or tolerance of the derivative strains. We confirmed the role of the SNP in *soxR* in the increased MICs of ICs; resistance to ampicillin, trimethoprim, chloramphenicol, tetracycline, kanamycin, novobiocin, norfloxacin, cephalexin, and nalidixic acid; and survival against lethal treatments with ICs in the carvacrol-selected strain. Furthermore, in the strain selected with citral, the hyperresistant phenotype might rely on SNPs in *marR* or on the intergenic region between *eptB* and *yhjX*. Finally, the insertion in *acrR* and the large deletions found in LIM were most likely responsible for the increased survival observed for this strain.

Although carvacrol, citral, and (+)-limonene oxide had dissimilar structures compared to those of the various antibiotic classes tested, the three ICs evoked mechanisms of defense similar to those observed for antibiotics and even analogous mechanisms of bacterial inactivation probably related to oxidative damage. These observations suggest that the physiological responses triggered by exposure to ICs and antibiotics share much in common. In addition, we showed that despite the particular IC driving selection, similar phenotypic profiles were obtained, suggesting that there might be multiple genetic responses to the applied selection pressure that nevertheless culminate in similar cellular adaptations to subvert antibiotic or IC stress.

Such valuable information increases our knowledge of the mechanisms of bacterial inactivation via carvacrol, citral, and (+)-limonene oxide derived from EOs. While here we present a detailed analysis suggesting a role of genes and mechanisms previously associated with antibiotic resistance, it is certainly possible that additional genes and pathways are involved in the acquisition of endogenous IC (and antibiotic) resistance and await discovery.

MATERIALS AND METHODS

Microorganisms and growth conditions. The strain used in this study was *Escherichia coli* MG1655. The culture was maintained in a cryovial at -80°C , from which plates of tryptic soy agar (Oxoid, Basingstoke, Hampshire, England) with 0.6% yeast extract (Oxoid) (TSAYE) were prepared on a weekly basis.

We prepared broth subcultures by inoculating 5 ml of sterile tryptic soy broth (Oxoid) and 0.6% yeast extract (TSBYE) with one single colony from a plate. After inoculation, the tubes were incubated overnight at 37°C . Flasks (250 ml) containing 50 ml of TSBYE were inoculated with the resulting subcultures to a final concentration of 10^5 CFU ml^{-1} . These flasks were incubated with agitation (130 rpm) (Rotabit; Selecta, Barcelona, Spain) at 37°C until the late stationary growth phase was reached (24 h). Tetracycline and kanamycin (Sigma-Aldrich, Steinheim, Germany) were used when needed at 10 and 30 $\mu\text{g ml}^{-1}$, respectively.

The strains used in this study are listed in Table 5.

Genome sequencing and SNP analysis. gDNA was prepared from cultures of parental WT *E. coli* strain MG1655 and the three isogenic IC-resistant derivative strains grown overnight in TSBYE at 37°C , as previously described (11). Solexa technology was used to sequence gDNAs of the four strains on an Illumina Hi-Seq 2500 genome analyzer instrument (Fasteris, SA, Geneva, Switzerland). The quality control-filtered paired-end reads (17.2 million 100-bp reads) were mapped onto the MG1655 genome sequence (NCBI accession number [NC_000913.2](#)) by using the Burrows-Wheeler alignment tool (63), giving a raw coverage depth of approximately 700-fold. Mapping covered 99.34%, 95.48%, 91.01%, and 90.28% of MG1655 for our stocked WT and CAR, CIT, and LIM isogenic strains. The generated consensus MG1655, CAR, CIT, and LIM sequences were then compared to detect SNPs and indel differences by Burrows-Wheeler alignment (63) together with SAMtools (64) and the CLC workbench. Sequence information was further analyzed by IGV (Broad Institute). All detected SNPs and indels were subsequently verified or rejected by PCR of relevant gDNA and capillary sequencing. Additional details are available upon request.

Generalized P1vir transduction. A method for the rapid, precise, and easy manipulation of phenotype-causing screenable mutations in *E. coli* was created in 1989 (60), comprising a library of selectable transposon insertion markers that can be used for mapping by replacement.

Phage P1vir is widely used for the generalized transduction of *E. coli* genome segments by replacing the homologous segment in the recipient with a sequence from the donor *E. coli* strain on which the phage was grown. To prepare transducing stocks, each single donor strain colony was grown overnight in TSBYE with 10 $\mu\text{g ml}^{-1}$ of tetracycline or 30 $\mu\text{g ml}^{-1}$ of kanamycin under agitation (130 rpm) for 24 h at 37°C . Cultures were diluted 1:100 into 2 ml TSBYE with 10 mM MgCl_2 (Probus, Badalona, Spain), 5 mM CaCl_2 (Sigma-Aldrich), and 0.2% glucose (VWR, Leuven, Belgium) and incubated at 37°C until an optical density at 595 nm (OD_{595}) of 0.25 was reached. Cultures were then infected with 80 μl of P1vir (previously grown on strain MG1655) and incubated for an additional 3 to 4 h at 37°C under agitation (130 rpm). When cultures were lysed, CHCl_3 (Panreac, Castellar del Vallés, Spain) was added at 50 $\mu\text{l ml}^{-1}$ per tube, and the tubes were vortexed. Insoluble debris was pelleted (MiniSpin Plus centrifuge at

14,000 $\times g$ for 2 min), and supernatant suspensions of phage P1vir particles (P1vir stocks) were filter sterilized (0.22- μ m pore; VWR), collected into sterile screw-cap glass tubes, and stored at 4°C.

For P1vir transductions, recipient *E. coli* strains were grown overnight in TSBYE, and cells were collected in one-third of the harvested volume in fresh TSBYE with 100 mM MgSO₄ (Panreac) and 5 mM CaCl₂. An equal volume of the P1vir lysate was added, and tubes were incubated for 30 min at 37°C. An equal volume of 1 M sodium citrate (Panreac) and 2 volumes of fresh TSBYE were added. Tubes were incubated for 1 h at 37°C under agitation (130 rpm). Cultures were then centrifuged at 2,000 $\times g$ for 5 min, and pellets were resuspended in 100 μ l of TSBYE supplemented with 100 mM sodium citrate and plated onto TSAYE with 10 μ g ml⁻¹ of tetracycline or 30 μ g μ l liter⁻¹ kanamycin and 10 mM sodium citrate. Plates were incubated for 24 h at 37°C, and 10 colonies were restreaked onto fresh TSAYE citrate plates in order to eliminate residual phage contamination.

Kanamycin- or tetracycline-resistant strains (CAG12156, CAG12164, and CAG18592) (Table 5) for P1vir phage cotransduction were obtained from the Singer-Gross collection (60). P1vir cotransduction relies on the proximity of the genes, and it was performed to move the tightly linked kanamycin or tetracycline resistance genes and regions of our interest into derivative strains or MG1655 by selecting for kanamycin or tetracycline resistance.

MIC determinations. MIC values of carvacrol (95%; Sigma-Aldrich), citral (95%; Sigma-Aldrich), and (+)-limonene oxide (97%; Sigma-Aldrich) for *E. coli* MG1655 were determined by the tube dilution method with an initial concentration of 10⁵ CFU ml⁻¹ (65). Tested concentrations were 50, 100, 200, 300, 500, 600, and 700 μ l liter⁻¹ of carvacrol; 200, 500, 1,000, 1,500, and 1,750 μ l liter⁻¹ of citral; and 200, 500, 750, 1,000, 1,500, 2,000, 2,250, and 2,500 μ l liter⁻¹ of (+)-limonene oxide. These ICs are practically immiscible in water; therefore, we applied a vigorous shaking method to prepare suspensions (66). For ICs, we also prepared negative controls containing TSBYE plus 2,500 μ l liter⁻¹ of ICs and positive controls containing TSBYE with microorganisms at a final concentration of 10⁵ CFU ml⁻¹. After 24 h of incubation at 37°C, the MIC was determined as the lowest concentration of each IC at which no visible changes could be detected in the broth medium (67).

Evaluation of resistance. (i) Lethal IC treatments. Prior to treatment, cultures were centrifuged at 6,000 $\times g$ for 5 min and resuspended at a final concentration of 2 \times 10⁷ CFU ml⁻¹ in 10 ml of McIlvaine citrate-phosphate buffer (pH 7.0) with 200 μ l liter⁻¹ of carvacrol, 300 μ l liter⁻¹ of citral, or 500 μ l liter⁻¹ of (+)-limonene oxide added. These treatment conditions were chosen in order to inactivate >3 log₁₀ cycles of the WT strain during the course of the challenge experiment according to the results of pilot experiments (data not shown). IC treatments were carried out at room temperature. Samples (0.1 ml) were taken at the determined intervals to enumerate survivors.

(ii) Lethal heat treatments. Heat treatment was carried out in a thermostated incubator (FX incubator, model ZE/FX; Zeulab, Zaragoza, Spain) at 55°C, with a thermocouple (Almemo 2450; Ahlborn, Holzkirchen, Germany) to monitor the heating temperature. Prior to treatment, cultures were centrifuged at 6,000 $\times g$ for 5 min and resuspended at a final concentration of 2 \times 10⁷ CFU ml⁻¹ in 450 μ l of McIlvaine citrate-phosphate buffer (pH 7.0) once the temperature had stabilized at 55°C. These treatment conditions were chosen according to pilot experiments (data not shown). After treatment of bacterial suspensions at this temperature for 26 min, 0.1-ml samples were taken at the determined intervals to enumerate survivors.

(iii) Enumeration of survivors. After treatment, samples were diluted in phosphate-buffered saline (pH 7.3) (Oxoid), and the samples (0.1 ml) were then pour plated onto TSAYE. Plates were incubated at 37°C for 24 h. After plate incubation, the colonies were counted with an improved image analysis automatic colony counter (Protos; Analytical Measuring Systems, Cambridge, United Kingdom) as previously described (68). Inactivation was expressed as the difference in log₁₀ counts prior to and after treatment in each case. The error bars in the figures indicate the means \pm standard deviations from the data obtained from at least three independent experiments carried out with different microbial cultures.

Antibiotic susceptibility testing. An agar disk diffusion assay was used for testing antibiotic susceptibility. An *E. coli* suspension (0.5 ml of 10⁷ CFU ml⁻¹) was spread onto the surface of TSAYE plates. After 10 min, blank disks (Oxoid ST6090 antimicrobial susceptibility disk dispenser; Thermo Scientific, Waltham, MA) were placed onto the surface of the inoculated plates and individually impregnated with 10 μ l of each antibiotic at the following concentrations: 5 mg ml⁻¹ ampicillin, 500 μ g ml⁻¹ trimethoprim, 3 mg ml⁻¹ chloramphenicol, 3 mg ml⁻¹ tetracycline, 6 mg ml⁻¹ rifampin, 3 mg ml⁻¹ kanamycin sulfate, 100 mg ml⁻¹ novobiocin sodium, 1 mg ml⁻¹ norfloxacin, 5 mg ml⁻¹ cephalixin, and 5 mg ml⁻¹ nalidixic acid sodium. All the antibiotics were provided by Sigma-Aldrich. These plates were incubated at 37°C for 18 to 24 h, and the diameters of the resulting inhibition zones were then measured (6.0-mm paper disk diameter included). The inner zone was considered for calculations in those cases where a double inhibition zone appeared after incubation.

Statistical analysis. Data for the evaluation of MICs, lethal treatments, and agar disk diffusion assays were obtained from at least three independent experiments carried out with different microbial cultures.

Data were analyzed and submitted to comparison of averages using analysis of variance (ANOVA) followed by a Tukey *post hoc* test and *t* tests with GraphPad PRISM (GraphPad Software, Inc., San Diego, CA, USA), and differences were considered significant if the *P* value was ≤ 0.05 . For MIC determination assays, the results were expressed as modal values because the MIC values were the same in all repetitions. For antibiotic susceptibility testing, results for derivative strains were compared to those for the WT strain by ANOVA.

Accession number(s). Complete genomic sequences of the CAR, CIT, and LIM strains have been deposited in the NCBI database under the accession numbers [NZ_CP026026.1](#), [NZ_CP026028.1](#), and [NZ_CP026027.1](#), respectively.

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