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Letter to the Editor

LETTER TO THE EDITOR – Biotechnology & Synthetic Biology

A simple technique for suppressor detection in *Escherichia coli*

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One sentence summary: A simple cloning technique can be used to detect low to high levels of suppressor activity that could explain viability and widespread dissemination of resistance isolates.

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ABSTRACT

To study the viability of a *gyrA* S83 stop mutation found in an *Escherichia coli* J53 ciprofloxacin-resistant strain (J53 CipR27), a pBR322 derivative was constructed with a TAG mutation in the *bla* gene knocking out ampicillin resistance. Ampicillin resistance was restored, suggesting that the strain contains tRNA suppressor activity able to suppress the UAG codon *gyrA* and allow viability. The method was applied to 22 unique clinical *E. coli* isolates, and all were found to have low-level suppressor activity.

Keywords: UAG mutations; *Escherichia coli* J53; *gyrA* Ser83Stop; tRNA suppressor

Quinolones target two essential bacterial type II toposiomerase enzymes, DNA gyrase and DNA topoisomerase IV. Single amino acid changes in the quinolone-resistance-determining region (QRDR) of GyrA can cause quinolone resistance in *Escherichia coli* (Hooper and Jacoby 2015). In the course of isolating ciprofloxacin-resistant mutants from *E. coli* J53, a common laboratory strain (Yi *et al.* 2012), a nonsense mutation was found unexpectedly at amino acid 83 in the QRDR region of GyrA in the J53 CipR27 strain. Despite the essentiality of gyrase, the growth curves of J53 and J53 CipR27 at 30°C, 37°C and 40°C showed no differences (data not shown), suggesting that J53 CipR27 contained a suppressor tRNA allowing translation of GyrA despite the stop codon at position 83. Thus, we sought to develop a simple method to detect this and other UAG suppressor mechanisms.

The widely used *E. coli* moderate-copy-number cloning vector pBR322 was chosen for this purpose. pBR322 is 4361 base pairs in length and harbors the origin of replication of plasmid pMB1, a close relative of the plasmid ColE1. It also contains the *bla* gene, encoding β-lactamase and conferring ampicillin resistance, and the *tet* gene, encoding the tetracycline resistance protein (Bolivar *et al.* 1977). Our strategy was to create a UAG mutation in the *bla* gene preventing full translation of β-lactamase and ampicillin resistance, which could be restored upon plasmid introduction into strains with suppressor activity. A similar approach cloning a nonsense codon within the chloramphenicol acetyltransferase (*cat*) gene in *E. coli* has been used in the past to prove the functionality of suppressors in mammalian cells (Capone *et al.* 1986), but the detection of naturally occurring nonsense suppressors was not demonstrated.

Site-directed mutagenesis was performed in the *bla* gene of pBR322 by using a Phusion site-directed mutagenesis kit (Thermo Scientific, MA, USA) to substitute a codon for serine at amino acid 220 for an TAG stop codon (TCG→TAG change at 662 bp). The forward primer, which included the desired mutation (shown in bold underline) Amber-F2 5′-GACCACTTCTGCGCT**A**GGCCCTTCCGGCTG-3′, Amber-R2, 5′-CTGCAACTTTATCCGCCTCCATCCAGTCTA-3′ and 10 ng of

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Table 1. Phenotype of ampicillin resistance determined by disk diffusion method and MIC in the laboratory strains.

Strains	Disk diffusion[a] zone diameter (mm)	MIC[b] (μg/ml)
BL21 (DE3)	20	2
BL21 (DE3) pBR322	<6	>256
BL21 (DE3) pBR322-Stop	21	2
MG1655	20	2
MG1655 pBR322	<6	>256
MG1655 pBR322-Stop	20	4
DH5 α	20	2
DH5 α pBR322	<6	>256
DH5 α pBR322-Stop	<6	>256
J53	20	4
J53 pBR322	<6	>256
J53 pBR322-Stop	<6	>256
J53 CipR27	21	0.5
J53 CipR27 pBR322	<6	>256
J53 CipR27 pBR322-Stop	<6	>256

[a]A disk of ampicillin 10μg (Becton, Dickinson and Company) was used.
[b]MIC was determined by agar dilution method (CLSI 2012).

Table 2. Relative *bla* transcript levels of pBR322 and pBR322-Stop in different strains.

Strains	Mean fold change relative to pBR322 (SEM)	
BL21 (DE3) pBR322	1.000	(0)
BL21 (DE3) pBR322-Stop	0.756	(0.130)
MG1655 pBR322	1.000	(0)
MG1655 pBR322-Stop	0.826	(0.040)
DH5 α pBR322	1.000	(0)
DH5 α pBR322-Stop	0.807	(0.165)
J53 pBR322	1.000	(0)
J53 pBR322-Stop	0.678	(0.094)
J53 CipR27 pBR322	1.000	(0)
J53 CipR27 pBR322-Stop	0.689	(0.081)

SEM: error standard of the mean.

pBR322 template (New England Biolabs), was used in a final volume of 50 μl with the following PCR conditions: 98°C 30 s; 24 cycles of 98°C 10 s, 65°C for 30 s, 72°C for 4 min and a final elongation step of 72°C for 5 min. After gel purification, amplified DNA was ligated using T4 DNA Ligase in a 5-min reaction. A portion of 1 μl of the ligated product was used to transform 20 μl of MAX Efficiency® DH5α Competent Cells (Invitrogen) and spread on tetracyline (10 μg/ml) LB plates for further selection. The mutated plasmid was verified by sequencing, using the primers bla-PCR-F (5′-AGTATTCAACATTTCCGTGTCG-3′) and bla-PCR-R (5′-TGCTTAATCAGTGAGGCACCTA-3′), and introduced into *E. coli* J53, J53 CipR27, DH5α (Grant *et al.* 1990), BL21 (DE3) (commercial) and MG1655, which is a wild-type K-12 *E. coli* control strain (Blattner *et al.* 1997), by electroporation for further testing of ampicillin resistance. A wild-type pBR322 was also electroporated into the same strains as a control. Ampicillin resistance was determined by agar dilution MIC on Mueller Hinton agar at 37°C with an inoculum of ~10^4 CFUs and by disk diffusion following in both cases CLSI recommendations and resistance breakpoints (MIC≥32 μg/ml and zone diameter ≤13mm, respectively) (CLSI 2012).

Escherichia coli BL21 (DE3) and MG1655, which are known to lack UAG suppressor activity (Nilsson and Rydén-Aulin 2003; O'Donoghue *et al.* 2012; Singaravelan, Roshini and Munavar 2010), exhibited an increase in ampicillin MIC with wild-type plasmid pBR322 but no increase with pBR322-Stop (Table 1). In contrast, DH5α and J53 with and without the *gyrA* CipR27 TAG mutation exhibited similar increases in ampicillin MIC in the presence of each plasmid. DH5α has a known UAG suppressor, but J53 was not previously known to have suppressor activity; the viability of the J53 *gyrA* CipR27 further validates suppressor activity in this strain background (Table 1).

To determine if *bla* trancript levels were similar in pBR322 and pBR322-Stop, we designed *bla* gene primers (*bla* gene: RT-bla-F; CGCCGCATACACTATTCTCA, RT-bla-R, AGTAAGTTGGCCGCAGTGTT, extracted RNA and determined expression levels by RT-qPCR (Vinué *et al.* 2015). Similar transcript levels were seen in all of the reference strain backgrounds (Table 2). Thus, differences in plasmid-encoded ampicillin resistance among the strains tested could not be attributed to changes in transcript levels and thus appear to be related to changes in translation, as expected for translational suppressor activity.

Although a *gyrA* Ser83stop mutation in *E. coli* J53 has been reported (Cesaro *et al.* 2008), suppressor activity was not otherwise documented. To determine if suppressor activity occurs in *E. coli* clinical isolates, we obtained 22 sequential clinical urine *E. coli* strains each from a different patient from the Massachusetts General Hospital Clinical Microbiology laboratory that were selected for susceptibility to ampicillin and tetracycline to allow ampicillin susceptibility testing using pBR322 and pBR322-Stop. These plasmids were introduced individually by electroporation into each strain, and plasmid-free and plasmid-containing strains were compared ampicillin MICs and disk diameters (Table 3). Although all strains tested showed a similar increase in MIC of ampicillin with introduction of pBR322 as seen in the reference strains, none showed a similar increase in ampicillin MIC with introduction of pBR322-Stop, indicating the absence of strong suppressor activity. Lower increases in ampicillin MIC of 4- to 64-fold, however, were seen in the presence of pBR322-Stop in all 22 strains, suggesting common but weak suppressor activity in clinical isolates. To our knowledge, suppressor activity has not been reported before in clinical isolates. UAG suppressor tRNAs vary greatly in their translational efficiency. Because all UAG suppressor tRNAs share the same codon–anticodon pairing, the differences must reside in other parts of the tRNA structure. Weak suppressors require changes at the first position of the anticodon (cardinal position) whereas strong suppressors have changes at the two other anticodon positions. Only when the cardinal nucleotide is embedded in a suitable extended anticodon and further embedded in an otherwise normal tRNA molecule, the anticodon performs as an efficient and accurate translational device (Yarus 1982). This occurrence could explain the weak suppressor activity found in the clinical isolates. Alternatively, several genes known as antisuppressors have been found to cause reduction in suppressor activity and affect mainly nucleotide modification enzymes involved in tRNA maturation and ribosomal proteins (Eggertsson and Söll 1988). Further studies are needed to elucidate the emergence of mutations in the tRNA and the presence of suppressors that could emerge and create low levels of resistance in a clinical setting.

This simple technique could be used to detect low to high levels of suppressor activity that could explain viability and widespread dissemination of resistance isolates.

Table 3. Effects of pBR322 and pBR322-Stop on ampicillin resistance in a set of ampicillin- and tetracycline-susceptible *E. coli* clinical isolates.

	No plasmid		pBR322		pBR322-Stop	
Strains	Zone diameter[a] (mm)	MIC[b] (μg/ml)	Zone diameter (mm)	MIC (μg/ml)	Zone diameter (mm)	MIC (μg/ml)
1	18	4	<6	>256	13	32
2	20	8	<6	>256	12	64
3	20	4	<6	>256	13	32
4	20	4	<6	>256	13	32
5	20	4	<6	>256	12	32
6	27	4	<6	>256	14	64
7	21	4	<6	>256	16	16
8	20	4	<6	>256	14	32
9	20	4	<6	>256	12	32
10	21	4	<6	>256	17	16
11	21	4	<6	>256	18	16
13	19	4	<6	>256	15	16
14	20	4	<6	>256	17	16
15	21	2	<6	>256	16	32
16	22	2	<6	>256	11	128
17	20	4	<6	>256	14	32
18	26	2	<6	>256	15	32
19	21	4	<6	>256	15	32
20	21	4	<6	>256	12	64
21	20	4	13	128	17	16
22	20	8	<6	>256	15	32
23	21	2	<6	>256	16	16

[a]A disk of ampicillin 10 μg (Becton, Dickinson and Company) was used.
[b]MIC was determined by agar dilution method (CLSI 2012).

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Conflict of interest. None declared.

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