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Genomewide Overexpression Screen for Fosfomycin Resistance in *Escherichia coli*: MurA Confers Clinical Resistance at Low Fitness Cost

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To determine whether the overexpression of chromosomal genes can confer fosfomycin resistance, genomewide screening of a complete set of 5,272 plasmid-expressed open reading frames of *Escherichia coli* (ASKA collection) was performed. Major results are that (i) no clinical level of resistance is achieved by overexpressing chromosomal genes, except *murA*; (ii) this level is reached at a low fitness cost; and (iii) this cost is much lower than that imposed by other mutations conferring fosfomycin resistance.

The emergence of antibiotic resistance mutants in a bacterial population is shaped by several factors, and both the mutation rate and the fitness cost of resistance are particularly relevant (2). If resistance came at a high fitness cost, the growth rate would not be enough to offset the clearance imposed by the host or to prevent the bacteria from being outcompeted by fitter susceptible bacteria once the antibiotic is removed (2).

Fosfomycin (Fos) is a broad-spectrum bactericidal antibiotic active against both Gram-positive and Gram-negative bacteria (7). Conveniently, Fos treatments have shown a relatively low likelihood that resistant mutants will persist *in vivo*, probably due to the high biological cost of resistance mutations, leading to good therapeutic effectiveness (19).

Fos resistance is acquired mainly by reducing the cell's drug uptake (4, 10, 11, 22), although active efflux, target alterations, and plasmid-encoded resistance have also been described in *Escherichia coli* and other species (18, 21, 23, 24). Resistant mutations usually entail a moderate to high fitness cost (1, 15, 19), as well as reduced virulence (8, 13, 15), which has been invoked to explain the low prevalence of resistant strains (19). Despite this, a significant increase in resistance has been recently described after antibiotic pressure in the community (20), suggesting that there may be other, unidentified, ways to attain less costly high-level Fos resistance.

The genome of *E. coli* harbors a substantial reservoir of resistance genes whose overexpression can decrease susceptibility (22). The complete *E. coli* open reading frame (ORF) ASKA library has already been screened for resistance to 237 toxins and antibiotics. However, Fos was not included among the drugs used to screen for resistance. Given the renewed interest in Fos treatment, we explored the capacity of the overexpression of chromosomal *E. coli* genes to confer clinical levels of Fos resistance by screening the complete ASKA library (12).

Genomewide overexpression screening for Fos resistance. The complete ASKA library was replicated in duplicate in 96-well plates containing Luria-Bertani (LB) broth plus chloramphenicol (50 μg/ml) with and without isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 100 μM and incubated overnight at 37°C. The transcription of the cloned genes of the ASKA collection is under the control of the P*tac* promoter, which is induced by the addition of IPTG (12). A 5-μl sample from each well was spotted onto an LB agar plate containing either 32 μg/ml Fos (the EUCAST breakpoint for *Enterobacteriaceae* [http://www.eucast.org/clinical_breakpoints/]) or no antibiotic. Plates were incubated for 24 h at 37°C. Only 1 of the 5,272 clones was able to grow on Fos. The clone, containing plasmid pCA24N-murA, was isolated from the original frozen 96-well plate and introduced by transformation into strain MG1655. Three independent transformants were isolated, and Fos resistance was verified as indicated above. The presence of the wild-type *murA* gene in the plasmid was verified by sequencing. Therefore, under our experimental conditions, no clinical level of Fos resistance can be achieved by the overexpression of chromosomal genes, except *murA*.

Effect of *murA* overexpression on Fos resistance level. MICs of Fos for strains MG1655(pCA24N) and MG1655(pCA24N-murA) were determined by the broth microdilution method as recommended by the CLSI (3), except that LB broth was used instead of Mueller-Hinton medium. Table 1 shows how increases in *murA* transcription, caused by rising IPTG concentrations, promote strong increases in the MIC of Fos.

Effect of *murA* overexpression on growth rate. To gain a quantitative insight into the fitness cost imposed by this type of resistance, growth curves in the presence of different concentrations of IPTG (3.3, 10, 33, and 100 μM) were recorded. Overnight cultures were diluted 1:100, and growth was resumed for 4 h, with and without different concentrations of IPTG. Cultures of the control and *murA*-carrying strains were then diluted 1:36 in LB broth plus IPTG in a final volume of 180 μl per well in a flat-bottom 96-well plate. The plate was covered with a lid to prevent evaporation and incubated at 37°C in a multiwell fluorimeter. Optical density at 595 nm was recorded every 10 min after 10 s of orbital shaking. Two controls were included, strain MG1655 harboring the vector pCA24N and the strain expressing *murA*, MG1655(pCA24N-murA), without IPTG. Figure 1A shows that the addition of IPTG, even at the higher concentration, did not produce any effect on the growth of the control strain in LB broth. On the contrary, when the expression of *murA* was increased by

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TABLE 1 Effect of *murA* transcription level on fosfomycin MIC for strain MG1655 containing either pCA24N (vector alone) or pCA24N-murA (expressing *murA*)

	MIC of fosfomycin (μg/ml)	
IPTG concn (μM)	pCA24N	pCA24N-murA
0	1	4
3.3	1	8
10	1	16
33.3	1	64
100	1	4,096

the addition of different concentrations of IPTG, the growth of MG1655(pCA24N-murA) was affected (Fig. 1B).

These results indicated that overproduction of MurA confers a fitness cost proportional to the level of transcription (Fig. 2), which opens the possibility for evolutionary adjustment of the resistance-fitness tradeoff. In particular, clinical resistance levels (32 μg/ml) can be achieved at a low fitness cost (about 5%, the result of interpolation in Fig. 2), whereas the fitness cost of the commonly found permeability mutants (with altered GlpT and/or UhpT functionality) has been estimated to be ~20% (19). A simple calculation can illustrate the meaning of this fitness costs, in terms of how long it will take for a wild-type strain to dominate the population. Assuming that both wild-type and mutant populations grow exponentially and excluding *de novo* mutations, it can be easily shown that the time needed to vary the ratio of mutant to wild-type bacteria by factor C (t_C) is equal to $\log_W C$, where w is the relative fitness of the mutant (ratio of the growth rates). Calculating t_C with fitness costs of 5% and 20%, respectively, shows that it will take *murA* overexpression mutants more than four times as long as permeability mutants to be outcompeted by the wild type in the absence of antibiotic pressure. The exact number of generations depends on C. For example, it will take the wild-type 51.6 generations to increase its frequency from 0.001% to 90% against permeability mutants, whereas it will take 224.5 generations against overexpression mutants.

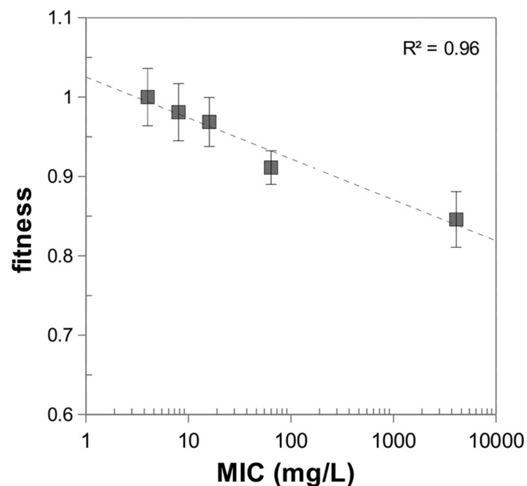

FIG 2 Tradeoff between fitness and resistance. Fitness and MICs were estimated for strain MG1655 with a plasmid harboring the *murA* gene at different levels of induction (expressed as concentrations of IPTG). The growth rate was estimated as the maximum slope of the natural logarithm of optical densities versus time. Curve fitting was done using a regression spline method (4, 24), and fitness was calculated as the ratio of the growth rate at each concentration of IPTG to that without IPTG (16). These measurements were determined from seven independent cultures, and a single mean relative fitness level was calculated for each condition. Error bars represent standard deviations.

Due to the current scarcity of new antibiotics, Fos has been proposed as an alternative treatment for infections caused by a wide variety of bacteria (7). Our genomewide screening has shown that of the 5,272 chromosomal genes tested, only 1, *murA*, is able to confer clinical levels of resistance when overexpressed. Even

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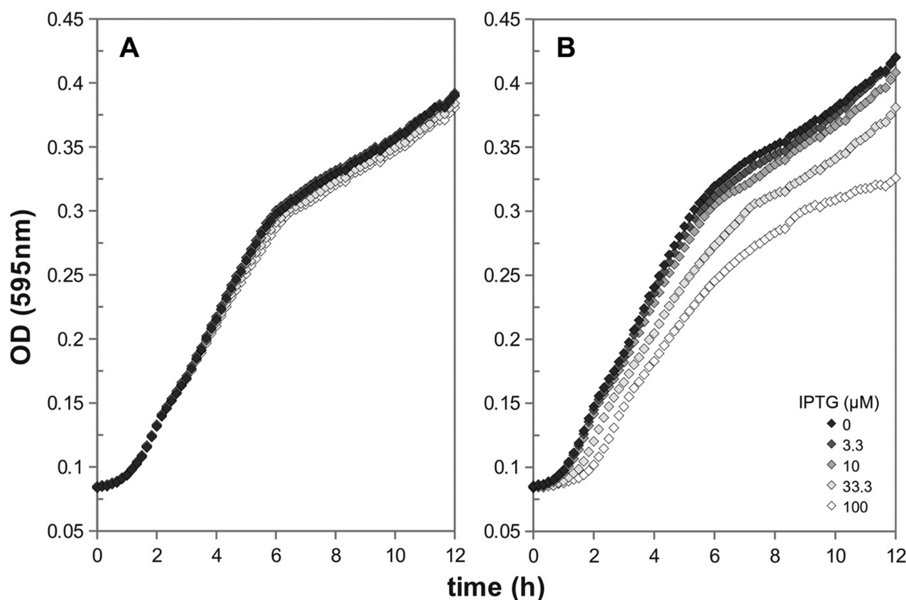

FIG 1 Growth curves at different IPTG concentrations. The values shown are averages of seven independent experiments. Different shades of gray represent different concentrations of IPTG. (A) Growth of control strain MG1655 carrying the cloning vector pCA24N. (B) Growth of the strain carrying *murA*-expressing plasmid pCA24N-murA.

though the effect of overexpression of *murA* on Fos resistance has been described already (9, 16, 19), our results indicate that no other chromosomal genes can produce Fos resistance by overexpression. In addition, we show that overproduction of MurA to a level high enough to produce clinical Fos resistance exacts a fitness cost significantly lower than that imposed by other mutations found in clinical isolates (19).

How likely are mutant bacteria overexpressing *murA* to be selected *in vivo*? Apart from the fitness cost, the mutation rate also plays a major role in determining this probability (2). Overexpression mutants could easily arise under natural conditions by increasing RNA polymerase binding affinity through promoter mutation, by disrupting gene control expression as described for other resistance determinants (6, 17), or by acquiring foreign DNA segments via, for instance, insertion sequences with strong promoter activity (5, 14). In any case, further clinical studies are necessary to determine the relative importance of this mechanism of resistance.

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