Shiga toxin-producing *Escherichia coli* (STEC) are often subjected to DNA damaging antibiotics during culturing in order to elicit the bacterial SOS response and up-regulation of bacteriophage-encoded proteins including Shiga toxin (Stx). However, such antibiotic exposure and stress may also have effects on protein expression.

Escherichia coli O157:H7 strain EDL933 was grown on Luria-Bertani agar (LBA) supplemented with

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bacterial host genomes by a process called horizontal or lateral gene transfer [7]. Expression of BP-encoded proteins (and Stx) occurs when the BP switches from its lysogenic cycle (a latent or dormant state in which BP DNA is replicated with host DNA) to the lytic cycle. The BP lysogenic/lytic switch is a consequence of a series of biological events in the bacterial host triggered by damage of its DNA, i.e., the SOS response [8]. Thus, some antibiotics, whose antimicrobial mode-of-action is to damage or disable bacterial DNA, e.g., by cross-linking DNA or inhibition of the replication fork, may also elicit a series of biological responses that lead to expression and release of Stx. In consequence, many antibiotics are contra-indicated for treatment of a suspected STEC infection.

A number of techniques have been developed to detect and characterize Stx from environmental and clinical isolates [9-13]. Monoclonal antibody (MAb)-based assays (e.g., ELISA, etc.) are very popular as (once developed and validated) they can be utilized in non-laboratory settings by end-users who require very little training or expertise in their use [10,11]. Another advantage is they can be used for screening a large number of samples. However, MAb-based approaches can also be plagued by non-specific binding especially for detection in complex matrices leading to false positives or a lack of detection. In addition, any initial positive result from such an assay must be considered *presumptive* until it is confirmed by another, typically orthogonal technique, i.e., bioassay or a chemical/physical-based technique such as mass spectrometry [9,12,13].

An additional complexity of Stx detection and identification is that an STEC strain may have multiple *stx* genes of different types and subtypes, e.g., *E. coli* O157:H7 strain EDL933 has stx_{1a} and stx_{2a} genes (both are expressed) [14,15]. PCR is often used to identify *stx* genes irrespective of whether the genes are actually expressed [16]. Clinical subtypes of Stx2 (a, c and d) require highly specific primers and

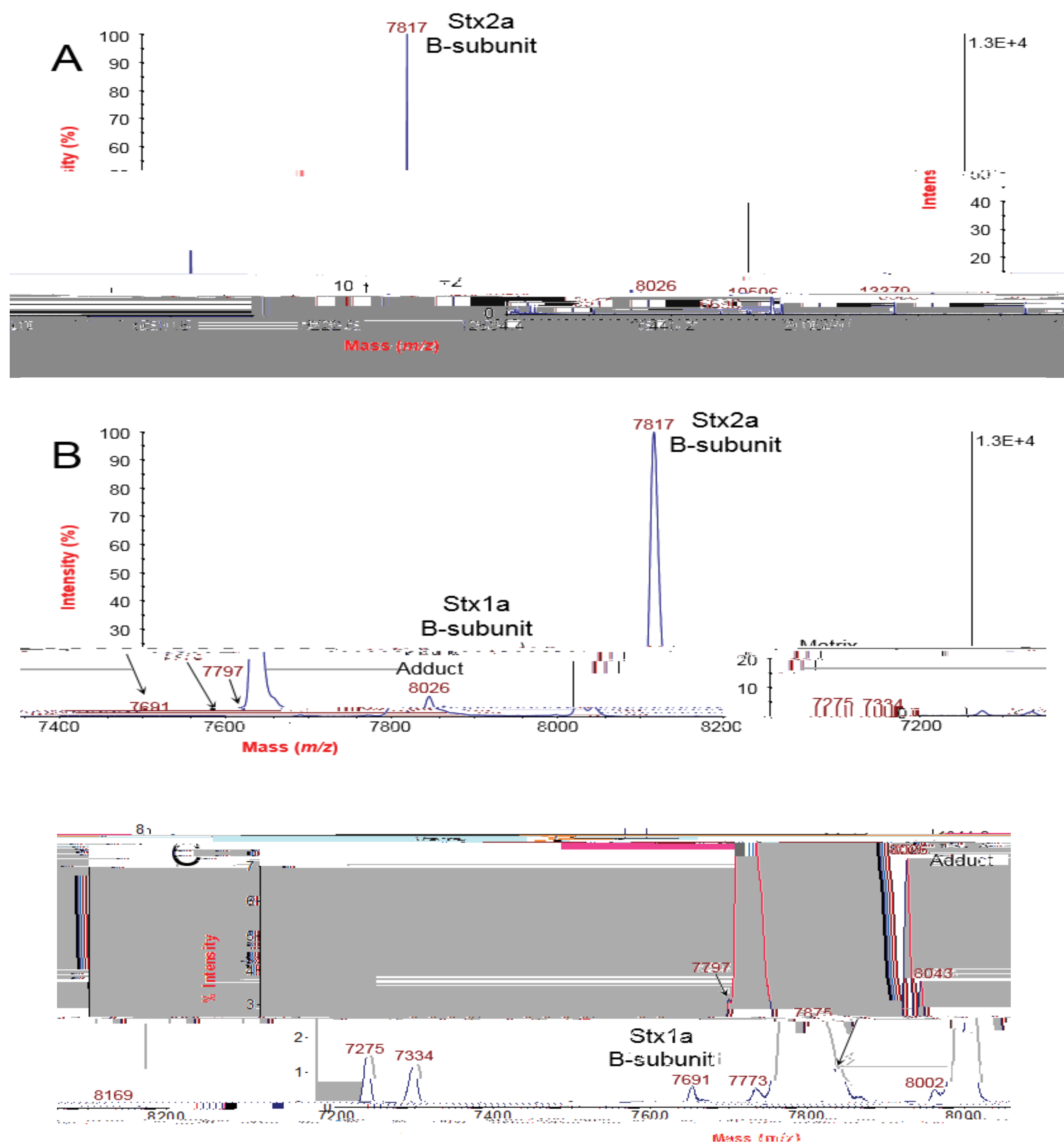
construct was then sub-cultured for multiple days at 37°C in order to cure the strain of the pKD46 plasmid.

MALDI-TOF mass spectrometry

E c e c a c O157:H7 strain EDL933 and the $_{2a_B\text{-}subunit}$ mutant strain (WJZ21) were grown overnight on Luria-Bertani agar (LBA) supplemented with a 20 ng/mL of cipro oxacin [12,13]. Bacterial cells were harvested, suspended in water, gently vortexed and centrifuged so as to avoid cell lysis [18]. Supernatants were analyzed by MALDI-TOF-MS as previously detailed [12,13]. Brie y, a 0.5 µL aliquot was spotted onto a stainless steel target and allowed to dry. is dried sample spot was then over-layed with a saturated solution (33% acetonitrile, 67% water and 0.1% formic acid) of sinapinic acid (Protea Biosciences, Morgantown, WV) and also allowed to dry. e instrument utilized was a 4800 MALDI-TOF-TOF mass spectrometer (Sciex). Mass data was collected in linear mode. Prior to data collection, the linear mode was calibrated with a standard mixture of proteins: cytochrome-C, myoglobin and lysozyme all obtained from the same source (Sigma-Aldrich, St. Louis, MO).

Nano-liquid chromatography-electrospray ionization-orbitrap mass spectrometry

Sample supernatants were also analyzed using nano-LC-ESI-Orbitrap mass spectrometry (Velos Pro LTQ-Orbitrap, emoFisher, San Jose, CA). e splitless nano-HPLC system used was an ekspert nanoLC400 (Eksigent, AB Sciex, Redwood City, CA). A third pump was installed on this system to allow three separate (but simultaneous) functions upon three analytical columns, i.e., elution, washing and loading. is nano-HPLC system was interfaced to a multi-column PicoSlide platform (New Objective, Woburn, MA). e analytical column was a PicoChip HALO Protein (C4, 3.4 µm, 400 Å, 105 mm)



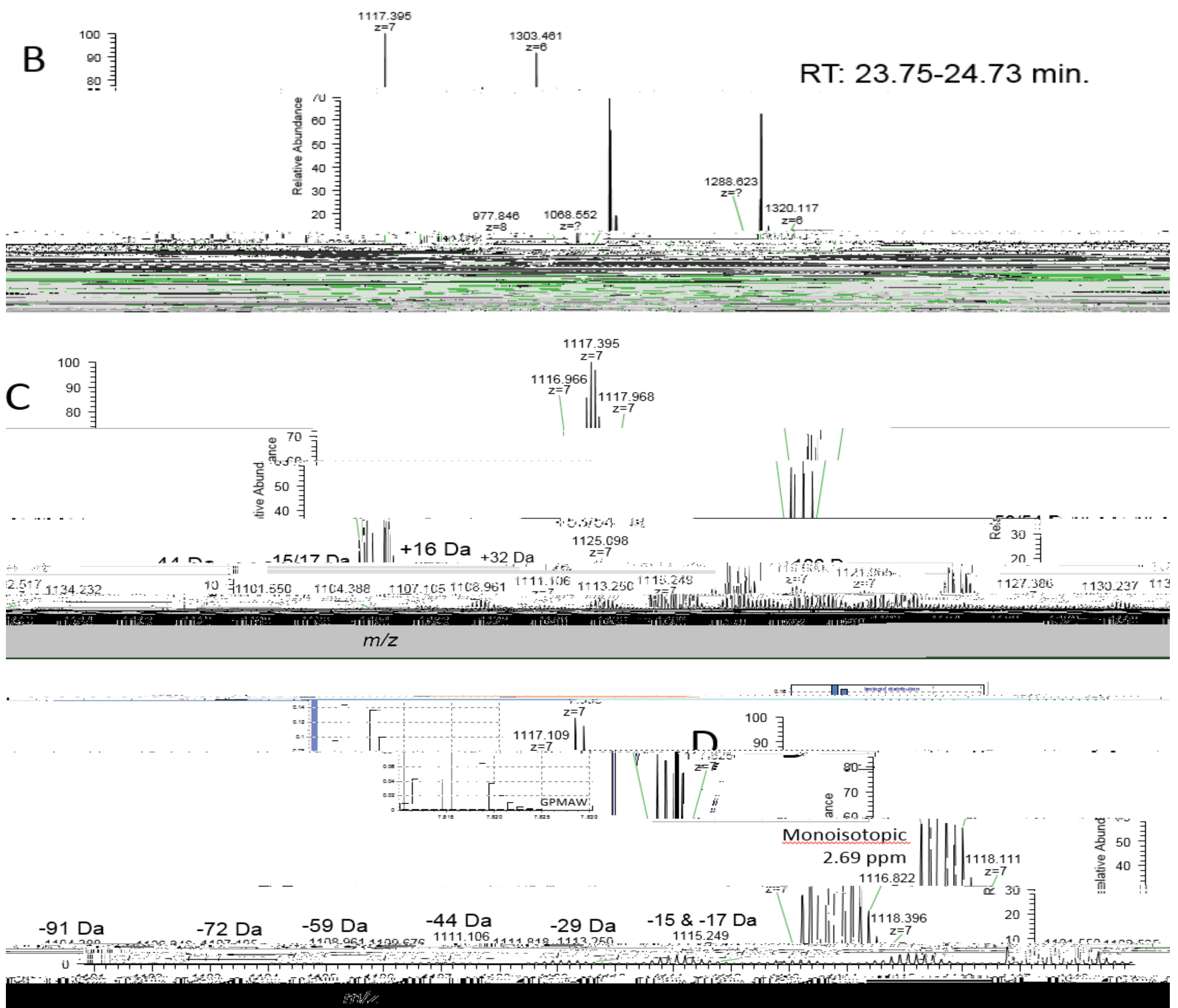
shows the MALDI-TOF-MS spectrum of *E. coli* O157:H7 strain EDL933 grown overnight on LBA supplemented with 20 ng/mL of ciprofoxacin. (shows an expanded mass-to-charge (*m/z*) range. shows a y-axis expansion (8%) of spectrum which allow a better view of less abundance peaks.

most abundant protein ion is an induced bacteriophage protein at m/z 13377 previously identi ed as an uncharacterized protein at locus tag 933Wp55 (synonym L0117) [18]. e peak at m/z 10507 was also previously identi ed as another uncharacterized bacteriophage protein: 933Wp74/L0136 [18]. e B-subunit of Stx1a is still detected at m/z 7693 as its gene was not deleted. Figure 2B shows an expanded m/z region that shows the complete absence of the B-subunit of Stx2a as well as the satellite peaks observed in Figure 1 including the matrix adduct peaks. us, the satellite peaks clustered around the Stx2a B-subunit peak in Figure 1 ultimately originate from the same B-subunit stx_{2a} gene. ese results strongly suggest that these additional peaks (mass variants) are due to mistranslation of Shiga toxin caused by antibiotic induction.

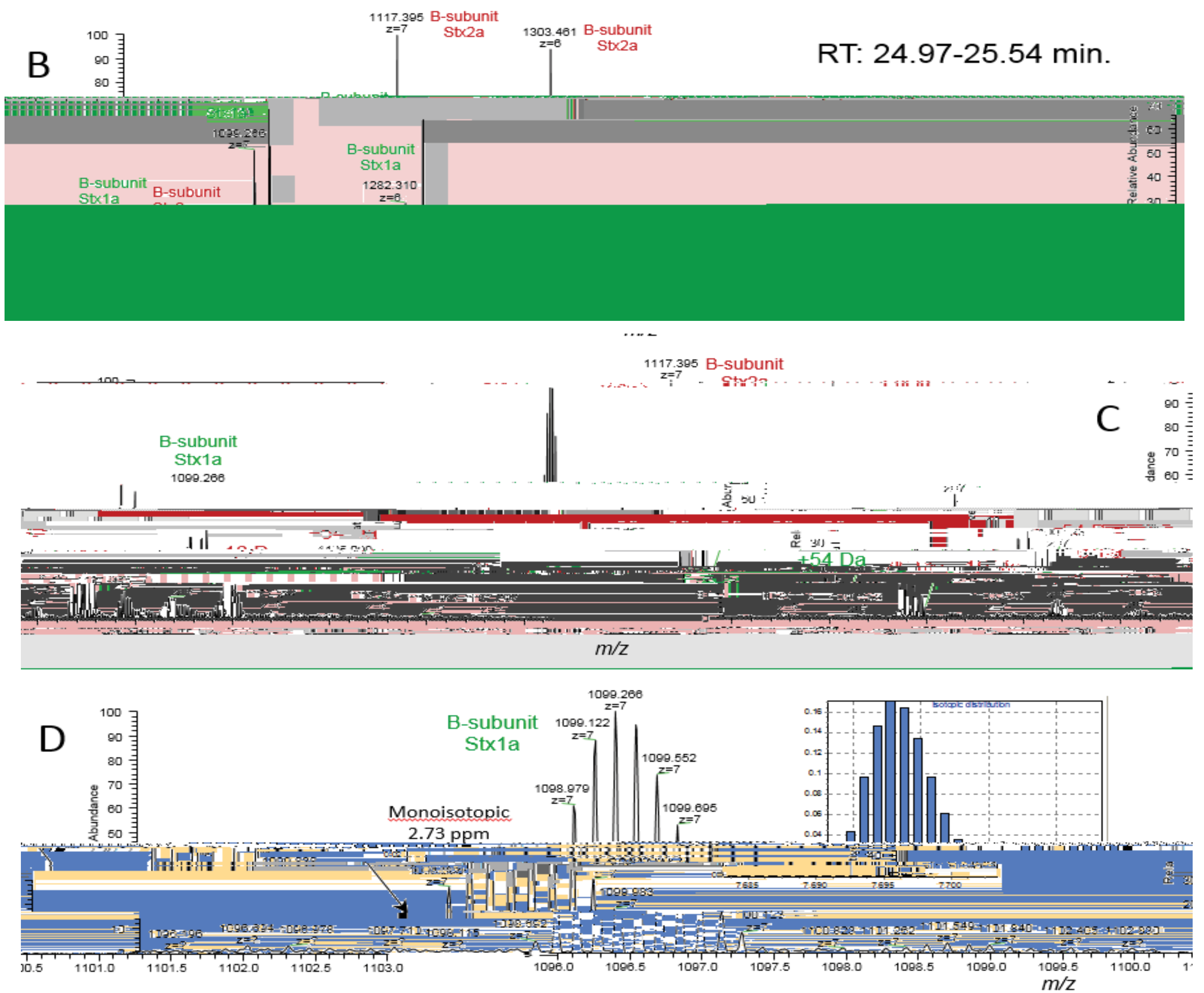
Figure 3A shows the nano-LC/MS chromatogram of *E. coli* O157:H7 strain EDL933 grown overnight on LBA supplemented with 20 ng/mL of cipro oxacin. e total ion chromatogram (TIC) and extracted ion chromatogram (XIC) of the +7 charge state of the

B-subunit of Stx2a are presented. As can be seen, the Stx2a B-subunit elutes around 24 minutes but it also has a significant elution tail. In Figure 3B, MS scans are summed across a major portion of the Stx2a B-subunit peak (23.75 to 24.73 minutes) revealing a narrow charge state envelope from +5 to +8 with major ion intensity protein ions at +7 and +6 charge states. This is not surprising Stx2a B-subunit is a relatively small protein, and since its disulfide bond is intact, it has a lasso-loop secondary structure as opposed to a linear chain structure [22]. The mature sequence of Stx2a B-subunit is shown in Figure 3. The cysteine residues of the disulfide bond are underlined. It should be noted that there are seven basic residues (highlighted in red) whose side-chains can each accommodate one ionizable proton. These basic residues are all located within the "loop". An eighth possible protonation site is at the N-terminus which is only a few residues away from two lysine residues (**K**). It is likely that once seven protonation sites have been occupied, the effects of Coulomb repulsion would inhibit (although not entirely eliminate) an eighth protonation which would explain the steep drop in intensity from +7 to +8 charge states.

Figure 3C expands the *m/z* region around the +7 charge state of Stx2a B-subunit from Figure 3B. Just as in Figure 1, where minor satellite peaks are observed clustered around the +1 charge state of Stx2a B-subunit peak, a similar appearance of minor intensity peaks are clustered around the +7 charge state of Stx2a B-subunit (as well as +6 charge state). The greater sensitivity and resolution of nano-


B
RT: 23.75-24.73 min.
1117.395
z=7
1303.461
z=6
1288.623
z=?
1320.117
z=6
977.846
z=8
1068.552
z=?
Relative Abundance
C
1117.395
z=7
1116.966
z=7
1117.968
z=7
+16 Da
+32 Da
1125.098
z=7
m/z
D
Monoisotopic
2.69 ppm
1116.822
1118.111
z=7
1118.396
z=7
GPMAW
-91 Da
-72 Da
-59 Da
-44 Da
-29 Da
-15 & -17 Da
1111.106
1115.249
m/z

the possible exception of the +16 Da mass variant, the chromatograms of the mass variants closely correlate with the chromatogram of the Stx2a B-subunit suggesting that the chemical characteristics of these variants are not signi cantly di erent from that of the Stx2a B-subunit


B
RT: 24.97-25.54 min.
1117.395 z=7 B-subunit Stx2a
1303.461 z=6 B-subunit Stx2a
B-subunit Stx1a
1282.310 z=6
C
B-subunit Stx1a
1099.266
+54 Da
m/z
D
B-subunit Stx1a
1099.266 z=7
1099.122 z=7
1099.552 z=7
1098.979 z=7
1099.695 z=7
Monoisotopic
2.73 ppm
m/z

below. Codon bases that are identical and have the same position in the substitution pair are highlighted in red.

r (**ACA**, **ACC**, **ACG**, **ACU**) Gly (**GGA**, **GGC**, **GGG**, **GGU**)

Met (**AUG**) Ser (**AGC**, **AGU**, **UCA**, **UCC**, **UCG**, **UCU**)

For the r Gly substitution, only the 3rd position (wobble position) is found to have any codon commonality. Met has only one codon: AUG. Its cognate $tRNA^{Met}$ anticodon is UAC. e anticodons of the rst two $tRNA^{Ser}$ are UCG and UCA, respectively. A Met Ser substitution involving the rst two $tRNA^{Ser}$ anticodons would require a U•C codon-anticodon mismatch in the middle position as well as a G•G or a G•A mismatch in the wobble position (3rd position) [24]. In a series of detailed measurements on the stability of complementary (and near-complementary) anticodon-anticodon tRNA pairs, Grosjean et al. reported that when uridine (U) occupies the middle position of an anticodon, it can result in stable mismatches for U•U and U•G in the middle position as well as UU and U•C in the wobble position [25]. Unfortunately, there is no information on the stability of a U•C mismatch in the middle position.

Kramer and Farabaugh have noted previously that the rate of mistranslation of a particular amino acid is dependent on the concentration of its corresponding $tRNA^{AA}$ [26]. ey also noted that since the rate constant for codon recognition is rst order, and therefore dependent on the concentration of the speci c $tRNA^{AA}$, i.e., a low abundant $tRNA^{AA}$ would result in an increase translational misreading of that speci c residue [25,26]. If the abundance of a particular $tRNA^{AA}$ is low, the rate of its mistranslation would increase. Met is a not a particularly abundant amino acid in bacterial proteins aside from its critical role as the rst residue of every protein. *E. c* utilizes a non-canonical pathway for methionine biosynthesis not shared by many other bacteria [27]. It is possible that translation of BP-encoded proteins during the lytic cycle results in a de cit of $tRNA^{Met}$, and the single Met present in the mature B-subunit sequence of Stx2a is replaced with a Ser residue despite codon-anticodon mismatches in the middle and wobble positions. It is also possible that cipro oxacin may facilitate misreading of the AUG codon. Kramer and Farabaugh have reported previously that aminoglycoside antibiotics can a ect translational misreading for speci c codons in *E c e c a c* as well as in

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