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Transposon-Sequencing Analysis Unveils Novel Genes Involved in the Generation of Persister Cells in Uropathogenic *Escherichia coli*

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Persister cells are highly tolerant to different antibiotics and are associated with relapsing infections. In order to understand this phenomenon further, we exposed a transposon library to a lethal concentration of ampicillin, and mutants that survived were identified by transposon sequencing (Tn-Seq). We determined that mutations related to carbon metabolism, cell envelope (cell wall generation and membrane proteins), and stress response have a role in persister cell generation.

Uropathogenic *Escherichia coli* (UPEC) is the main etiologic agent of urinary tract infections worldwide, generating approximately 80% of clinical cases every year. Most of these infections are chronic and represent a worldwide public health threat (1). Relapsing infections have been associated with the generation of persister cells (2, 3). This subpopulation is characterized by a transient nonhereditary dormant state that leads to survival in lethal concentrations of different antibiotics. The molecular mechanisms reported to be involved in the generation of persister cells include stochastic processes, toxin-antitoxin (TA) modules, and the stringent response (4–6). However, a deeper understanding of the physiological and genetic regulation of this process is lacking.

Aiming to identify global regulators or metabolic pathways that *E. coli* might downregulate to enter into a dormant persister state, we used a high-throughput genetic screen to recognize mutants with increased fitness during exposure to a lethal concentration of the cell wall-active antibiotic ampicillin in order to identify novel regulators and/or metabolic pathways involved in the generation of persister cells. This method has previously been used to identify metabolic pathways associated with the generation of persister cells in stationary-phase cultures of *E. coli* exposed to gentamicin (7).

We used a library of UPEC strain CFT073 containing approximately 360,000 random insertions of the EZ-Tn*5* <R6Kγ*ori*/KAN-2> transposon (8). A 1:100 dilution of an overnight culture of the library was grown at 37°C in Luria-Bertani (LB) broth with aeration and when the optical density at 600 nm (OD_{600}) reached 0.3, the culture was split in two flasks, and ampicillin was added to a final concentration of 125 μg ml^{-1} (10 times the MIC). After 6 h of incubation at 37°C with shaking, we enriched the survivors by overnight growth in fresh LB lacking ampicillin (approximately 45 generations of outgrowth).

Total DNA, corresponding to three independent experiments, was extracted from the input and ampicillin-treated output cultures using a QIAamp DNA minikit (Qiagen).

Transposon insertions in mutants showing increased survival in cultures exposed to ampicillin were identified by transposon sequencing (Tn-Seq) using the homopolymer tail-mediated ligation PCR (HTML-PCR) method followed by sequencing on a HiSeq 2500 system (Illumina) (9, 10). Briefly, DNA was fragmented by sonication, and poly(C) tails were added to all 3′ termini using terminal deoxynucleotidyl transferase. Then, a nested PCR strategy was used to amplify the genomic DNA adjacent to transposon insertions, while at the same time the index sequences required for multiplex sequencing were added. Data were analyzed using the Tufts University Core Facility Galaxy server as described previously (11). Mutants were considered positively selected if the following requirements were met: (i) at least three unique insertions were present in each gene in all three input samples, (ii) a Dval genome value (defined as the number of reads of each gene divided by predicted number of reads) of ≥0.01 in each input, which is a criterion used to discard mutants in the library with severely impaired growth, was found in each input, and (iii) an average survival index (Dval genome output/input) of ≥2 was observed.

We detected ~68,000 mutants in the input samples and ~55,000 in the output samples. Among those, we identified 50 genes under positive selection in our screen; i.e., transposon insertions in these genes led to increased survival upon ampicillin exposure (Table 1). Among these are genes encoding citric acid cycle enzymes AcnB and FumA, arginine catabolism-related YdjS (12), fructose transport FruA (13), maintenance of NAD^+/NADH balance UdhA (14), and gluconeogenic fructose-1,6-bisphosphatase. In addition, we found a putative pyruvate formate-lyase 3-activating enzyme, which might catalyze the conversion of pyruvate into formate in anaerobic respiration (15), and the *N*-acetylglucosamine repressor NagC, which is necessary for the utilization of *N*-acetylglucosamine as a carbon source (16). Also, the YfcC (hypothetical protein) has been related to the glyoxylate shunt, which might contribute to the generation of carbon skeletons that can be used as energy in the absence of glucose (17). These results suggest that mutants with impaired metabolism and/or an inability to use

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TABLE 1 Proteins of mutants under positive selection displaying increased survival when exposed to a lethal concentration of ampicillin

Locus	Protein	Gene	Mean SI[a]
c0147	Aconitate hydratase 2	*acnB*	87.405
c0608	Hypothetical protein		5.706
c0751	*N*-Acetylglucosamine repressor	*nagC*	6.602
c0764	Putative pyridoxine phosphate biosynthetic protein		5.446
c0861	Hypothetical protein YbhK	*ybhK*	11.288
c0909	Putative pyruvate formate-lyase 3-activating enzyme		11.155
c0943	Hypothetical protein		7.865
c1174	Hypothetical protein		2.832
c1176	Hypothetical protein		10.699
c1179			9.273
c1254	Putative glucosyltransferase	*iroB*	8.533
c1303	Hypothetical protein		16.152
c1560	Outer membrane porin protein NmpC precursor	*nmpC*	12.465
c1572	Putative capsid assembly protein of prophage		8.870
c1610	Conserved hypothetical protein		2.817
c1822	Outer membrane protein N precursor	*ompN*	7.103
c1884	Hypothetical protein		6.538
c1888	Conserved hypothetical protein		5.430
c1925	Hypothetical ABC transporter ATP-binding protein YddA	*yddA*	3.138
c1927	Putative sulfatase YdeN precursor	*ydeN*	4.039
c1945	Hypothetical protein YneF	*yneF*	3.146
c1956	Putative outer membrane protein YieC precursor		4.482
c2004	Fumarate hydratase class I, aerobic	*fumA*	3.121
c2034	Transcriptional regulator SlyA	*slyA*	15.285
c2144	Succinylglutamate desuccinylase	*ydjS*	3.865
c2199	Hypothetical protein YeaP	*yeaP*	8.016
c2232	Hypothetical protein YobF	*yobF*	62.546
c2377	Hypothetical protein YedI	*yedI*	7.341
c2385	Protein YedU	*yedU*	3.832
c2465	Hypothetical protein		12.476
c2471	Hypothetical protein		16.751
c2475	Hypothetical protein		55.491
c2615	Hypothetical protein		3.164
c2677	SanA protein	*sanA*	3.147
c2702	PTS[b] system, fructose-specific IIBC component	*fruA*	6.573
c2841	Hypothetical protein YfcC	*yfcC*	2.660
c3028	Exodeoxyribonuclease VII large subunit	*xseA*	4.108
c3174	Prophage QSR′ DNA packaging protein NU1 homolog	*nohB*	5.787
c3255	Membrane-bound lytic murein transglycosylase B precursor	*mltB*	6.485
c3721	Hypothetical ATP-binding protein YghR	*yghR*	5.162
c3888	PTS system, *N*-acetylgalactosamine-specific IIB component 2	*agaV*	5.440
c4475	Guanosine-3′,5′-bis(diphosphate) 3′-pyrophosphohydrolase	*spoT*	7.415
c4704	Undecaprenyl-phosphate alpha-*N*-acetylglucosaminyltransferase	*rfe*	3.950
c4725	Adenylate cyclase	*cyaA*	76.484
c4804	Thiol:disulfide interchange protein DsbA precursor	*dsbA*	437.265
c4923	Soluble pyridine nucleotide transhydrogenase	*udhA*	52.361
c5177	Hypothetical protein in IS[c]		2.712
c5207	Hypothetical protein		37.124
c5320	Hypothetical protein YtfP	*ytfP*	2.534
c5329	Fructose-1,6-bisphosphatase	*fbp*	6.908

[a] SI, survival index.
[b] PTS, phosphotransferase system.
[c] IS, insertion element.

different carbon sources might enter into a dormant state that is characteristic of the persister subpopulation (18, 19). These results are supported by recent findings showing that the generation of persister cells is associated with depletion of ATP in *Staphylococcus aureus* (20).

Moreover, we also identified genes encoding envelope proteins, i.e., peptidoglycan turnover and membrane proteins. Among these, increased survival indices were observed for *nmpC*, *sanA*, *mltB*, *agaV*, *rfe*, and *dsbA* mutants (Table 1). Additionally, transposon insertions in the *ybhK* gene were identified in our screen. It has been shown that YbhK participates in gluconeogenesis, and a role in the generation of cell wall precursor molecules has been proposed (21). These results indicate that decreasing the turnover of peptidoglycan and/or inhibiting the ex-

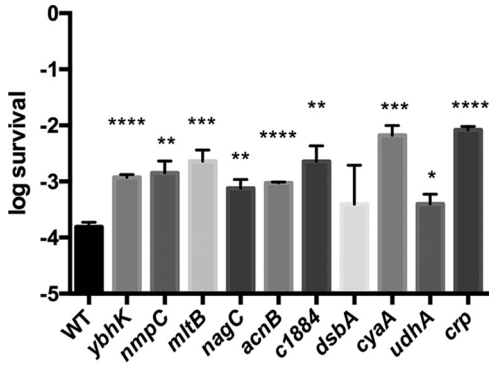

FIG 1 Increased survival of ampicillin exposure by *E. coli* strains deficient in genes identified by Tn-Seq. The ability to generate persister cells of mutant strains in the background CFT073 was assessed by CFU counting. Error bars denote standard errors corresponding to 3 independent trials. Statistical significance was determined using a two-tailed Student *t* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$). WT, wild-type strain.

pression of certain membrane proteins might induce tolerance to ampicillin through envelope stabilization. However, it is also possible that these mutations might in addition or alternatively generate dormancy through some mechanism such as decreasing the rate of multiplication.

Our selection also identified three genes, *yedU*, *xseA*, and the transcriptional regulator *slyA*, which are related to different kinds of stress responses (22–24). We speculate that insertion mutants with an impaired ability to respond to different types of cell damage might, in the presence of ampicillin, exhibit growth arrest, dormancy, and a persister phenotype.

We identified transposon insertions in the noncoding regions upstream of the *c1884* and *rfe* genes, likely in their respective promoters. This observation is consistent with the results of Table 1 showing that insertions in the same genes are positively selected, which indicates that either knocking out or inhibiting the expression of those genes generates increased tolerance to ampicillin. This result was further validated by showing that a clean deletion in *c1884* increased survival compared to that of the wild-type strain (Fig. 1).

Consistent with the known role of the stringent response in the persister phenotype, we identified *spoT*, which encodes the enzyme that synthesizes the stringent response alarmone (p)ppGpp. Based on our results, we cannot exclude the possibility that a secondary mutation(s) in *relA* and/or another gene(s) that affects the (p)ppGpp level contributes to the observed phenotype, since (i) it has been determined that in *E. coli spoT* is essential in an *relA*$^+$ background (25) and (ii) it has been shown that strains deficient in *spoT* and *relA* display an impaired ability to generate persister cells (26) Alternatively, the transposon insertions in *spoT* may have created truncated proteins that retained the essential activity.

Similarly, we identified *cyaA* and *crp* promoter mutants in our screen. The effector molecule cyclic AMP (cAMP) synthetized by adenylate cyclase (CyaA) and cAMP receptor protein (CRP) regulate a plethora of biological processes in response to the energy status of the cell (27). It has been previously established that cAMP is involved in the persistence of *E. coli* through the regulation of indole levels (28). These findings further support our results and the important role of this molecule in the persistence phenomenon.

Most of the genes identified in our selection are hypothetical, which does not allow a deeper interpretation. However, since these results were validated as shown in Fig. 1, a role for those genes in persister generation is proposed.

Finally, insertions in *udhA* (Table 1 and Fig. 1), which encodes a soluble pyridine nucleotide transhydrogenase, were found to have increased tolerance to ampicillin. We observed that a mutant with this gene deleted grows slower than the wild-type strain (data not shown), and this slow growth might explain the associated increased survival.

To validate our findings we randomly chose and constructed deletions in 10 of the 50 genes identified using the FRT (FLP recombination target)-FLP and lambda Red systems as described previously (29, 30). Each mutation was back-crossed to the wild-type background by generalized transduction using phage φEB49 (31). Finally, the kanamycin resistance cassette was deleted by expression of the FLP resolvase in *trans*, leaving behind an FRT scar in place of each gene. Tables S1 and S2 in the supplemental material contain the bacterial strains/plasmids and DNA sequences of the primers used to construct these mutations.

The ability to generate persister cells in the deletion strains was evaluated by measuring survival of a lethal dose of ampicillin. Briefly, an overnight culture was diluted 100-fold in fresh LB and incubated at 37°C with aeration; when the OD_{600} reached ~0.3 (~3×10^8 CFU/ml), it was exposed to 125 μg/ml ampicillin for 6 h at 37°C with aeration. For determination of CFU, aliquots were serially diluted in phosphate-buffered saline (PBS) and plated on LB agar plates supplemented with 20 mM $MgSO_4$ and 2 mg/ml sodium pyruvate as previously described (32). Persister cell levels were determined by the ratio of the number of CFU/ml in the culture after 6 h of exposure to ampicillin to the number of CFU/ml before addition of the antibiotic. Increased survival was observed for 9 of 10 mutants tested: *ybhK*, *mltB*, *nmpC*, *acnB*, *c1884*, *cyaA*, *udhA*, *crp*, and *nagC* (Fig. 1). This validates our genetic selection and Tn-Seq analysis. No changes in MICs were observed for any mutants except for *cyaA* and *crp*, which showed 2-fold increases compared to that for the wild-type strain. Similarly, no differences in the growth rates were observed for the other mutants evaluated except for the *udhA* strain as stated above.

This study demonstrates that analysis of a complex pool of mutants under positive selection is a powerful method to study biological phenomena such as persistence, where genetic redundancy is relevant (33).

Taken together, our results suggest that *E. coli* should be able to enter into a persister state by downregulating pathways involved in central metabolism, catabolism of alternative carbon sources, peptidoglycan turnover, and stress response genes. This adds to our knowledge of how persister cells may be generated and suggests that processes in addition to the stringent response and TA modules may be involved in this important phenotype.

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