Fluoroquinolone resistance in *Elizabethkingia bruuniana*

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ABSTRACT

The Gram-negative bacterial genus *Elizabethkingia* displays natural multiple antimicrobial resistance and is an emerging pathogen. One of the therapeutic agents still effective in the treatment of the *Elizabethkingia* are the fluoroquinolones (e.g. ciprofloxacin). Even though these drugs are effective, the emergence of target gene mutation-mediated resistance has been reported in *E. anophelis* and *E. meningoseptica*. Fluroquinolones target DNA biosynthesis by inactivating the DNA gyrase and topoisomerase IV which are produced by the *gyrAB* and *parCE* genes. I now intend to determine the mechanisms by which *Elizabethkingia* species become resistant to the fluoroquinolone ciprofloxacin. I hypothesized that any fluoroquinolone-resistant mutants would exhibit compromised growth, elevated MICs, and mutations in the quinolone resistance-determining regions (QRDRs) of *gyrAB* and/or *parCE*. First, I isolated fluoroquinolone-resistant mutants via single-step selection in media containing ciprofloxacin. Standard minimum inhibitory concentration assays were performed to compare suspected mutants to the parent. I also performed checkerboard assays to assess potential synergistic interactions between ciprofloxacin and the cell wall active antibiotic vancomycin. Changes in the growth of the mutants were compared to the parent strains using standard growth curves. Finally, I utilized cloning and Sanger sequencing to determine the sequences of the QRDRs in *gyrAB* and *parCE* in the confirmed fluoroquinolone-resistant mutants of *E. ursingii.* I determined that fluoroquinolone-resistant mutants showed MICs elevated up to 32-fold compared to their parent strains. Checkerboard assays showed that the drug interaction was not synergistic. I demonstrated slowed growth in almost all fluoroquinolone-resistant mutants compared to their respective parent strains, suggesting a growth fitness cost from the acquisition of fluoroquinolone resistance mutations. Sanger sequencing of the QRDRs in *E. bruuniana* did not reveal the presence of mutations common to other fluoroquinolone-resistant Gram-negative

bacteria. Further sequencing and experimentation are necessary to determine the mechanisms by which *E. bruuniana* acquires fluoroquinolone resistance.

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INTRODUCTION

Elizabethkingia is a genus of Gram-negative bacteria of the family *Flavobacteriaceae* which was first delineated and defined in 2005 [1]. *Elizabethkingia spp* have been linked to cases of septicemia and neonatal meningitis since the earliest reported case in 1944 [2, 3]. It is an important emerging pathogen due to its intrinsic antibiotic resistance and ability to survive in unfriendly environments, such as those in hospitals and medical devices [4]. *Elizabethkingia* spp have caused clusters of outbreaks with high mortality rates in both community and healthcare settings, and readily infect and colonize hospital equipment such as ventilator tubing and faucet aerators [5].

Elizabethkingia spp can be broadly divided into two groups. The first group, which is most clinically relevant, includes *E. anophelis* and *E. meningoseptica. E. meningoseptica* is the most widely-known species of *Elizabethkingia,* having been blamed for most of the severe cases and outbreaks in the last 75 years [6]. However, this began to change when *E. anophelis* was isolated from the gut bacteria of an anopheles mosquito in the Central African Republic in 2011. The first case report of an infection attributed to *E. anophelis* was published in 2013: meningitis in an 8 day old infant female in the Central African Republic [7]. Cerebrospinal fluid aspirate grew a Gram-negative rod identified as *E. meningoseptica* by mass-assisted laser desorption-ionization time-of-flight mass spectroscopy (MALDI-TOF); however, the authors utilized 16S RNA sequencing techniques to demonstrate that this bacteria, as well as the preserved bacteria from a similar case in 2006, was actually *E. anophelis.* Later studies suggest that *E. anophelis* is actually the most prevalent infectious agent of the genus [8].

This case is a critical example of a known weakness of MALDI-TOF systems, which are common in clinical laboratory settings due to their efficiency. MALDI-TOF systems come from

the factory with a database of known bacteria which is used to compare to mass spectroscopy results [9]. The MALDI-TOF systems presently on the market do not have the full complement of *Elizabethkingia* species in their databases, so correct and specific identification of the bacteria in question is unlikely [10]. This has important implications for physicians because the efficacy of empirical antimicrobial therapy depends heavily on what bacteria is causing the infection in a patient. Adding to this difficulty is that *Elizabethkingia* species are not easily distinguishable by normal microbiological techniques, so more complex assays such as 16S RNA sequencing, whole genome sequencing, pulse-field gel electrophoresis, and/or *in silico* DNA hybridization are often used to establish a positive identity [1, 11].

The species of the Miricola group of *Elizabethkingia* include *bruuniana, occulta, ursingii,* and *miricola.* These four were originally considered one species, *miricola,* but were differentiated into species based on core genome comparative analysis [11]. The Miricola grouping is unofficial, but the four species are grouped together due to their genetic similarity. While *E. anophelis* and *E. meningoseptica* are the most relevant species from a clinical perspective, the other four species have all been implicated as the causative agent of infection in cases of septicemia, pneumonia, and cystic fibrosis around the world [11, 12].

In many cases, *Elizabethkingia* infections have been treated with vancomycin because in early reports, *Elizabethkingia* infection showed susceptibility *in vivo* to vancomycin. However, more recent studies have presented evidence that the *in vitro* efficacy of vancomycin is diminished, and newer strains show heightened resistance [13]. In many strains, however, there is still susceptibility to other antibiotics, such as ciprofloxacin, which has shown varying levels of effectiveness over other antibiotics, such as rifampin and piperacillin/tazobactam [14]. Ciprofloxacin is a heterocyclic, fat-soluble antibiotic of the fluoroquinolone class which

indirectly inhibits DNA synthesis. Ciprofloxacin is a derivative of the first quinolone antibiotic, nalidixic acid, which was first isolated in 1962 as a byproduct of the synthesis of chloroquines, a class of antimalarial drugs [15]. Nalidixic acid was mostly used for urinary tract infections until further research led to a second generation of quinolones. The substitution of a fluorine at C6 and the addition of a large ring substituent improved the pharmacokinetics and therapeutic index of the drug, and ciprofloxacin is currently used to treat a variety of Gram-negative bacterial infections [16, 17].

Ciprofloxacin's specific targets are the enzymes gyrase and topoisomerase IV. Both are type II topoisomerases which break phosphodiester bonds in the DNA strand and bind covalently to the strands to aid in winding and unwinding of the bacterial genome [18]. Both enzymes use energy from ATP to break DNA phosphodiester bonds using catalytic divalent metallic ions, and a tyrosine residue in the active site which binds covalently to the DNA. The two enzymes have different functions. Gyrase works with other topoisomerases (including the ω protein, a type I topoisomerase) and is responsible for controlling DNA supercoiling, which must be strictly controlled during DNA replication and transcription [19]. Topoisomerase IV is responsible for untangling the DNA strands which may become knotted during normal cellular function in a process known as decatenation [20]. Gyrase and topoisomerase IV are structural homologues with a heterotetrameric structure. Each protein has two subunits, A and B. Therefore, the subunits of Gyrase are written as GyrA and GyrB [21]. In Gram-negative topoisomerases, the proteins are abbreviated as ParC and ParE. In both cases, the B subunit is responsible for ATP binding and hydrolysis [19], while the A subunit defines the specificity of the enzyme [22, 23]. Poor conservation of sequence between gyrase and topoisomerase means that only Gyrase can

affect supercoiling [19]. It is important to note that the active site of both gyrase and topoisomerase are identical in structure and sequence.

In the normal course of action, gyrase and topoisomerase move along the genome and are activated whenever necessary for genome maintenance and DNA replication. However, because their job is to fragment and stabilize the genome, the genome is destabilized whenever the enzymes are present [19].

Ciprofloxacin, like all quinolones, binds noncovalently to a serine residue (Ser83) at the active site of both enzymes and an acidic residue four amino acids downstream, mediated by a divalent cation [21-23] Ciprofloxacin may target either gyrase or topoisomerase IV and the specificity is not related to Gram status [24].The fluoroquinolone chelates the calcium ion with its C3 and C4 keto acids, and the magnesium ion then forms a hydrogen bond with the amino acid residues [21]. Binding of drug to gyrase and the fragmented DNA strand reversibly locks the enzyme in place on the DNA strand and inhibits the catalytic activity of the enzyme by preventing re-ligation of the DNA molecule [25]. Tension on the genome causes more topoisomerase enzymes to be dispatched, and they are in turn locked into place on the enzyme. When any DNA-scanning enzyme complex (such as helicase, the leading enzyme in a replication fork) collides with the inactivated type II topoisomerase, the genome is fragmented, and the cell is unable to repair this damage due to the inability to replicate the fragmented genome [26].

Fluoroquinolones have a high therapeutic index because they are only effective against bacterial type II topoisomerases, and not human type II topoisomerases. This is because the human enzymes are not homotetramers but instead homodimers, with A and B subunits fused into a single protein. In addition, human type II topoisomerases lack the serine and acidic residues present in bacterial type II topoisomerases, preventing fluoroquinolones from chelating

to the enzyme [27]. While fluoroquinolones do not inhibit DNA synthesis in humans, they have been associated with CNS effects such as seizures, and they inhibit the CYP1A2 enzyme pathway [28].

Fluoroquinolone resistance may result from three processes: mutations in the genes which encode fluoroquinolone targets, such as *gyrAB* and *parCE* [29, 30], horizontal transfer of genes conferring resistance phenotypes [26, 31], and genomic regulation of fluoroquinolone entry via downregulation of porin protein expression and upregulation of multi-drug efflux pump protein expression [32-34].

While the cause of ciprofloxacin resistance in Gram-negative species is well-understood, there is only one article in the literature about the efficacy of fluoroquinolones against *Elizabethkingia*, and that paper deals only with *E. anophelis* and *E. meningoseptica* and uses clinical isolates and their associated case reports [35]. However, cases of infection and disease caused by every known species of *Elizabethkingia* have been reported [12, 35] Furthermore, Lin et al., (2018) acknowledge that a handful of their clinical isolates were *E. bruuniana* but excluded these isolates from their experiments. Lin et al., (2018) identified 44 isolates of *E. anophelis* and *meningoseptica* resistant to levofloxacin and sequenced the genes encoding GyrA, GyrB, ParC, and ParE. They found mutations in the Ser83 position of GyrA (Ser83Ile and Ser83Arg), Ser95 (Ser95Pro), Lys102 (Lys102Gln); and in GyrB at positions 425 (Ile425Lys), 452 (Arg452Ser), and 470 (Glu470Asp). No mutations in topoisomerase IV were observed in the fluoroquinolone-resistant strains examined [35].

In this study, I sought to characterize ciprofloxacin-resistant mutant strains of the Miricola group. In particular, I sought to characterize the effects that fluoroquinolone resistance mutations had on the fitness and antimicrobial resistance profiles of resistant strains. I also

attempted to find whether the mutations conferring fluoroquinolone resistance were similar to those in *E. anophelis* and *E. meningoseptica.* It has been demonstrated in other species that the mutations typically responsible for fluoroquinolone resistance also negatively affect bacterial growth and fitness [36]. I therefore sought to determine these effects. I hypothesized that a common mechanism of ciprofloxacin resistance, mutations in one or more of the genes encoding the GyrAB and ParCE proteins, would be the associated with resistance in the Miricola group, as is the case in *E. anophelis* and *E. meningoseptica* [35]. Furthermore, I hypothesized that the mutations causing ciprofloxacin resistance would have a negative impact on the fitness of each strain.

MATERIALS AND METHODS

Mutant Isolation

Four bacterial strains were used in this study: *E. ursingii* G4122^T [37], *E. miricola* G4071^T [38], *E. bruuniana* ATCC33958^T [39], and *E. occulta* G4070^T [38].

Each strain was revived from freezer stock in 20% glycerol at -80°C on heart infusion agar (HIA) (Remel, San Diego, CA, USA) supplemented with defibrinated rabbit blood (Hemostat Laboratories, Dixon, CA, USA) and grown overnight at 37°C. Colonies were taken from this plate and incubated overnight at 37°C with shaking in 3 mL Mueller-Hinton Broth (MHB) (Beckton Dickinson and Company, Cockeysville, MD, USA).

To isolate ciprofloxacin-resistant mutants, culture plates were prepared with Mueller-Hinton Broth powder, granulated agar powder (Beckton Dickinson and Company, Cockeysville, MD, USA), and ciprofloxacin HCl (Sigma Aldrich, St. Louis, MO, USA) to create a variety of ciprofloxacin concentrations, ranging from 0.25 mg/L to 2 mg/L (final concentration). This was necessary in case inoculated plates did not produce any colonies after inoculation. The plates were inoculated using a spread-plating technique from the overnight MHB culture tubes and incubated for up to 48 h at 37°C. Any colonies which did grow on ciprofloxacin-containing media were suspected to be mutants. Suspected mutants were arbitrarily named "[Parent strain]-CRS#," e.g. G4122-CRS5. After naming, the mutant colonies were streaked onto drug-free MHA plates and incubated at 37°C for 24-48 h before re-streaking on fresh media. This was repeated three times for three total drug-free plates per suspected ciprofloxacin-resistant mutant strain ("passages").

Minimum growth-inhibitory concentration determination

Mutant strains were tested for phenotypic confirmation of mutation by broth microdilution MIC assays to CLSA standards. Five mg/mL ciprofloxacin HCl was added to MHB to a concentration of 64 mg/L. MHB was placed in the wells of a 96 well plate. and the antibiotic was serially diluted across the plate using two-fold dilutions, so that ciprofloxacin concentrations tested ranged from 32 mg/L (2^5) to 0.0625 mg/L (2^{-4}), with a positive control column (left-most column) containing no drug, and a negative control row containing no bacteria. One hundred μL of overnight culture in MHB at $OD_{600nm} = 0.010 \pm 0.002$ was added to each well, excluding the bacteria-free negative control rows. The plates were incubated at 37°C for 24-48 h and examined visually to determine MIC.

Synergy (checkerboard) assays

Mutant strains of *E. bruuniana* were tested using a checkerboard assay to determine whether a synergistic interaction between ciprofloxacin and vancomycin was observed in the mutant strains. Plates were set up similar to the MIC assays above, and vancomycin (Sigma Aldrich, St. Louis, MO, USA) (starting concentration 128 mg/L) was then serially diluted vertically down the plate, forming a final concentration range from 32 mg/L to 0.5 mg/L, with the bottom row containing no vancomycin. Each well was then inoculated with 100 μL culture as described above and incubated at 37°C for 24-48 h and examined visually to determine MIC and FIC. The fractional inhibitory concentration index (FICI) was calculated as the sum of the ratios of each drug's FIC to its MIC, and standard conventions for breakpoints were followed (FICI < 0.5 synergistic, 0.5 < FICI < 1.0 additive, 1.0 < FICI < 2.0 indifferent, FICI > 2.0 antagonistic).

Growth Curves

To determine the growth characteristics of verified ciprofloxacin-resistant mutants, overnight cultures of the mutant strains were grown in MHB at 37℃ with shaking, alongside the parent strains as a control. The cultures were diluted to OD_{600nm} = 0.010 ± 0.002 and 25 mL of culture were transferred to sterile 50 mL beveled Erlenmeyer flasks. The cultures were then grown at 37℃ with shaking at 200 RPM. OD_{600nm} readings were taken hourly for eight hours, then at 16 h and 24 h. At the end of the 24 h period, the cultures were Gram stained to check for contamination and the bacteria were again challenged with ciprofloxacin as necessary. Because ciprofloxacin resistance is typically associated with decreased fitness, any strain which exhibited growth equivalent to or more vigorous than the parent strain in a 24 h growth curve was again challenged with ciprofloxacin. Ten μL of culture was withdrawn from the 50 mL growth flask and added to 3 mL MHB containing a concentration of ciprofloxacin equivalent to the mean of the parent and mutant's MICs. For example, G4122-CRS1 exhibited elevated growth and an MIC of 4 mg/L compared to G4122-P, which exhibited an MIC of 0.5 mg/L. If bacteria grew, the strain retained its resistance, and if there was no growth after 48 h, the strain was assumed to have reverted to the parent phenotype.

Primer Design, Genomic DNA extractions, and PCR

Publicly available FASTA files containing the whole genome sequences of parent strains ATCC33958^T[40], G4122^T [38], G4070^T [41], and G4071^T [38] were downloaded and annotated using the RAST database [42-44]. The sequences of the genes for the A and B subunits of gyrase (GyrA and GyrB), and the C and E subunits of topoisomerase IV (ParC and ParE) were downloaded along with 500-750 base pairs on either side of the gene.

Sequencing primers for the QRDR regions were designed using NCBI's PrimerBlast tool and optimized for melting temperature. Sequencing primers were designed to encompass all known *Elizabethkingia* QRDR mutations reported by Lin et al [35] with ample margin on each side of the genome to account for inaccuracies inherent to Sanger sequencing. The primer sequences and their T_m values are listed in appendix B. Primers were received from Integrated DNA Technologies (Coralville, IA, USA) in lyophilized tubes and reconstituted to 100μM before dilution to 10μM with ultrapure H_2O.

Chromosomal DNA of the parent strains were extracted using QIAGEN Genomic-tips (QIAGEN, Hilden, DE) according to manufacturer's protocol and nanodropped. Genomes of the mutant strains were prepared via phenol:chloroform:isoamyl alcohol extraction [45] and dissolved in ultra-pure water. Samples of genomic DNA were assessed for purity and yield using a nanodrop spectrophotometer.

PCR reactions were prepared so that each 200 μL tube contained 25 μL of One*Taq* Hot Start Quick-Load 2x Master Mix with GC Buffer (New England Biosciences, Boston, MA, USA), 23 μL ultra-pure water, 1μL each of forward and reverse primers, diluted to 10 μM in Ultra-pure water, and 1 μL of genomic DNA. PCR protocols were developed to be specific to the T_m of each primer. PCR protocols used are listed in appendix D.

Following amplification, the contents of the reaction tubes were electrophoresed through agarose gel. All gels used were 100 mL, 1% agarose with 1.5 μL of 10 mg/mL ethidium bromide (final concentration 0.15 mg/L), and all electrophoresis was carried out at constant 120V for 20 minutes. Gel images were captured with a Bio-Rad Gel Doc XR+ imager and Image Lab 4.1 software. Gel bands were excised using a razor blade and DNA was purified using New England Biosciences Gel Purification Kit #T1020 per manufacturer's protocol, and DNA was eluted in

ultra-pure water and stored at 4℃. Samples of the purified amplicons were assessed for purity and yield using a nanodrop spectrophotometer. In some cases, the PCR product was merely sampled for successful amplification and the remainder was purified using New England Biosciences PCR Cleanup Kit #T1030 per manufacturer's protocol to prevent product loss.

Sequencing primers were diluted to 5 μM and purified DNA was submitted at no less than 5 ng/μL for sequencing, which was carried out using an ABI sequencer by the staff of the OSU CORE Laboratory.

RESULTS

Mutation frequency, MICs, and synergy

Colonies appeared on drug-containing plates at a rate of 10^{-8} for all four strains (Table 1). Analysis of ciprofloxacin MICs revealed that in all cases, the suspected fluoroquinolone-resistant mutants appeared to have increased fluoroquinolone resistance compared to respective parent strains (Table 1). In all cases, checkerboard assay results determined that for all *E. bruuniana* mutants, the drug combination of vancomycin and ciprofloxacin had an indifferent effect, meaning that the combination performed no better than each drug individually (Table 2).

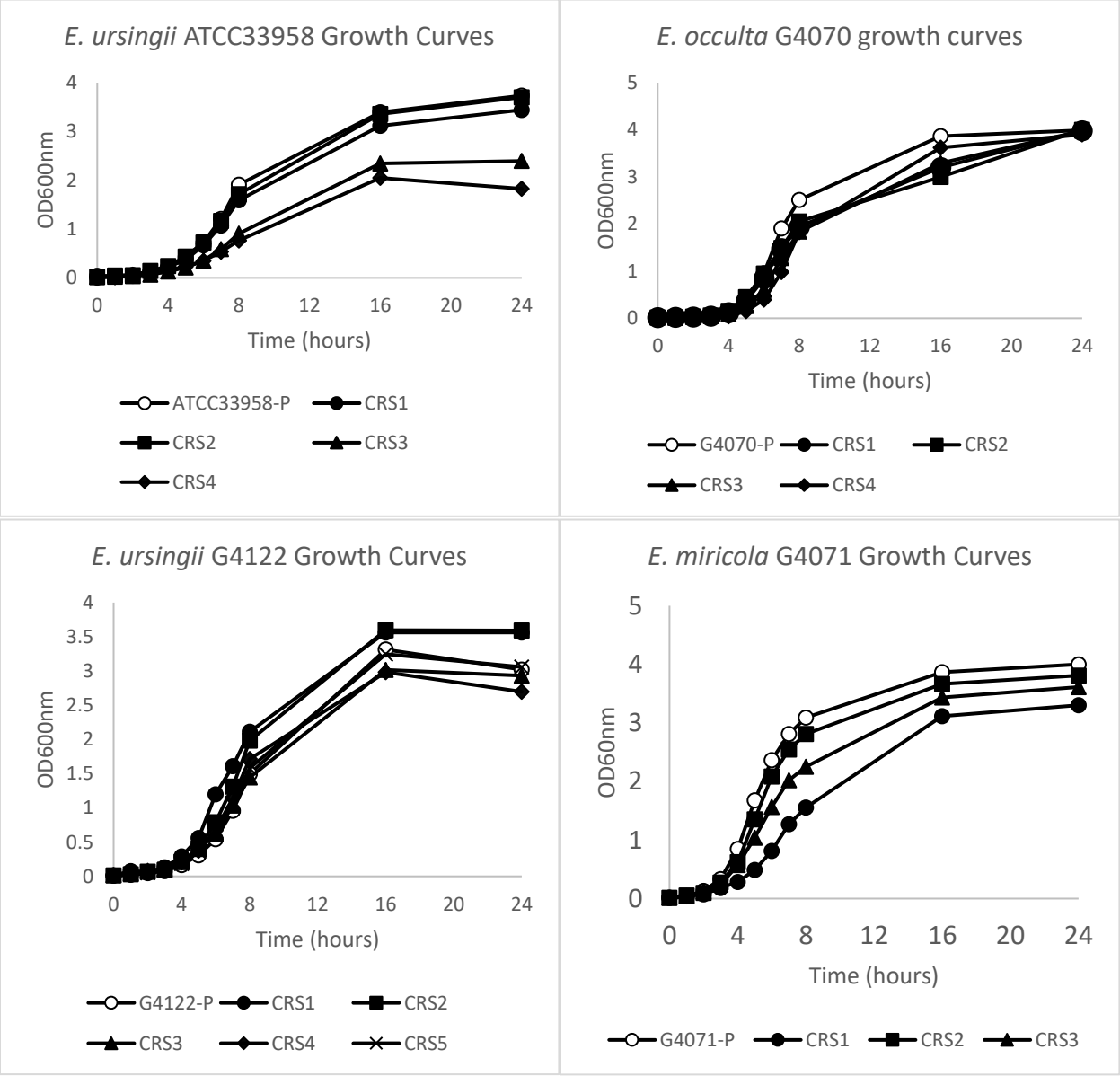
Table 1: Selection Concentration, Mutation Frequency, and Ciprofloxacin MICs

Isolate	Species	Ciprofloxacin resistance selection concentration, mg/L	Mutation Frequency	(MIC), mg/L
G4122-P	*ursingii*	1.5	3.13×10^{-8}	0.5
G4122-CRS1	*ursingii*			4
G4122-CRS2	*ursingii*			2
G4122-CRS3	*ursingii*			8
G4122-CRS4	*ursingii*			4
G4122-CRS5	*ursingii*			1
G4071-P	*miricola*	2	9.04×10^{-8}	1
G4071-CRS1	*miricola*			4
G4071-CRS2	*miricola*			8
G4071-CRS3	*miricola*			8
ATCC33958-P	*bruuniana*	1	9.38×10^{-8}	0.5
ATCC33958-CRS1	*bruuniana*			2
ATCC33958-CRS2	*bruuniana*			2
ATCC33958-CRS3	*bruuniana*			8
ATCC33958-CRS4	*bruuniana*			8
G4070-P	*occulta*	1.5	4.77×10^{-8}	0.25
G4070-CRS1	*occulta*			16
G4070-CRS2	*occulta*			2
G4070-CRS3	*occulta*			4
G4070-CRS4	*occulta*			4

Table 2: Synergy Assays with ciprofloxacin and vancomycin (n=3)

	Ciprofloxacin MIC	Ciprofloxacin FIC	Vancomycin MIC	Vancomycin FIC	FICI	Interpretation
ATCC-P	1.25	0.75	12	2	1.3125	Indifferent
ATCC-CRS1	9.3	3.3	18.7	6.7	1.083	Indifferent
ATCC-CRS2	9.3	6.7	13.3	4	1.167	Indifferent
ATCC-CRS3	5.7	5.7	10.7	5.3	1.5	Indifferent
ATCC-CRS4	9.3	6.7	10.7	8	1.667	Indifferent

Growth Curves



Standard growth curves showed that, in general, mutant strains grew slower than their parent strains. However, some strains (*E. ursingii* G4122-CRS1 and G4122-CRS2, *E. ursingii* ATCC33958-CRS2) grew as well as or better than the parent. G4122-CRS1 and G4122-CRS2 were re-challenged with ciprofloxacin as discussed earlier and retained their ciprofloxacin resistance.

QRDR translated sequences

Sequences of the sense and antisense strands of the QRDRs of *gyrA, gyrB, parC,* and *parE* revealed no mutations in the QRDRs when compared to known wild-type sequences. In all GyrA forward sequences, at least two substitutions are visible at the beginning of the sequence, but comparing the forward and reverse sequences revealed nucleotide mismatches in these positions of the forward strand. The protein sequences (translated from the raw sequences) of all 16 genes can be found in Appendix D.

DISCUSSION

Like other species of *Elizabethkingia*, the Miricola group readily acquires resistance to ciprofloxacin with mutation frequencies of 10^{-8} mutants (Table 1) and shows elevated MICs (Table 1) well beyond the maximum serum concentration of a maximum oral dose of ciprofloxacin HCl [28], making ciprofloxacin ineffective as a therapeutic agent in cases of infection with organisms expressing similar levels of ciprofloxacin resistance. Synergy assays with ciprofloxacin and vancomycin in fluoroquinolone-resistant *E. bruuniana* ATCC33958 (Table 2) indicate an indifferent relationship between the two drugs and suggest that the two drugs are eliminated from the cell by the same system, which functions comparably in the parent and fluoroquinolone-resistant strains as shown by their comparable vancomycin MICs.

Examination of the growth of fluoroquinolone-resistant mutants revealed that growth was often compromised, but not consistently within or across species. *E. occulta* G4070 fluoroquinolone-resistant strains demonstrated slower growth than the parent by 8 and 16 h, but by 24 h had caught up to the parent. This is not the case for *E. ursingii* G4122, where three of the five strains show completely compromised growth and see their OD_{600nm} decline from 16 to 24 h. Curiously, G4122-CRS1 and -CRS2 both out-grew the parent, indicating that some sort of compensatory mutation might have been acquired which negates the effect on growth that the mutations causing ciprofloxacin resistance might have caused. The growth curves of *E. bruuniana* ATCC33958 are also unique, as they indicate that ATCC33958-CRS3 and ATCC33958-CRS4 show significantly compromised growth when compared to the parent and ATCC33958-CRS1 and ATCC33958-CRS2. *E. miricola* G4071 shows the most consistent growth of the four species tested, but the range of values between the four strains suggests that none of the three fluoroquinolone-resistant strains has the same set of mutationsWhile compromised growth is consistent with gyrase or topoisomerase IV mutations, the causal

relationship is not elucidated by these data. In fact, the ability of some strains to keep pace with or exceed the parent strain's growth would indicate that whatever mutations confer fluoroquinolone-resistance are not in the QRDRs of gyrase or topoisomerase IV. In fact, it is reasonable to assume that in all *Elizabethkingia* strains tested, the variety of growth profiles could be due to a variety of mutations. Furthermore, the difference in growth being based on the nature of the mutation suggests that various mutations have different energetic costs to the cell and thus compromise growth by requiring excess energy which could be used for replication.

However, the growth curves may be flawed for a number of reasons. First, measurements of OD_{600nm} were used as a stand-in for CFUs/mL. A more accurate way of determining CFUs/mL would have been to serially dilute samples from the flasks and inoculate the samples onto agar plates. Optical density measurements were used because they were simpler and more economical. However, optical density measurements assume that the culture's optimal absorbance remains at 600nm for the duration of the assay and assumes that no changes in phenotype (such as a stress phenotype, where the cells elongate into filaments) affect the absorbance. Furthermore, a spectrophotometer cannot tell between viable cells, apoptotic cells, or cell debris. Therefore, it is likely that CFUs/mL were likely lower than a related OD_{600nm} value would suggest. Second, no replicates of the growth curve experiments were performed due to time constraints. Replicates might have painted a clearer picture about the nature of the growth of all fluoroquinolone-resistant strains.

The validity of OD_{600nm} compared to CFUs/mL notwithstanding, it is clear that some form of mutation has occurred in the majority of fluoroquinolone-resistant strains which has imparted that drug resistance and simultaneously affected cell growth. One likely candidate for mutation would be the QRDRs of gyrase and topoisomerase IV. However, the complete absence

of mutations within the QRDR sequences of *E. bruuniana* rules this out, contrary to my hypothesis. If gyrase and topoisomerase are unaffected in fluoroquinolone-resistant mutants, then ciprofloxacin likely still has its standard effect on the enzymes: the drug chelates to the 83Ser residue, freezing the enzyme in place on the DNA strand. However, the increase in MIC for all strains would suggest that resistance is not due to mutations in the target genes.

An alternative mechanism for fluoroquinolone resistance is based on drug influx and efflux. Fluoroquinolones enter Gram-negative cells through porin proteins via diffusion and may be forced back out by active transport using one or more multi-drug efflux pumps [32]. *Elizabethkingia* are thought to have several classes of drug efflux pumps based on putative RAST annotation, but this has not been confirmed in vitro [33]. Regulation of the expression of such proteins could explain the increased drug resistance. One final mechanism, though far-fetched, may be possible: chemical modification of ciprofloxacin. Limited evidence suggests that fluoroquinolone-degrading enzymes exist in nature[46]. *In silico* modeling of many bacterial proteins has suggested that mutations or modifications in some oxidoreductases and decarboxylases would cause pyrolysis or decarboxylation of fluoroquinolones, a topic of interest in sewage treatment as fluoroquinolone metabolites form micropollutants in sewage[47]. It is possible that *Elizabethkingia* chemically modifies ciprofloxacin to reduce toxicity using a nonspecific oxidoreductase or decarboxylation pathway, but further testing is needed to determine if this is the case.

Future directions of this study should be divided into four phases. First, the QRDRs of *E. occulta, E ursingii,* and *E. miricola* should be sequenced to find gyrase and/or topoisomerase IV mutations. It was my intent to do this work for *E. ursingii* due to its unusual growth curves, but due to time constraints and factors beyond my control I was unable to complete this task.

Second, quantitative RT-PCR could be used to determine how *Elizabethkingia* upregulates and downregulates porins and efflux pumps under ciprofloxacin challenge. Third, sequencing the full lengths of *gyrAB* and *parCE*, and whole genome sequencing could be used to identify mutations outside the QRDRs which may contribute to ciprofloxacin resistance. Finally, an investigation of any chemical modifications of ciprofloxacin by *Elizabethkingia* might be warranted.

Appendix A: Strains used

Isolate	Species	Source and Date	ciprofloxacin selection concentration (mg/L)	Reference
ATCC33958^T	*bruuniana*	Contaminated commercial enzyme preparation; California, USA; 1982	N/A	[39]
ATCC33958-CRS1	*bruuniana*	Selected from ATCC33958^T	1	This study
ATCC33958-CRS2	*bruuniana*	Selected from ATCC33958^T	1	This study
ATCC33958-CRS3	*bruuniana*	Selected from ATCC33958^T	1	This study
ATCC33958-CRS4	*bruuniana*	Selected from ATCC33958^T	1	This study
G4071^T	*miricola*	Tracheal exudate; Strasbourg, France; 1978	N/A	[38]
G4071-CRS1	*miricola*	Selected from G4071^T	2	This study
G4071-CRS2	*miricola*	Selected from G4071^T	2	This study
G4071-CRS3	*miricola*	Selected from G4071^T	2	This study
G4122^T	*ursingii*	Soil; Odense, Denmark; 1964	N/A	[37]
G4122-CRS1	*ursingii*	Selected from G4122^T	1.5	This study
G4122-CRS2	*ursingii*	Selected from G4122^T	1.5	This study
G4122-CRS3	*ursingii*	Selected from G4122^T	1.5	This study
G4122-CRS4	*ursingii*	Selected from G4122^T	1.5	This study
G4122-CRS5	*ursingii*	Selected from G4122^T	1.5	This study
G4070^T	*occulta*	Sputum; Melbourne, Australia; 1977	N/A	[38]
G4070-CRS1	*occulta*	Selected from G4070^T	1.5	This study
G4070-CRS2	*occulta*	Selected from G4070^T	1.5	This study
G4070-CRS3	*occulta*	Selected from G4070^T	1.5	This study
G4070-CRS4	*occulta*	Selected from G4070^T	1.5	This study

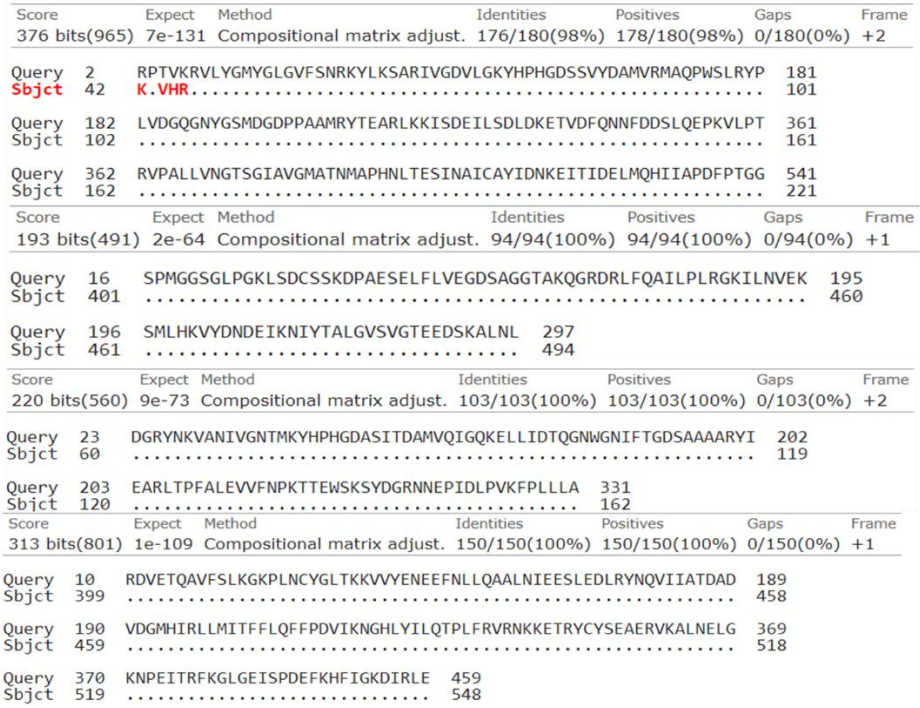
Appendix B: Primers
All amplification and sequencing reactions were performed using the same set of primers.

Primer	Residues	Start/stop bp	Sequence	Amplicon length	T_m
ATCC *GyrA* F	65-120	195-360	GAGCGTTACCGGACGTAAGA	570	60
ATCC *GyrA* R			TACCTCCGGTTGGGAAGTCT		60
ATCC *GyrB* F	420-470	1260-1410	GACAGGCAGCTAAGAAGGCT	322	60
ATCC *GyrB* R			AGGTTAAGTGCCTTGCTGTCT		60
ATCC *ParC* F	75-120	225-360	GACTGAAACCCGTACAGCGA	359	60
ATCC *ParC* R			TGCCAGAAGCAAAGGGAACT		60
ATCC *ParE* F	420-470	1260-1410	GAGGGAGATTCCGCATCAGG	486	60
ATCC *ParE* R			CAAGTCCCTTGAATCGCGTG		60

Appendix C: PCR protocol
All PCR reactions were performed under the following conditions:

1. Melt at 94℃ for 4:30
2. Melt at 94℃ for 0:30
3. Anneal at 55℃ for 0:30
4. Extend at 68℃ for 5:00
5. Repeat steps 2-4 30 times
6. Final extension at 68℃ for 5:00 (total of 10:00)
7. Cool to 4℃ and hold indefinitely

Appendix D: Sequences of GyrA, GyrB, ParC, ParE from *E. bruuniana* ATCC33958 fluoroquinolone-resistant strains



Score	Expect	Method	Identities	Positives	Gaps	Frame
376 bits(965)	7e-131	Compositional matrix adjust.	176/180(98%)	178/180(98%)	0/180(0%)	+2

```
Query  2    RPTVKRVLYGMYGLGVFSNRKYLKSARIVGDVLGKYHPHGDSSVYDAMVRMAQPWSLRYP  181
Sbjct  42   K.VHR.....................................................  101

Query  182  LVDGQGNYGSMDGDPPAAMRYTEARLKKISDEILSDLDKETVDFQNNFDDSLQEPKVLPT  361
Sbjct  102  ............................................................  161

Query  362  RVPALLVNGTSGIAVGMATNMAPHNLTESINAICAYIDNKEITIDELMQHIIAPDFPTGG  541
Sbjct  162  ............................................................  221
```

Score	Expect	Method	Identities	Positives	Gaps	Frame
193 bits(491)	2e-64	Compositional matrix adjust.	94/94(100%)	94/94(100%)	0/94(0%)	+1

```
Query  16   SPMGGSGLPGKLSDCSSKDPAESELFLVEGDSAGGTAKQGRDRLFQAILPLRGKILNVEK  195
Sbjct  401  ............................................................  460

Query  196  SMLHKVYDNDEIKNIYTALGVSVGTEEDSKALNL  297
Sbjct  461  .................................  494
```

Score	Expect	Method	Identities	Positives	Gaps	Frame
220 bits(560)	9e-73	Compositional matrix adjust.	103/103(100%)	103/103(100%)	0/103(0%)	+2

```
Query  23   DGRYNKVANIVGNTMKYHPHGDASITDAMVQIGQKELLIDTQGNWGNIFTGDSAAAARYI  202
Sbjct  60   ............................................................  119

Query  203  EARLTPFALEVVFNPKTTEWSKSYDGRNNEPIDLPVKFPLLLA  331
Sbjct  120  ........................................  162
```

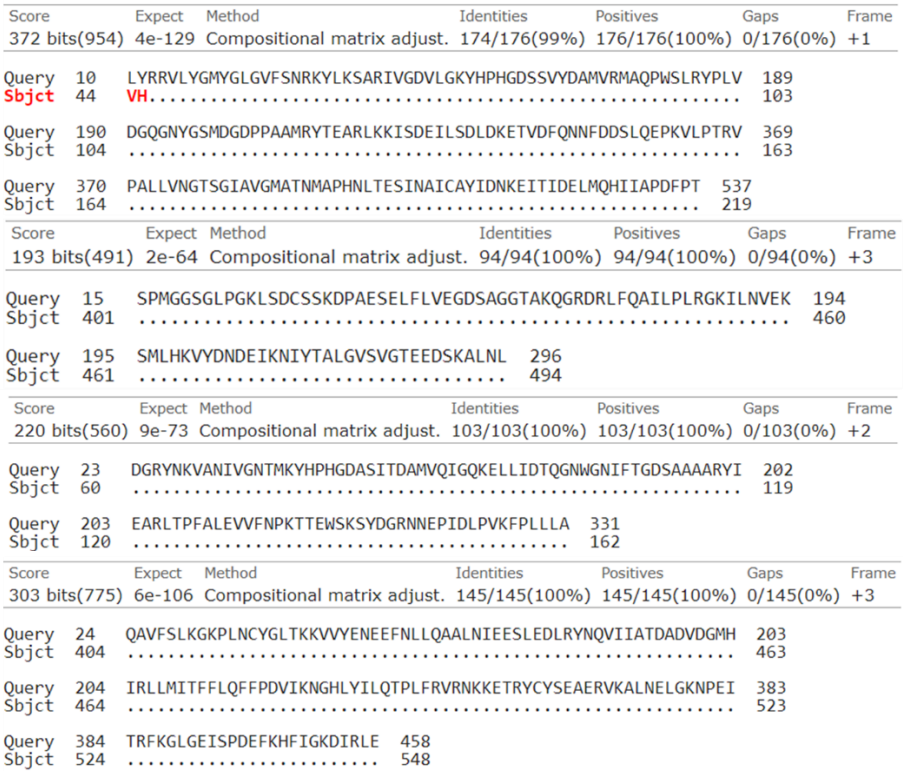
Score	Expect	Method	Identities	Positives	Gaps	Frame
313 bits(801)	1e-109	Compositional matrix adjust.	150/150(100%)	150/150(100%)	0/150(0%)	+1

```
Query  10   RDVETQAVFSLKGKPLNCYGLTKKVVYENEEFNLLQAALNIEESLEDLRYNQVIIATDAD  189
Sbjct  399  ............................................................  458

Query  190  VDGMHIRLLMITFFLQFFPDVIKNGHLYILQTPLFRVRNKKETRYCYSEAERVKALNELG  369
Sbjct  459  ............................................................  518

Query  370  KNPEITRFKGLGEISPDEFKHFIGKDIRLE  459
Sbjct  519  ..............................  548
```

E. bruuniana ATCC33958-CRS1 (from top to bottom) GyrA, GyrB, ParC, ParE



Score	Expect	Method	Identities	Positives	Gaps	Frame
372 bits(954)	4e-129	Compositional matrix adjust.	174/176(99%)	176/176(100%)	0/176(0%)	+1

```
Query  10   LYRRVLYGMYGLGVFSNRKYLKSARIVGDVLGKYHPHGDSSVYDAMVRMAQPWSLRYPLV  189
Sbjct  44   VH..........................................................  103

Query  190  DGQGNYGSMDGDPPAAMRYTEARLKKISDEILSDLDKETVDFQNNFDDSLQEPKVLPTRV  369
Sbjct  104  ............................................................  163

Query  370  PALLVNGTSGIAVGMATNMAPHNLTESINAICAYIDNKEITIDELMQHIIAPDFPT  537
Sbjct  164  ........................................................  219
```

Score	Expect	Method	Identities	Positives	Gaps	Frame
193 bits(491)	2e-64	Compositional matrix adjust.	94/94(100%)	94/94(100%)	0/94(0%)	+3

```
Query  15   SPMGGSGLPGKLSDCSSKDPAESELFLVEGDSAGGTAKQGRDRLFQAILPLRGKILNVEK  194
Sbjct  401  ............................................................  460

Query  195  SMLHKVYDNDEIKNIYTALGVSVGTEEDSKALNL  296
Sbjct  461  .................................  494
```

Score	Expect	Method	Identities	Positives	Gaps	Frame
220 bits(560)	9e-73	Compositional matrix adjust.	103/103(100%)	103/103(100%)	0/103(0%)	+2

```
Query  23   DGRYNKVANIVGNTMKYHPHGDASITDAMVQIGQKELLIDTQGNWGNIFTGDSAAAARYI  202
Sbjct  60   ............................................................  119

Query  203  EARLTPFALEVVFNPKTTEWSKSYDGRNNEPIDLPVKFPLLLA  331
Sbjct  120  ........................................  162
```

Score	Expect	Method	Identities	Positives	Gaps	Frame
303 bits(775)	6e-106	Compositional matrix adjust.	145/145(100%)	145/145(100%)	0/145(0%)	+3

```
Query  24   QAVFSLKGKPLNCYGLTKKVVYENEEFNLLQAALNIEESLEDLRYNQVIIATDADVDGMH  203
Sbjct  404  ............................................................  463

Query  204  IRLLMITFFLQFFPDVIKNGHLYILQTPLFRVRNKKETRYCYSEAERVKALNELGKNPEI  383
Sbjct  464  ............................................................  523

Query  384  TRFKGLGEISPDEFKHFIGKDIRLE  458
Sbjct  524  ........................  548
```

E. bruuniana ATCC33958-CRS2 (from top to bottom) GyrA, GyrB, ParC, ParE

Score	Expect	Method	Identities	Positives	Gaps	Frame
372 bits(954)	3e-129	Compositional matrix adjust.	174/176(99%)	176/176(100%)	0/176(0%)	+3

```
Query  9    LYRRVLYGMYGLGVFSNRKYLKSARIVGDVLGKYHPHGDSSVYDAMVRMAQPWSLRYPLV  188
Sbjct  44   VH.....................................................  103

Query  189  DGQGNYGSMDGDPPAAMRYTEARLKKISDEILSDLDKETVDFQNNFDDSLQEPKVLPTRV  368
Sbjct  104  ............................................................  163

Query  369  PALLVNGTSGIAVGMATNMAPHNLTESINAICAYIDNKEITIDELMQHIIAPDFPT  536
Sbjct  164  ..........................................................  219
```

Score	Expect	Method	Identities	Positives	Gaps	Frame
193 bits(491)	2e-64	Compositional matrix adjust.	94/94(100%)	94/94(100%)	0/94(0%)	+3

```
Query  15   SPMGGSGLPGKLSDCSSKDPAESELFLVEGDSAGGTAKQGRDRLFQAILPLRGKILNVEK  194
Sbjct  401  ............................................................  460

Query  195  SMLHKVYDNDEIKNIYTALGVSVGTEEDSKALNL  296
Sbjct  461  .................................  494
```

Score	Expect	Method	Identities	Positives	Gaps	Frame
220 bits(560)	8e-73	Compositional matrix adjust.	103/103(100%)	103/103(100%)	0/103(0%)	+3

```
Query  21   DGRYNKVANIVGNTMKYHPHGDASITDAMVQIGQKELLIDTQGNWGNIFTGDSAAAARYI  200
Sbjct  60   ............................................................  119

Query  201  EARLTPFALEVVFNPKTTEWSKSYDGRNNEPIDLPVKFPLLLA  329
Sbjct  120  ........................................  162
```

Score	Expect	Method	Identities	Positives	Gaps	Frame
312 bits(799)	2e-109	Compositional matrix adjust.	150/152(99%)	151/152(99%)	0/152(0%)	+2

```
Query  5    KAGDVETQAVFSLKGKPLNCYGLTKKVVYENEEFNLLQAALNIEESLEDLRYNQVIIATD  184
Sbjct  397  .SR.....................................................  456

Query  185  ADVDGMHIRLLMITFFLQFFPDVIKNGHLYILQTPLFRVRNKKETRYCYSEAERVKALNE  364
Sbjct  457  ............................................................  516

Query  365  LGKNPEITRFKGLGEISPDEFKHFIGKDIRLE  460
Sbjct  517  ................................  548
```

E. bruuniana ATCC33958-CRS3 (from top to bottom) GyrA, GyrB, ParC, ParE

Score	Expect	Method	Identities	Positives	Gaps	Frame
372 bits(954)	3e-129	Compositional matrix adjust.	174/176(99%)	176/176(100%)	0/176(0%)	+2

```
Query  8    LYRRVLYGMYGLGVFSNRKYLKSARIVGDVLGKYHPHGDSSVYDAMVRMAQPWSLRYPLV  187
Sbjct  44   VH.....................................................  103

Query  188  DGQGNYGSMDGDPPAAMRYTEARLKKISDEILSDLDKETVDFQNNFDDSLQEPKVLPTRV  367
Sbjct  104  ............................................................  163

Query  368  PALLVNGTSGIAVGMATNMAPHNLTESINAICAYIDNKEITIDELMQHIIAPDFPT  535
Sbjct  164  ..........................................................  219
```

Score	Expect	Method	Identities	Positives	Gaps	Frame
193 bits(491)	2e-64	Compositional matrix adjust.	94/94(100%)	94/94(100%)	0/94(0%)	+3

```
Query  15   SPMGGSGLPGKLSDCSSKDPAESELFLVEGDSAGGTAKQGRDRLFQAILPLRGKILNVEK  194
Sbjct  401  ............................................................  460

Query  195  SMLHKVYDNDEIKNIYTALGVSVGTEEDSKALNL  296
Sbjct  461  .................................  494
```

Score	Expect	Method	Identities	Positives	Gaps	Frame
220 bits(560)	9e-73	Compositional matrix adjust.	103/103(100%)	103/103(100%)	0/103(0%)	+1

```
Query  22   DGRYNKVANIVGNTMKYHPHGDASITDAMVQIGQKELLIDTQGNWGNIFTGDSAAAARYI  201
Sbjct  60   ............................................................  119

Query  202  EARLTPFALEVVFNPKTTEWSKSYDGRNNEPIDLPVKFPLLLA  330
Sbjct  120  ........................................  162
```

Score	Expect	Method	Identities	Positives	Gaps	Frame
303 bits(775)	7e-106	Compositional matrix adjust.	145/145(100%)	145/145(100%)	0/145(0%)	+1

```
Query  25   QAVFSLKGKPLNCYGLTKKVVYENEEFNLLQAALNIEESLEDLRYNQVIIATDADVDGMH  204
Sbjct  404  ............................................................  463

Query  205  IRLLMITFFLQFFPDVIKNGHLYILQTPLFRVRNKKETRYCYSEAERVKALNELGKNPEI  384
Sbjct  464  ............................................................  523

Query  385  TRFKGLGEISPDEFKHFIGKDIRLE  459
Sbjct  524  .........................  548
```

E. bruuniana ATCC33958-CRS4 (from top to bottom) GyrA, GyrB, ParC, ParE

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