Polymorphic Variation in Susceptibility and Metabolism of Triclosan-Resistant Mutants of *Escherichia coli* and *Klebsiella pneumoniae* Clinical Strains Obtained after Exposure to Biocides and Antibiotics

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Exposure to biocides may result in cross-resistance to other antimicrobials. Changes in biocide and antibiotic susceptibilities, metabolism, and fitness costs were studied here in biocide-selected *Escherichia coli* and *Klebsiella pneumoniae* mutants. *E. coli* and *K. pneumoniae* mutants with various degrees of triclosan susceptibility were obtained after exposure to triclosan (TRI), benzalkonium chloride (BKC), chlorhexidine (CHX) or sodium hypochlorite (SHC), and ampicillin or ciprofloxacin. Alterations in antimicrobial susceptibility and metabolism in mutants were tested using Phenotype MicroArrays. The expression of AcrAB pump and global regulators (SoxR, MarA, and RamA) was measured by quantitative reverse transcription-PCR (qRT-PCR), and the central part of the *fabI* gene was sequenced. The fitness costs of resistance were assessed by a comparison of relative growth rates. Triclosan-resistant (TRI^r) and triclosan-hypersusceptible (TRI^{hs}) mutants of *E. coli* and *K. pneumoniae* were obtained after selection with biocides and/or antibiotics. *E. coli* TRI^r mutants, including those with mutations in the *fabI* gene or in the expression of *acrB*, *acrF*, and *marA*, exhibited changes in susceptibility to TRI, CHX, and antibiotics. TRI^r mutants for which the TRI MIC was high presented improved metabolism of carboxylic acids, amino acids, and carbohydrates. In TRI^r mutants, resistance to one antimicrobial provoked hypersusceptibility to another one(s). TRI^r mutants had fitness costs, particularly *marA*-overexpressing (*E. coli*) or *ramA*-overexpressing (*K. pneumoniae*) mutants. TRI, BKC, and CIP exposure frequently yielded TRI^r mutants exhibiting alterations in AraC-like global regulators (MarA, SoxR, and RamA), AcrAB-TolC, and/or FabI, and influencing antimicrobial susceptibility, fitness, and metabolism. These various phenotypes suggest a trade-off of different selective processes shaping the evolution toward antibiotic/biocide resistance and influencing other adaptive traits.

Biocides are commonly used in hospitals, farms, agriculture, a wide variety of industries, and in households for inhibiting or killing potentially pathogenic bacteria (1). Exposure to biocides can cause a decrease in bacterial susceptibility to different antimicrobial compounds due to the adaptive expression of different mechanisms influencing efflux pump activity, membrane permeability, lipid and cell wall synthesis, and cellular respiration (2, 3). Some of these metabolic routes are also involved in stress response, virulence, and even invasiveness (4–11). Frequently, antibiotics and biocides are simultaneously used at homes, hospitals, and farms, which may increase the risk of cross-selection of resistant variants (1, 12–14). Multidrug-resistant (MDR) *Enterobacteriaceae*, mainly *Escherichia coli* and *Klebsiella pneumoniae*, are currently considered a serious public health threat by the Centers for Disease Control and Prevention (CDC) (http://www.cdc.gov/drugresistance/threat-report-2013/), and the use of biocides, such as chlorhexidine (CHX), is now recommended for decontaminating areas of high risk of MDR *Enterobacteriaceae* (13–15).

The overproduction of efflux pumps is the most commonly described mechanism of resistance to biocides and is associated with low-level resistance to both biocides and antibiotics (β-lactams, chloramphenicol, fluoroquinolones, tetracyclines, and macrolides) (6, 16, 17). AcrAB-like efflux pumps are intrinsically expressed in different species of *Enterobacteriaceae*; the overexpression of *acrAB* or *acrEF* is controlled by global transcriptional regulators, such as MarAB, RamA, and SoxR. Besides controlling

the expression of efflux pumps, these regulators also control the levels of porins when they are induced by oxidative stress (18). Besides classical Qac efflux proteins, some SMR-type efflux pumps, such as KpnEF and SugE, have also recently been involved in antibiotic and biocide resistance (19). Furthermore, alterations in FabI, an enoyl-acyl carrier protein reductase that acts on fatty acid synthesis, constitute the main mechanism of resistance to triclosan (TRI) in *E. coli*; however, cross-resistance to antibiotics has not been proven (20).

The effects of biocides on antibiotic susceptibility, the expres-

sion of different pumps, or growth kinetics have been reported mainly in *Salmonella enterica* strain SL1344 (2, 3, 21). However, only a few studies with limited data have been performed with either *E. coli* or *K. pneumoniae*, and less is known in the case of clinical isolates (19, 22–27). The aim of this study was to determine the effect of preexposure to the more commonly used biocides in hospitals and domestic settings (TRI, CHX, benzalkonium chloride [BKC], and sodium hypochlorite [SHC]), or to antibiotics of wide consumption (ampicillin [AMP] and ciprofloxacin [CIP]) on the selection of biocide-resistant variants and to explore the associated changes in metabolism and bacterial growth rate. For this purpose, instead of using classic strains from laboratory collections, which are already "domesticated" and may not have all the capabilities of natural isolates to cope with the exposure to antimicrobials, we used clinical *E. coli* and *K. pneumoniae* strains belonging to relevant phylogroups to achieve observations closer to those in the clinical setting.

MATERIALS AND METHODS

Bacterial strains. The clinical *E. coli* HEC30 strain belonging to the B2$_3$ phylogenetic group, recovered at Ramón y Cajal University Hospital from a blood culture in 2010, and the *K. pneumoniae* 39.11 strain, belonging to phylogroup KpI, recovered from a urinary tract infection in an outpatient in 2011, were used for the experimental selection of biocide resistance. Phylogroup B2 *E. coli* comprises human-adapted strains associated with extraintestinal diseases and containing a high number of adaptive traits enhancing its ability to colonize and, often, to infect (28). The *K. pneumoniae* phylogroup KpI is the most frequent *K. pneumoniae* phylogenetic group detected in clinical isolates (29). Both isolates investigated in this work were susceptible to both first- and last-line antibiotics, including third-generation cephalosporins, carbapenems, fluoroquinolones, aminoglycosides, and tetracyclines, and also to chloramphenicol. Both isolates were also susceptible to biocides, according to suggested epidemiological cutoff (ECOFF) values (30). The *E. coli* isolate was resistant to ampicillin, streptomycin, and sulfonamides, while the *K. pneumoniae* isolate was resistant to ampicillin and fosfomycin, according to EUCAST breakpoints (http://mic.eucast.org/Eucast2/).

Selection of mutants. As biocides have different degrees of affinity toward multiple bacterial targets, we facilitated the isolation of a variety of diverse mutants by using preconditioning broth cultures with different antimicrobials at subinhibitory concentrations. These cultures were seeded and incubated overnight in rich medium (Columbia blood agar) plates. A single colony from each plate was inoculated into plain Luria-Bertani broth (LB) and into LB supplemented with subinhibitory concentrations (0.5× the MIC) of biocides (TRI, CHX, BKC, and SHC, with one biocide per tube) (Sigma-Aldrich, Inc., St. Louis, MO) or antibiotics (AMP and CIP) and further incubated overnight at 37°C with shaking at 150 rpm. Aliquots of 100 μl were plated onto LB plates, each containing biocide or antibiotic at concentrations between 2.5× and 33× MIC and incubated at 30°C. The plates were examined for growth for 7 days. Three colonies were randomly picked from each plate and stored for subsequent analyses. Colonies capable of growing in plates containing inhibitory concentrations of biocides (TRI, CHX, and BKC) and antibiotics (AMP and CIP) were submitted to subcultures in medium without selection (3 daily passages) to determine whether the observed phenotype was stable or was just due to transient resistance induction after challenge.

As biocides are rarely used for treating infections, there is not a clear definition of resistance based on breakpoints for these compounds. For this reason, we define as resistant any mutant able to grow in plates with a higher biocide concentration than that of the wild-type parental strain for a given biocide (31). Mutants were designed by the abbreviation of the compound used for exposure before plating, followed by the name and concentration of the compound used in the selective plates from which it had been retrieved. Mutants obtained without antimicrobial preexposure

were named nonexposed (NE). The morphologies of mutant colonies and their corresponding parental strains were compared in LB and Columbia blood agar plates to establish the colony morphotype. The genomic digestion profiles of diverse mutants and parent strains were also assessed by pulsed-field gel electrophoresis (PFGE) of XbaI-digested genomic DNA (see Fig. S1 and S2 in the supplemental material).

Antimicrobial susceptibility testing. The susceptibilities of parental and mutant strains against biocides (TRI, CHX, BKC, and SHC) and antibiotics (AMP, CIP, ceftazidime [CAZ], erythromycin [ERY], gentamicin [GEN], chloramphenicol [CLO], and tetracycline [TET]) (bioMérieux, Marcy l'Etoile, France) were determined by both broth microdilution and agar diffusion using Etest strip methods, respectively, according to CLSI guidelines (60). *E. coli* ATCC 10536 and *S. aureus* ATCC 6538 were used as control strains (3). Susceptibility to biocides was assessed in at least two independent experiments, and only consistent variations in two or more double dilutions were considered significant for a definition of biocide variants with altered susceptibility.

Susceptibility to 240 cell growth-inhibiting chemical compounds (using the Phenotype MicroArray; Biolog, Hayward, CA, USA) was performed using tryptic soy broth (TSB) and tetrazolium dye at a final concentration of 1%, according to the manufacturer's instructions for Gram-negative bacteria (32). Mutant growth at sub-MICs was performed in 96-well microtiter plates sealed with gas-permeable sealing membranes (Breathe-Easy; Diversified Biotech, Boston, MA) and incubated at 37°C for 24 h in a thermostatic kinetic microplate reader; the data were recorded using an OmniLog instrument (VersaMax; Molecular Devices, Sunnyvale, CA). The optical density at 590 nm (OD$_{590}$) was measured every 10 min. The plates were gently shaken for 2 s prior to each reading. An increase in the kinetic curve area of 33% was taken as a parameter for the identification of significant phenotypic differences.

Metabolic profile assay. An analysis of growth using different carbon and nitrogen sources was performed using Biolog Phenotype MicroArray panels PM1 and PM2A for carbon sources and PM3B for nitrogen sources (Biolog, Inc., Hayward, CA, USA). Carbon and nitrogen utilization pathways were tested for a variety of *E. coli* HEC30 and *K. pneumoniae* 39.11 mutants and the respective wild-type strains, according to the manufacturer's instructions. The goal was to assess changes in the physiological response associated with biocide resistance in *E. coli* and *K. pneumoniae*.

Growth kinetics. The growth kinetics of 14 mutants with different phenotypes against biocides (5 from *E. coli* strain HEC30 and 9 from *K. pneumoniae* strain 39.11) and their corresponding parental strains were measured by inoculating 400 μl of a 1:1,000 dilution of an overnight culture in fresh LB broth (approximately 10^4 CFU/ml). The samples were analyzed in triplicate in a microtiter plate, and the optical density at 600 nm was measured every 10 min for 24 h in Bioscreen C (Labsystems, Helsinki, Finland) at 37°C, adapting the method described by Foucault et al. (33). The data were averaged for each strain, and the fitness cost (FC) was determined by calculating the relative growth rates.

RNA isolation and qRT-PCR. The expression of components of the AcrAB, AcrCD, and AcrEF efflux pumps (encoded by the *acrB*, *acrD*, and *acrF* genes, respectively), related to resistance to both biocides and antibiotics, and the expression of global transcriptional factors that regulate the expression of efflux pumps and other pathways (*marA*, *ramA*, and *soxS*) were evaluated by reverse transcription-quantitative PCR (qRT-PCR), using *gapA* as the reference gene.

Three independent cultures of wild-type strains and their corresponding mutants were grown in 25 ml of plain LB at exponential phase and cooled rapidly on ice before centrifugation. All cultures were analyzed in duplicate. Five milliliters from each duplicate inoculum was mixed and centrifuged at 8,000 rpm and 4°C for 20 min. RNA was isolated by means of the RNeasy commercial kit (Qiagen, GmbH, Hilden, Germany), according to the manufacturer's instructions, treated with Turbo DNase (Ambion) to remove genomic DNA contamination, and cleaned using the Qiagen kit. The quality and concentration of RNA were assessed using a NanoDrop (ND-1000; Labtech). cDNA was synthesized using the High-

TABLE 1 Oligonucleotides used for the study of gene expression in this study

Species	Gene	Primer direction[a]	Primer sequence (5′ to 3′)	Amplicon size (bp)	Melting temp (°C)	Annealing temp (°C)
E. coli and *K. pneumoniae*	acrB	F	TTTGCGTGGGTGATCGC	192	54	50
		R	GTTATCGATACCGTTCATA		52	
E. coli	acrF	F	TGGCCATTATYCTGATGA	179	50	48
		R	GTTATCGATACCGTTCATA		52	
K. pneumoniae		F	TTCATCGACCGGTCTTCG	208	56	52
		R	AGATTGTCGATGCCGTTC		54	
E. coli and *K. pneumoniae*	acrD	F	GTGCTGGCAATCCTGTT	181	52	50
		R	TATCGAGGCCGGTCATAT		54	
E. coli	marA	F	TCATAGCATTTTGGACTGG	172	54	52
		R	TTGCGCGATTTCCGTCAT		54	
K. pneumoniae		F	AGCGCTCCGGTTACTCTAA	171	58	56
		R	GCTGCGACTCGAAACCATA		58	
E. coli	soxS	F	ATTGACCAGCCGCTTAACAT	197	58	54
		R	ACATAACCCAGGTCCATTG		56	
K. pneumoniae		F	TAGTCGCCAGAAAGTCAGG	190	58	
		R	GAGAAGGTTTGCTGCGAGA		56	
K. pneumoniae	ramA	F	CTGCAACGGCTGTTTTTACA	180	58	56
		R	ATTGAAGGTCCGGGTGAAG		58	
E. coli	gapA	F	GCTAACCTGAAATGGGACG	189	58	56
		R	GTCCTGGCCAGCATATTTG		58	
K. pneumoniae	gapA	F	GAAAGGCGTTCTGGGTTAC	184	58	
		R	GATGTGGGCAATCAGATCC		58	

[a] F, forward; R, reverse.

Capacity cDNA reverse transcriptase kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. The qRT-PCR experiments were carried out in the 7500 and 7300 Fast real-time PCR devices (Applied Biosystems) using Power SYBR green master mix (Applied Biosystems) on 50 ng of initially isolated RNA with 100 nM primers. The primers used are listed in Table 1. The quantities of initial cDNA in each sample were inferred from cycle threshold (C_T) values. The data were normalized against both the value of the corresponding reference gene (*gapA*) and the value of the analyzed gene in the parent strain. The amount of RNA was calculated according to the $2^{-\Delta\Delta C_T}$ method (34).

Analysis of FabI amino acid changes. Amplified DNA from both parental strains and mutants showing different MICs against TRI were analyzed for mutations in the *fabI* gene, which codes for the enoyl-acyl carrier protein reductase within bacterial fatty acid synthesis. Primers FabIF (5′-ATGGGTTTTCTTTCCGGTAAG-3′) and FabIR (5′-AATGC TGAANCCGCCGTCAA-3′) were designed using consensus sequences from *fabI* of *E. coli* and *K. pneumoniae* strain MGH 78578. The *fabI* amplicons (759 bp) were sequenced by the Sanger method (Macrogen Korea, Seoul, Republic of Korea), and both nucleotides and amino acid sequences of the parental strains and their corresponding mutants were aligned by Clustal W for comparison.

RESULTS

Selection of biocide mutants presenting different phenotypes. Considering only variants with susceptibility changes involving two or more double dilutions, the only biotypes consistently obtained were triclosan resistance or triclosan hypersusceptibility (Table 2). In *E. coli*, triclosan-resistant (TRI^r) variants were recovered from plates that contained TRI, BKC, or CIP after preconditioning broth exposure with TRI, BKC, or CIP and without preexposure to antimicrobials. Similarly, in *K. pneumoniae*, TRI^r variants were obtained not only from plates containing TRI, BKC, or CIP, after being preexposed to subinhibitory concentrations of TRI, BKC, or CIP, but also to SHC, CHX, and AMP. In *K. pneumoniae*, TRI-hypersusceptible (TRI^hs) variants were recovered

from plates containing CHX as a selective agent. Of note, half of the TRI^r *E. coli* variants showed slight increases (less than two double dilutions) in susceptibility to CHX (hypersusceptibility to CHX [CHX^hs]), which suggests negative epistasis between the mechanisms of resistance to these two compounds. In *K. pneumoniae*, TRI^r variants appeared to be slightly more resistant to both BKC and CHX than *E. coli* mutants, but the difference with the wild-type strain MIC was not sufficient to ascertain the robustness of these trends under our experimental conditions.

A large number of colonies of *E. coli* and *K. pneumoniae* mutants was obtained in plates supplemented with CIP (either the culture was previously exposed to antibiotics or not), in cases when the same compound was applied during preexposure and plate selection, and when low concentrations of TRI or BKC were used (Table 2).

Antibiotic susceptibility of triclosan mutants. As described above, TRI resistance was achieved after selection with TRI but also under selection in plates containing BKC or CIP. Table 2 shows the antibiotic susceptibility profile of the TRI^r mutants obtained. It is noteworthy that one of the *E. coli* TRI^r variants showing hypersusceptibility to CHX (CHX^hs) also presented slightly increased susceptibility to CAZ, GEN, and CLO (Table 2). Some *K. pneumoniae* TRI^r variants showed a remarkable decrease in susceptibility to AMP (1.5- to ≥8-fold MIC increases), CIP (2- to 16-fold), CLO (32- to ≥128-fold), TET (2.6- to ≥16-fold), and CAZ (1.3- to 4-fold). One of them (*Kpn*_CIP/BKC2) also presented a loss of susceptibility to ERY (≥16-fold) but increased GEN susceptibility (5-fold).

An extended analysis of susceptibility to a wide variety of antimicrobial substances using Phenotype MicroArrays (Biolog, Inc.) was performed not only for TRI^r mutants (4 *E. coli* and 2 *K. pneumoniae*) but also for mutants for which the MIC of TRI (MIC_{TRI}) showed either a slight increase (only a one-dilution difference) or

TABLE 2 Diversity of mutants and their biocide and antibiotic susceptibility

Species and biocide phenotype[a]	Preconditioning agent[b]	Biocide MIC (mg/liter)			Antibiotic MIC (mg/liter)[c]							Mutant designation[d]	No. of CFU by plate[e]	No. of mutants
		TRI	BKC	CHX	AMP	CAZ	CIP	ERY	GEN	CLO	TET			
E. coli														
Parental strain HEC30		0.06	16	8	≥256	0.25	0.023	16	1–1.5	6	3			
TRI^r	TRI	4	16	2	≥256	0.13	0.032	16	0.75	3	3	Ec_TRI/TRI2	2	3
	CIP	1	32	8	≥256	0.75	0.75	16	1	12	0.75	Ec_CIP/CIP	7	
	BKC	0.25	16	8	≥256	0.5	0.032	16	1.5	4–12	3	Ec_BKC/BKC2	1	
TRI^r CHX^s	NE	1	16	4	≥256	0.38	0.023	16	1–1.5	6	2	Ec_NE/TRI1	UC	1
K. pneumoniae														
Parental strain 39,11		0.5	16	32	32	0.38	0.047	16	1	2	3			
TRI^r	AMP	2	32	64	64	0.75	0.094	16	1	128	4	Kpn_AMP/TRI2	7	16
	NE	2	32	64	128	0.5	0.5	16	1	>256	12	Kpn_NE/CIP	126	
	TRI	2	32	64	≥256	0.5	0.5	16	1.5	>256	8	Kpn_TRI/CIP	177	
	CHX	2	32	64	≥256	0.38	0.38	32	0.75	>256	8	Kpn_CHX/CIP	59	
	BKC	2	32	64	≥256	1	0.38	96	0.75	64	>128	Kpn_BKC/CIP	53	
	SHC	2	32	64	128–192	0.75	0.5	32–48	1.5	>256	16–24	Kpn_SHC/CIP	31	
	NE	2	32	32	32	0.5	0.38	16	1	>256	4	Kpn_NE/TRI1	UC	
	TRI	2	32	32	64	0.5	0.38	12	1	128	6	Kpn_TRI/TRI2	272	
	BKC	2	32	32	48	0.5	0.38	12	1	128	4	Kpn_BKC/TRI2	12	
	CHX	2	32	32	32	0.5	0.5	16	1	>256	4	Kpn_CHX/TRI2	6	
	CIP	2	32	32	32	0.38	0.5	32	1	>256	24–32	Kpn_CIP/TRI2	20	
	TRI	2	32	32	≥256	0.38	0.5	16	0.5	>256	16–24	Kpn_TRI/BKC1	UC	
	BKC	2	32	32	≥256	1.5	0.38	16	0.5	192	32	Kpn_BKC/BKC1	UC	
	CIP	2	32	32	≥256	1.5	0.75	>256	0.19	96	12	Kpn_CIP/BKC2	11	
	AMP	2	32	32	≥256	0.5	0.5	16	0.5	>256	8	Kpn_AMP/CIP	152	
	CIP	2	32	32	192	0.5	0.38	16	1	>256	12	Kpn_NE/CIP	126	
TRI^hs	NE	0.12	8	32	48	0.25	0.094	16	1.5	3	2	Kpn_NE/CHX2	3	2
	CIP	0.12	16	16	32	0.25–0.38	0.094	16	1	4	1.5	Kpn_CIP/CHX2	UC	

[a] TRI, triclosan; CHX, chlorhexidine.
[b] CIP, ciprofloxacin; BKC, benzalkonium chloride; NE, no exposure; AMP, ampicillin; SHC, sodium hypochlorite.
[c] CAZ, ceftazidime; ERY, erythromycin; GEN, gentamicin; CLO, chloramphenicol; TET, tetracycline.
[d] When the selecting agent was TRI, the numbers in the nomenclature of mutants refer to the concentrations of the compounds in the plates as follows: 1 = 1 mg/liter and 2 = 1.5 mg/liter. When the selecting agent was CHX, the concentration in the plate was as follows: 1 = 64 mg/liter or 2 = 128 mg/liter. In the case of BKC, the numbers 1 to 3 refer to the concentrations in plates as follows: 1 = 32 mg/liter, 2 = 64 mg/liter, and 3 = 128 mg/liter. The concentration of CIP was 0.12 mg/liter in all plates.
[e] UC, uncountable.

a slight decrease in comparison with that of the wild parental strain (2 *E. coli* and 3 *K. pneumoniae* strains, respectively). The parental strains *Ec*_HEC30 and *Kpn*_39.11 were also included. The results are shown in Tables S1 and S2 in the supplemental material. Some common patterns were observed, even though the phenotypes of susceptibility were diverse in the different mutants.

All six *E. coli* TRI^r mutants analyzed were more resistant to cefoxitin, cefuroxime, tetracycline, and dodine (a fungicide) but showed enhanced susceptibility to highly diverse antimicrobials, remarkably, those that increase membrane permeability (such as poly-L-lysine, polymyxin B, and metaborate, a herbicide), and to many others. The five *K. pneumoniae* mutants analyzed (2 TRI^r and 3 triclosan susceptible [TRI^s]) were more tolerant to penicillin, the tetracycline derivative penimepicycline, and novobiocin and more susceptible to compounds acting on membrane permeability, such as colistin (a polypeptide polycationic antibiotic), methyl viologen or paraquat (oxidant herbicide), and cobalt (a toxic cation). The two TRI^r *K. pneumoniae* mutants (*Kpn*_CIP/BKC2 and *Kpn*_TRI/TRI2) exhibited decreased antimicrobial susceptibility to a wide panel of agents (to 57 and 58 chemicals, respectively, with 52 compounds being commonly affected), which comprised several antibiotic classes (see Table S2 in the supplemental material). On the contrary, TRI^hs *K. pneumoniae* variants were mainly susceptible to several more antimicrobials than their parental strain. These results indicate that the acquisition of resistance to biocides can either decrease or increase susceptibility to a wide variety of different cell growth-inhibiting agents.

Acquisition of resistance to biocides alters the physiology of *E. coli* and *K. pneumoniae*. One interesting issue derived from testing some of the analyzed mutants was the possible alteration of their normal physiology. Alterations in colony morphology were observed for some of the TRI^r mutants (*Ec*_CIP/CIP, *Kpn*_CIP/BKC2, *Kpn*_AMP/TRI2, and *Kpn*_TRI/CIP), as reflected in the smaller size exhibited by their colonies in comparison with that of the corresponding parental strains, whereas other TRI^r and TRI^hs mutants (*Kpn*_AMP/Tri2, *Kpn*_BKC/BKC1, and *Kpn*_SHC/CHX2) formed mucous colonies (see Fig. S1 in the supplemental material) with enhanced biofilm formation (not shown). All of them exhibited the same XbaI-digested genomic DNA profile as their respective parental ancestor, suggesting that the alterations in physiological behavior are not due to large genomic reorganizations (see Fig. S2 in the supplemental material).

To further understand the effect of acquiring biocide resistance on the physiology of *K. pneumoniae* and *E. coli*, the use of carbon sources in each mutant was compared to that in the parental strains. The clustering of metabolic profiles of both the mutants and their respective wild-type strains is shown in Fig. 1 and in Tables S3 and S4 in the supplemental material. Among *E. coli* TRI^r mutants, the most remarkable finding was the enhanced use of D-malic acid by all strains analyzed and the more efficient growth of most mutants in arbutin (β-D-glucopyranoside). Some TRI^r mutants (*Ec*_TRI/TRI2 and *Ec*_CIP/CIP), characterized by a similar antibiotic susceptibility profile, were able to use some amino acids (hydroxyl-L-proline), carboxylic acids, carbohydrates, and alcohols more efficiently than the parental strain. However, they differ in their ability to metabolize different substrates (adonitol, D-glucosaminic acid, *m*-inositol, D-psicose, L-lyxose, D-allose, salicin, α-hydroxyglutaric acid γ-lactone, and dihydroxyacetone).

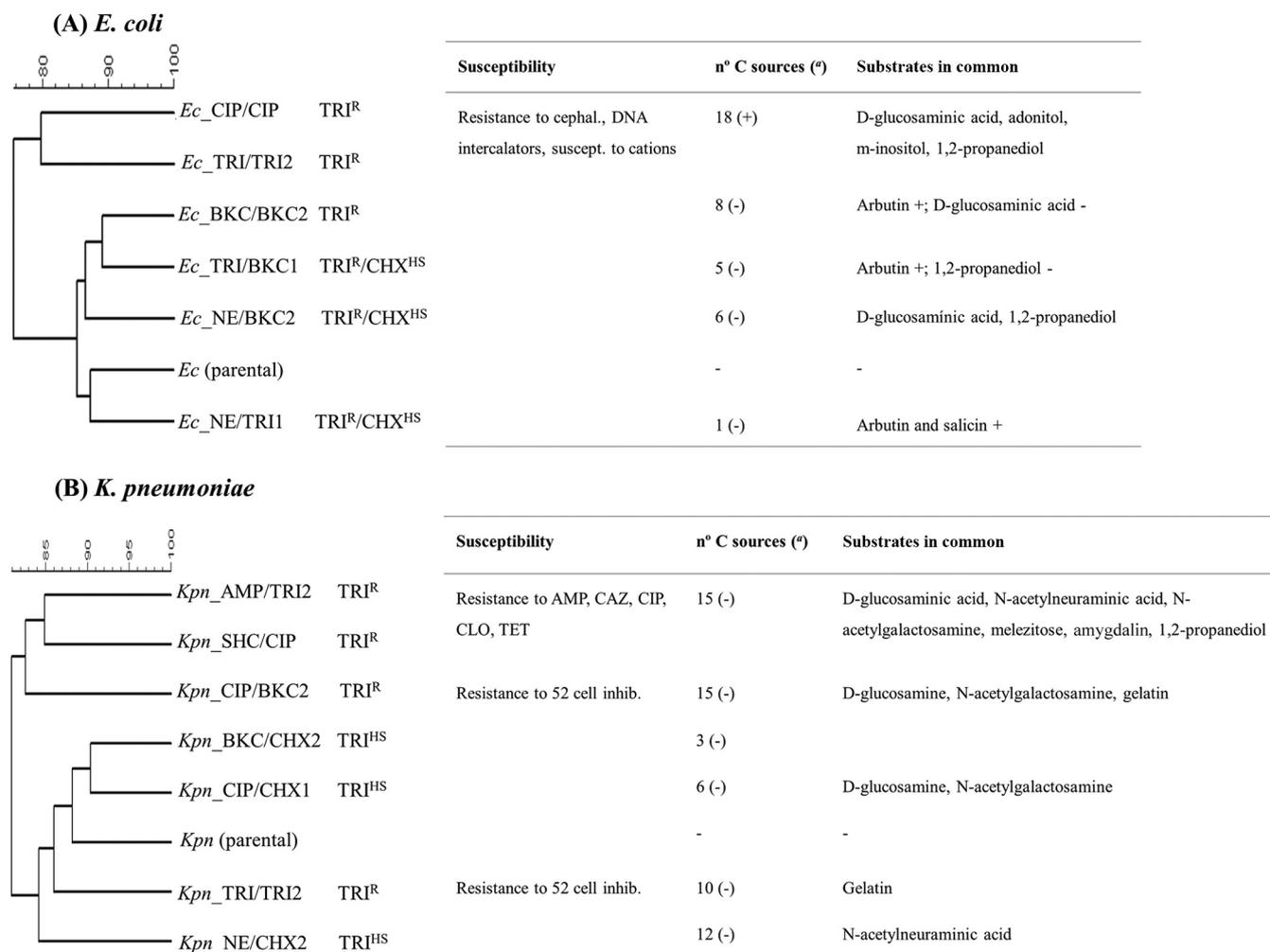All *K. pneumoniae* mutants studied were unable to use Tween 80, γ-cyclodextrin, and 3-methylglucuronic acid compared to the wild-type strain, but almost all metabolized some amides (D-alaninamide), amino acids (L-ornithine and D-aspartic acid), sugars (D-fructose, D-arabinose, *N*-acetylneuraminic acid, and glycogen), and carboxylic acids (propionic acid) (Fig. 1; see also Table S4 in the supplemental material) better than the parental strain. Two clusters of *K. pneumoniae* mutants were inferred. One grouped TRI^r mutants (*Kpn*_AMP/TRI2, *Kpn*_SHC/CIP, and *Kpn*_CIP/BKC2), which used some sugars (D-melezitose and D-amygdalin), carboxylic acids (citramalic, oxalic, and oxomalic), amino sugar components of peptidoglycan (D-glucosaminic acid and *N*-acetylneuraminic acid), and alcohols (1,2-propanediol) less efficiently than their parental strain. The second group comprises both TRI^r (*Kpn*_CIP/BKC2) and TRI^hs mutants with disparate antibiotic susceptibility but a similar metabolic profile consisting of enhanced use (for ornithine) or diminished use (for β-D-allose, D-melezitose, amygdalin, D-arabinose, and sedoheptulosan) in comparison with that of the wild-type strain. The TRI^hs CHX^hs mutants metabolized some peptidoglycan amino sugars (D-glucosaminic acid/*N*-acetyl-D-glucosamine, and *N*-acetylneuraminic acid) less efficiently than the parental strain. Mutants with remarkable and distinct antibiotic susceptibility changes (*Kpn*_CIP/BKC2 and *Kpn*_SHC/CIP) only showed impaired use of L-glucose.

Fitness costs associated with the acquisition of biocide resistance in *E. coli* and *K. pneumoniae*. Various FC values were observed for TRI^r mutants of both *E. coli* (1 to 75%) and *K. pneumoniae* (4 to 66%) (Table 3). For *E. coli*, in the TRI^r mutants with the highest FC values (26 and 75%, respectively) and increased lag phases, notable changes were shown in the susceptibility to TRI or CIP (10-fold increase in MIC), as was an increased metabolism of some carbon sources (*Ec*_TRI/TRI2 and *Ec*_CIP/CIP).

Seven out of 9 TRI^r *K. pneumoniae* mutants showed variable FC (4 to 66%). The *Kpn*_CIP/BKC2 mutant with striking reduced susceptibility to several antibiotics (CAZ, AMP, CIP, ERY, CLO, and TET) showed the highest FC value and the longest lag phase (Fig. 2). This variant was unique in presenting such an increase in the susceptibility to GEN. Together with other two mutants that demonstrated appreciable FCs (*Kpn*_AMP/TRI2 and *Kpn*_SHC/CIP, with FC values of 11 and 15%, respectively), an impaired ability to metabolize a high number of carboxylic acids, amino acids, sugars, and amino sugars involved in peptidoglycan synthesis was observed in comparison with parental strains. Finally, *K. pneumoniae* TRI^hs variants (*Kpn*_NE/CHX2 and *Kpn*_CIP/CHX2), with phenotypes of increased susceptibility, showed low FC.

A comparison of the mutants reveals that decreased susceptibility to TRI accompanied by a remarkable decreased susceptibility to CIP or ERY is associated with higher physiological costs in both *E. coli* and *K. pneumoniae*. The finding that mutants with high FC show increased metabolism of some carbon sources, in the case of *E. coli*, or decreased metabolic ability for other substrates, in the case of *K. pneumoniae*, suggests that FC may depend on the environment and available nutrients.

Gene expression of *acrAB* and *marA*, and *soxS* and *ramA*. Gene expression of *acrAB*, *acrEF*, and *acrCD* and *marA*, *soxS*, and *ramA* was evaluated in different TRI^r and TRI^hs mutants exhibiting different FC values (Table 3). Increased expression of both *acrF* and *marA* was detected for one *E. coli* mutant (*Ec*_CIP/CIP), with notable changes in the susceptibility to TRI or CIP (10-fold increase in MIC) and an increased metabolism of some carbon

(A) *E. coli*

	Susceptibility	nº C sources (^{a})	Substrates in common
*Ec*_CIP/CIP TRI^R	Resistance to cephal., DNA intercalators, suscept. to cations	18 (+)	D-glucosaminic acid, adonitol, m-inositol, 1,2-propanediol
*Ec*_TRI/TRI2 TRI^R			
*Ec*_BKC/BKC2 TRI^R		8 (-)	Arbutin +; D-glucosaminic acid -
*Ec*_TRI/BKC1 TRI^R/CHX^{HS}		5 (-)	Arbutin +; 1,2-propanediol -
*Ec*_NE/BKC2 TRI^R/CHX^{HS}		6 (-)	D-glucosamínic acid, 1,2-propanediol
Ec (parental)		-	-
*Ec*_NE/TRI1 TRI^R/CHX^{HS}		1 (-)	Arbutin and salicin +

(B) *K. pneumoniae*

	Susceptibility	nº C sources (^{a})	Substrates in common
*Kpn*_AMP/TRI2 TRI^R	Resistance to AMP, CAZ, CIP, CLO, TET	15 (-)	D-glucosaminic acid, N-acetylneuraminic acid, N-acetylgalactosamine, melezitose, amygdalin, 1,2-propanediol
*Kpn*_SHC/CIP TRI^R			
*Kpn*_CIP/BKC2 TRI^R	Resistance to 52 cell inhib.	15 (-)	D-glucosamine, N-acetylgalactosamine, gelatin
*Kpn*_BKC/CHX2 TRI^{HS}		3 (-)	
*Kpn*_CIP/CHX1 TRI^{HS}		6 (-)	D-glucosamine, N-acetylgalactosamine
Kpn (parental)		-	-
*Kpn*_TRI/TRI2 TRI^R	Resistance to 52 cell inhib.	10 (-)	Gelatin
*Kpn*_NE/CHX2 TRI^{HS}		12 (-)	N-acetylneuraminic acid

FIG 1 Cluster of metabolic profiles of biocide mutants and respective wild-type strains of *E. coli* (A) and *K. pneumoniae* (B). Clusters of mutants according to their ability to metabolize carbon and nitrogen substrates by the unweighted-pair group method using average linkages (UPGMA) are shown. In the number of carbon (C) sources column, (+) indicates carbon substrates that were used more efficiently by mutants than by parental strains, and (−) indicates carbon substrates that were used less efficiently by mutants than by parental strains. cephal., cephalosporins; suscept., susceptible; inhib., inhibitors.

sources, as well as a high FC value (26%). The mutant *Ec*_TRI/TRI2 presented the highest FC and increased metabolism of certain carbon sources, although the expression of the tested genes was not modified. Conversely, decreased expression of *acrB*, *acrF*, *marA*, and *soxS* (fold changes and standard deviations: 0.26 ± 0.17, 0.18 ± 0.15, 0.32 ± 0.18, and 0.21 ± 0.18, respectively) was observed in a TRI^r mutant (4-fold increase in MIC for *Ec*_BKC/BKC) that showed 6% FC and a 2-fold increase in the MIC of CAZ (MIC$_{CAZ}$).

Mutants with a low fitness cost, corresponding to phenotypes of decreased (4-fold increase in MIC$_{TRI}$ for *Kpn*_TRI/TRI2) and increased susceptibility to TRI (4-fold decrease in MIC$_{TRI}$ for *Kpn*_NE/CHX2 and *Kpn*_CIP/CHX2), showed increased expression of *acrB* and *acrF* and either down- or overexpression of *acrB* and *marA*, respectively. The mutant *Kpn*_SHC/CIP, with a 15% FC, showed increased *acrF* expression. However, the variant with the highest FC had enhanced expression of the transcriptional regulator *ramA* (fold change, 45.01 ± 40.81). Overexpression of *ramA* was previously involved in ciprofloxacin resistance of *S. enterica* obtained after increasing passages in CIP (35).

Analysis of FabI amino acid changes. Different amino acid changes were identified in TRI^r *E. coli* mutants with MICs of 4 (Gly93Val in *Ec*_TRI/TRI2 mutant) and 1 mg/liter (Leu94Phe in *Ec*_NE/TRI1 mutant), both located in the binding site of FabI and the second reported here for the first time. Another isolate showing up to five mutations did not show any TRI resistance phenotype. Mutations in the *fabI* gene were not observed in *K. pneumoniae* mutants.

DISCUSSION

The effect of biocides in antibiotic resistance has been explored in different studies specifically focused on particular situations of cross-resistance or induction of resistance (2, 23, 36–39). In this study, we went one step further and comprehensively analyzed the high diversity of effects exerted by several biocides and antibiotics on the most relevant enterobacterial pathogenic species, *E. coli* and *K. pneumoniae*. For this purpose, we studied not only cross-resistance to a limited set of drugs but also other aspects, such as fitness costs and the effect of the selected mutations on susceptibility to a large number of compounds and on bacterial metabo-

TABLE 3 Possible mechanisms of resistance and phenotype alterations to biocides

Species and biocide phenotype[a]	Colony morphology	Fitness cost (%)	FabI mutation	Differential gene expression	Mutant designation[b]
E. coli					
TRI[r]	Changed	75	ND[c]	*acrF* (1.99 ± 0.81) *marA* (4.31 ± 0.71)	*Ec*_CIP/CIP
	Not changed	26	1 nt, Gly93→Val	Not changed	*Ec*_TRI/TRI2
	Not changed	6	ND	*acrB* (0.26 ± 0.17) *acrF* (0.18 ± 0.15) *marA* (0.32 ± 0.18) *soxS* (0.21 ± 0.18)	*Ec*_BKC/BKC2
TRI[r] CHX[hs]	Not changed	1	1 nt, Leu94→Phe (*E. coli* K-12 F)[d]	Not changed	*Ec*_NE/TRI1
K. pneumoniae					
TRI[r]	Changed	11	0	ND	*Kpn*_AMP/TRI2
	Not changed	9	ND	ND	*Kpn*_NE/CIP
	Changed	8	0	ND	*Kpn*_TRI/CIP
	Not changed	ND	0	ND	*Kpn*_CHX/CIP
	Not changed	ND	ND	ND	*Kpn*_BKC/CIP
	Not changed	15	ND	*acrF* (357.44 ± 246.36)	*Kpn*_SHC/CIP
	Not changed	ND	ND	ND	*Kpn*_NE/TRI1
	Not changed	4	0	*acrB* (62.62 ± 40.01) *acrF* (2,923.37 ± 1,101.81) *marA* (1.64 ± 0.26)	*Kpn*_TRI/TRI2
	Not changed	ND	ND	ND	*Kpn*_BKC/TRI2
	Not changed	ND	ND	ND	*Kpn*_CHX/TRI2
	Not changed	ND	ND	ND	*Kpn*_SHC/TRI2
	Not changed	ND	ND	ND	*Kpn*_CIP/TRI2
	Not changed	ND	ND	ND	*Kpn*_TRI/BKC1
	Not changed	ND	ND	ND	*Kpn*_BKC/BKC1
	Changed	66	0	*ramA* (45.01 ± 40.81)	*Kpn*_CIP/BKC2
	Not changed	ND	ND	ND	*Kpn*_AMP/CIP
	Not changed	9	ND	ND	*Kpn*_NE/CIP
TRI[hs]	Not changed	NC[e]	0	*acrB* (0.29 ± 0.11) *acrF* (0.01 ± 0.00) *marA* (2.21 ± 0.18)	*Kpn*_NE/CHX2
	Not changed	NC	ND	*acrB* (35.50 ± 13.52) *acrD* (3.02 ± 0.83) *marA* (0.28 ± 0.13)	*Kpn*_CIP/CHX2

[a] TRI, triclosan; CHX, chlorhexidine.

[b] CIP, ciprofloxacin; BKC, benzalkonium chloride; NE, no exposure; AMP, ampicillin; SHC, sodium hypochlorite.

[c] ND, not determined.

[d] This amino acid change was identified here for the first time.

[e] NC, no cost.

lism; these are aspects that were not usually taken into consideration in previous studies in this field. Further, in order to obtain more applicable results, we used clinical isolates instead of the domesticated collection strains regularly used in previous studies on this topic. The biocides tested in this study (the bisphenol TRI, the biguanide CHX, the quaternary ammonium BKC, and the oxidizer SHC) are representatives of the four main classes of biocides commonly used in the food industry, hospitals, farms, and households (39). To the best of our knowledge, this is the first study including preconditioning antimicrobial agents at subinhibitory concentrations in the selection of mutants of *E. coli* and *K. pneumoniae* resistant to several biocides and antibiotics (40).

As in other works, TRI was the biocide that led more consistently to the selection of resistant mutants. Also, in accordance with what was already reported, TRI resistance is frequently associated with reduced susceptibility to different antimicrobials (4, 41). The finding that TRI resistance could be selected by low con-

centrations of either BKC or CIP in addition to TRI is disturbing, due to the extensive use of several of these agents in different settings. Nevertheless, our results show that the acquisition of resistance to this biocide may increase susceptibility to other antimicrobial compounds, which may reflect negative epistasis between the involved mechanisms of resistance. This is also suggested by the fact that TRI[r] entails more susceptibility to CHX, and TRI[hs] mutants were selected with CHX, a phenomenon reported for TRI[r] *S. enterica* mutants (42). Our results support the presence of different mechanisms simultaneously conferring resistance phenotypes to a diverse number of compounds and enhanced susceptibility to other antimicrobials. Whereas the risks of using biocides for the development of resistance have been discussed in several studies, the possibility that biocides may enhance the activity of some antibiotics has never been explored and is a topic that may require more attention.

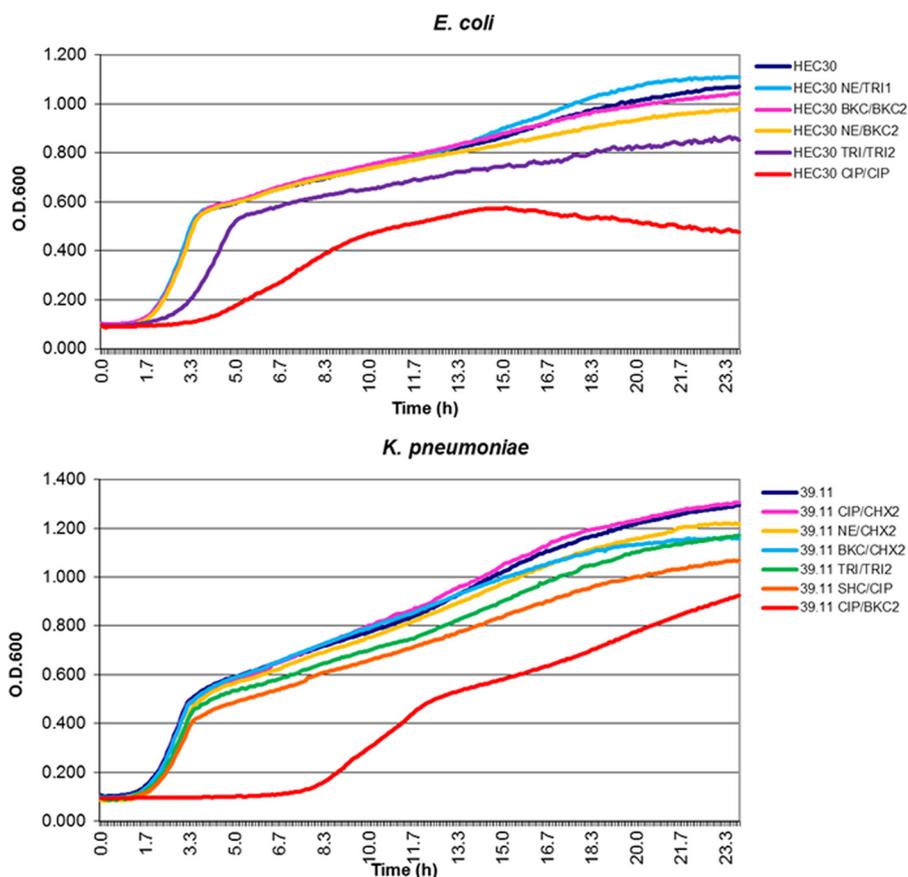This work reports that changes in AraC-like global regulators

FIG 2 Growth kinetics of *E. coli* and *K. pneumoniae* mutants and their parental strains. The parental strains are HEC30 (*E. coli*) and 39.11 (*K. pneumoniae*). The average data are represented in the graphics. Growth rates were determined in plain LB broth at 37°C for 24 h. O.D.600, optical density at 600 nm.

(MarA, SoxR, and RamA), AcrAB-TolC, and FabI are involved in biocide/antibiotic alterations in susceptibility and result in variations in the metabolism and bacterial fitness of *E. coli* and *K. pneumoniae*. Overall, our data indicate that selection by biocides results in the emergence of a complex variety of mutants affecting not only susceptibility to TRI but also to other antimicrobials. Previous studies have documented the involvement of efflux pumps and AraC global regulators on resistance and virulence (43) and the frequent contribution of different mechanisms of resistance to both biocides (such as TRI, BKC, and CHX) and antibiotics (44), some of them associated with metabolic changes (45). In contrast to previous works that are focused on the effect of specific antimicrobials on laboratory strains (46–49), our work is based on the analysis of mutants derived from clinical isolates belonging to predominant *E. coli* and *K. pneumoniae* infective clones. A subset of AraC regulators belonging to the AraC/XylS family, including MarA, SoxS, and RarA, confers an MDR phenotype in *K. pneumoniae* through the upregulation of the AcrAB, AcrCD, and AcrEF efflux pumps and downregulation of the OmpF porin (50). We identified only induced expression of the transcriptional activator *ramA*, which controls a wealth of genes associated with cellular metabolism and virulence in different species of *Enterobacteriaceae* (46–49, 51, 52). While MarA, RamA, and SoxS can be simultaneously expressed (down- and/or upregulated) in response to different signals, as seems to have happened in our study, RarA seems to be associated with the increased ex-

pression of both the AcrAB and OqxAB efflux pumps in *Klebsiella* and is seemingly independent of other AraC-type regulators (24). To explain the lack of altered expression of AcrAB-like efflux pumps in some mutants, we cannot discard the possible late expression of these during stationary phase, its expression in the presence of antimicrobials, or even a more remarkable effect on *acrA* or *tolC* genes not tested here. Mutants with the highest FC showed increased expression of *marA* (*E. coli*) or *ramA* transcriptional regulators (*K. pneumoniae*). They correspond to mutants with remarkable changes in their antibiotic susceptibility and metabolic profiles.

Known mechanisms of TRI resistance, such as modification of FabI or modified expression of AcrAB-TolC under the control of specific regulators, such as AcrR, or global regulators, such as MarA and SoxA, have been identified for different *E. coli* mutants (53, 54). However, mutations in FabI do not completely justify TRI^r in *E. coli* or in *K. pneumoniae* mutants. Enhanced expression of the *acrF* gene was observed for TRI^r *E. coli* and *K. pneumoniae* mutants selected in TRI or CIP showing remarkable changes in susceptibility to a broad range of antimicrobials, which suggests that AcrEF also had a role here, as already reported for *Salmonella* TRI^r mutants (2, 42). While enzymatic modification of FabI alters the synthesis of lipids and membrane permeability, the effects of increased expression of efflux pumps and their regulators may result in an alteration of metabolic pathways linked to enhanced stress response, persistence phenotypes, and eventually, pathoge-

nicity (11, 55, 56). Despite most mutants showing fitness costs when growing under laboratory conditions, some of them have improved capabilities for degrading some compounds, including amino acids and glycosidic carbohydrates, which are present in the intestinal mucus layer. This improved metabolic capability may enhance the ability of such mutants to colonize and eventually infect the host (57). This might be also the case of mutants able to improve the metabolism of 1,2-propanediol, adonitol, D-glucosaminic acid, D-psicose, arbutin, *m*-inositol, or *N*-glucosaminic acid (Table 3). 1,2-Propanediol is a three-carbon glycol that is produced during the catabolism of rhamnose and fucose, which are abundant sugars in mammalian glycoconjugates. In this respect, it is worth mentioning that operons of genes for 1,2-propanediol utilization have been identified in *Salmonella* and *Listeria monocytogenes* and are suggested to be virulence mechanisms (58). Along the same line, the *m*-inositol-positive phenotype seems to be highly related to enhanced pathogenicity, as the ability to use some inositol derivatives is associated with an inflammatory host response or fluid secretion (59).

In summary, this work reports the effects of exposure to biocides and antibiotics in selected mutants showing polymorphic changes, including resistance to antimicrobial compounds, differential fitness costs, and changes in the metabolic profiles in *E. coli* and *K. pneumoniae*. The complex variety of antimicrobial mechanisms of action for biocides affecting different basic networks of bacterial physiology is consequently mirrored by the complex variety of resistance mechanisms, determining secondary effects on susceptibility to other compounds, metabolism, and ultimately environment-dependent influences on fitness and colonization/virulence.

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