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Evaluation of the electron transfer flavoprotein (ETF) as an antibacterial target in *Burkholderia cenocepacia*.

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Abstract

There are hundreds of essential genes in multidrug resistant bacterial genomes, but only a few of their products are exploited as antibacterial targets. An example is the electron flavoprotein (ETF) which is required for growth and viability in *Burkholderia cenocepacia*. Here, we evaluated ETF as an antibiotic target for *Burkholderia cepacia* complex (Bcc). Depletion of the bacterial ETF during infection of *Caenorhabditis elegans* significantly extended survival of the nematodes, proving that ETF is essential for survival of *B. cenocepacia* in this host model. In spite of the arrest in respiration in ETF mutants, the inhibition of *etf* expression did not increase the formation of persister cells, when treated with high doses of ciprofloxacin or meropenem. To test if *etf* translation could be inhibited by RNA interference, antisense oligonucleotides that target the *etfBA* operon were synthesized. One antisense oligonucleotide was effective in inhibiting *etfB* translation *in vitro* but not *in vivo*, highlighting the challenge of reduced membrane permeability for the design of drugs against *B. cenocepacia*.

This work contributes to the validation of ETF of *B. cenocepacia* as a target for antibacterial therapy and demonstrates the utility of a *C. elegans* liquid killing assay to validate gene essentiality in an *in vivo* infection model.

Keywords

Burkholderia cenocepacia; cystic fibrosis; electron transfer flavoprotein (ETF); essential genes; antibacterial targets; persister cells; antisense oligonucleotides; *C. elegans*

Burkholderia cepacia complex (Bcc) is a group of Gram-negative bacteria that causes a substantial detriment in pulmonary function in patients with the genetic disease cystic fibrosis (CF) (Mahenthiralingam et al. 2005). In 20% of the cases, Bcc infections result in sepsis and death. Further, Bcc bacteria can survive in the presence of disinfectants (Kim et

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al. 2015, Ahn et al. 2016) causing occasional outbreaks in hospitals worldwide (Lee et al. 2013). Unfortunately, there are very limited options to treat Bcc infections as these bacteria are intrinsically resistant to most antibiotics (Avgeri et al. 2009). Thus, novel antibiotics are needed to treat Bcc infections.

The targets of effective antibiotics are typically the products of ‘essential genes’, which are those coding for components required for bacteria to grow in rich medium (Arigoni et al. 1998, Akerley et al. 2002). Bacterial genomes encode hundreds of essential genes; however, only a few of those gene products are currently used as targets of antibiotics (Fields et al. 2016). Among the challenges for validating such essential products as novel antibiotic targets is the possibility that essential genes may code for functions that are essential in laboratory conditions, which differ from the host environment (Brinster et al. 2009). Indeed, certain metabolic pathways have been ruled out as drug targets due to the ability of bacteria to bypass the essential requirement by utilizing bioproducts from the host (Brinster et al. 2009). Another challenge for validating an essential product as an antibacterial target is whether targeting an essential process can increase persister cell formation (Lewis 2007, Kwan et al. 2013). Persistence is a phenotypic state of dormancy experienced by up to 1% of the bacterial population that allows surviving under high concentrations of antibiotic (Lewis 2007). Persistence has been strongly linked to the recalcitrant CF infections and failure of treatment (Mulcahy et al. 2010). Previous studies have demonstrated that a decrease in ATP intracellular levels leads to an increase in persister cell formation in *Staphylococcus aureus* (Conlon et al. 2016). On the other hand, loss of respiration in *Escherichia. coli* showed a ~1000 fold reduction in the number of persisters in stationary phase (Orman and Brynildsen 2015).

To search for potential antibacterial targets, we previously built a conditional growth mutant library of the clinically relevant strain *B. cenocepacia* K56–2 (Table 1), in which essential genes were placed under the control of a rhamnose-inducible promoter (Bloodworth et al. 2013). In one of these mutants, rhamnose-dependent downregulation of an electron transfer flavoprotein (ETF), coded for by *etfBA* caused the strongest defect in cell viability and abolition of aerobic respiration (Bloodworth et al. 2015). Analysis of the mechanisms that resulted in loss of cell viability showed a pleiotropic effect that included abolition of aerobic respiration and changes in cell morphology. The essentiality of the *B. cenocepacia* ETF was surprising as ETF has a non-essential role as an electron acceptor for dehydrogenases involved in fatty acid degradation in other organisms (Matsuoka et al. 2007, Fujita et al. 2007). Identification of essential genes in other bacteria also show that ETF is essential in relevant human pathogens such as *Acinetobacter baylyi* (Berardinis et al. 2008), *Mycobacterium tuberculosis* (Griffin et al. 2011), *Porphyromonas gingivalis* (Klein et al. 2012), *Burkholderia pseudomallei* (Moule et al. 2014), and *Pseudomonas aeruginosa* (Lee et al. 2015). These findings prompted us to investigate whether the essential ETF could be considered as an antibacterial target. Although the reasons for ETF essentiality are unknown in Bcc, many ETFs are components of the fatty acid metabolism pathway (Fujita et al. 2007). Therefore, it is possible that fatty acids produced by the host could bypass the bacterial requirements for survival under ETF depletion. In addition, because of the known function of ETF as an electron transporter, ATP depletion caused by downregulation of ETF

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could cause an increase in the formation of persister cells, as observed in *S. aureus* (Conlon et al. 2016). Both aspects would rule out ETF as a promising antibacterial target.

In this work, we used the *Caenorhabditis elegans* host infection model (Desalermos et al. 2011, Kim et al. 2017), to determine that worms infected with an ETF knockdown mutant overcome the infection when ETF is downregulated. We also demonstrate that ETF depletion, which causes lack of aerobic respiration, does not increase the formation of persister cells. In an attempt to look for venues to inhibit expression of *etfBA* in wild type cells as a mean of treatment, we designed antisense oligonucleotides that effectively interfered with expression *in vitro*.

We first hypothesized that a *C. elegans* liquid killing assay (Selin et al. 2015), where infected worms are suspended in liquid medium with or without the addition of rhamnose would allow us to modulate the bacterial ETF expression inside the host. If this was the case, ETF depletion should cause a decrease in bacterial intestinal growth increasing the survival of the worms. Briefly, L4 worms grown on *E. coli* OP50 lawns were transferred to plates containing lawns of *B. cenocepacia* K56–2 WT or the *B. cenocepacia* knockdown mutant of ETF (CG*etf*) (Table 1) grown in nematode growth medium (NGM) or NGM supplemented with trimethoprim 100 μg/mL and 0.2% rhamnose, respectively. The plates were incubated at 25°C for 16 hours, to allow bacterial intestinal colonization (Law et al. 2008). Worms were then washed from the plate with M9 buffer, and resuspended in liquid killing medium (80% M9 and 20% NGMII). Twenty-30 worms per well, using 4 wells per condition, were spotted in a 96-well plate containing liquid killing medium with or without addition of rhamnose. We then tracked worm survival during 7 days by visually counting dead and live worms for each well and the average was calculated for each condition. Fig. 1 illustrates a representative survival curve of three biological replicates. When the worms were kept in liquid medium supplemented with 0.2% rhamnose (Fig. 1A) *C. elegans* infected with either WT or *CGetf* bacterial strains showed similar survival rates, less than 50% individuals survived after four days. This result indicates that rhamnose reached the nematode’s gut and induced ETF expression, allowing bacterial proliferation in the gut. Infection in these conditions was comparable to that of the wild type strain. Conversely, in the absence of rhamnose, the individuals infected with *CGetf* recovered from the infection with a survival rate close to 100% (Fig. 1B). These results suggest that *B. cenocepacia CGetf* cells are not able to bypass the ETF depletion in the *C. elegans* host model by metabolizing the contents of the intestinal lumen or components of the intestinal cells.

To determine the effect of ETF inhibition on persister cell formation, we quantified the cell viability of the *CGetf* mutant after treatment with 50X the minimal inhibitory concentration (MIC) of ciprofloxacin or meropenem. Briefly, *B. cenocepacia* WT and *CGetf* were cultured in LB broth in the presence of 0.2% rhamnose at 37°C for 16 hours. Cells were collected and washed, inoculated to an OD_{600} of 0.05 in LB broth with or without addition of 0.2% rhamnose, and incubated in a shaker at 37°C until late exponential phase. The cells were then standardized to an OD_{600} of 0.7 in the same media with the addition of 100 μg/mL ciprofloxacin or 800 μg/mL meropenem. 100 μl aliquots were removed at time 0 (before treatment), 3, 4, 5, 20 and 24 hours’ post-treatment, washed with fresh LB to remove residual antibiotic, and plated onto LB agar plates (WT) or LB agar plates containing 100

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μg/mL trimethoprim and 0.2% rhamnose *(CGetf)*. Plates were incubated at 37°C for estimation of colony forming units. Fig. 2 shows that after three hours of treatment with ciprofloxacin (Fig. 2A) or meropenem (Fig. 2B), the viability of the WT strain dropped to approximately 0.01%, which is typical of the formation of a persister population. Cells depleted of ETF showed the same killing profile as the WT strain under ciprofloxacin treatment with a survival lower than 0.0001% after 24 h of treatment. Meropenem treatment seemed to have a delayed killing effect in CG *etf* cells, reaching the WT killing rate after 24 h post treatment. This effect was not due to ETF depletion because the delayed killing was also observed in ETF-replete cells (CG*etf* plus rhamnose). While the reasons for the delayed killing of the *CGetf* cells in the presence of meropenem are unknown in both cases, we were able to demonstrate that the inhibition of ETF does not increase the formation of persister cells, despite its effect on aerobic respiration.

A limitation of the use of ETF as a drug target is that the human mitochondrial electron transport chain contains an ETF homolog (Roberts et al. 1996). Inhibition of human ETF could cause metabolic disorders (Frerman and Goodman 2013), which emphasizes the need of selective inhibition of the bacterial ETF. Antisense therapy is a powerful tool for development of new drugs that inhibit the translation of a target mRNA (Kole et al. 2012). The main advantage of this technology is target specificity, which minimizes side effects on commensal bacteria and host (Sully and Geller 2016). Antisense oligonucleotides can inhibit gene expression through different mechanisms such as steric hindrance, and RNase H- or RNase P-induced degradation of the mRNA (Rasmussen et al. 2007). In this case, the antisense sequence was designed to target the initiation of translation region by steric hindrance. To test the potential of ETF as a target for antisense inhibition, we synthesized four antisense oligodeoxynucleotides (ODNs) complementary to the 5'end of the *etfB* coding gene (See ODN sequences in Fig. 3A) and evaluated the effect of these ODNs on *etfBA* expression in an *in vitro* expression system (Lopez et al. 2015).

In vitro translation of *etfB* was carried out by adding a 941 bp PCR fragment including ribosome binding site (RBS) and coding sequence of the *etfB* gene to the *E. coli* S30 Cell Free Extract System for Circular DNA kit (Promega). The amino acid mixture (minus methionine) plus 10 μCi of radiolabeled [^{35}S] methionine (Perkin-Elmer) were added to the reaction to facilitate subsequent detection. 6.6 μM of the different ODNs were added for translation inhibition when indicated. An unrelated ODN complementary to the alkaline phosphatase *phoA* was included as a control. The products were visualized by autoradiography in 18% SDS-PAGE after treatment with En3hance (Perkin Elmer). ETF *in vitro* synthesis produced the expected 26.6 KDa protein band that corresponds to the EtfB subunit (Fig. 3C). When the protein synthesis was carried out in the presence of any of the four ODNs tested, no protein band was observed in the gel. All the four ODNs were able to inhibit the production of ETF protein *in vitro*. However, practical utilization of antisense oligonucleotides as drugs require them to resist the action of the ubiquitous nucleases and to penetrate the cell membrane to reach the bacterial cytosol. The former problem can be solved by utilizing nucleotide analogs so that the oligonucleotides composed of units of one analog or different analogs are resistant to most nucleases but still form duplexes with the target mRNA (Rasmussen et al, 20017). We have shown before hybrid oligomers that are nuclease-resistant (Soler Bistue et al. 2009) and good inhibitors of translation by steric

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hindrance (Lopez et al. 2015). Furthermore, the antisense compounds must penetrate the cell wall, reach the cytosol, and be active inside the cells. Conjugation of antisense analogs to permeabilizer peptides has been proposed as a solution to this problem in several bacteria (Boisguerin et al., 2015). Therefore, one of the ODNs, ODN4, was selected for further analysis and an isosequential oligonucleotide analog containing 2',4'-bridged nucleic acid (BNA) and deoxynucleotide residues with the configuration A+GGAT+TTTC+ATGC +ACAC+GCTC+C, where the "+" before the base indicates that it is BNA residue, was covalently bound to the cell penetrating peptides $(RXR)_4XB$ or $(RFF)_3RXB$ (purchased from Biosynthesis Inc) (Greenberg et al. 2010). These compounds should be nuclease resistant and capable of reaching the cytosol where they should interact with the mRNA and inhibit translation. Fig. 3D shows that BNA4- $(RXR)_4XB$ was active in the cell-free *in vitro* system and inhibited *etfB* translation at the same level as the original ODN4. This result demonstrated that *etfB* expression can be turned off by short antisense oligonucleotide analogs covalently bound to permeabilizing peptides. However, neither BNA4-$(RXR)_4XB$ nor BNA4-$(RFF)_3RXB$ inhibited bacterial growth when added to cultures of the WT strains K56–2 and J2315 at a concentration of up to 20 μM (Table 2). Both strains belong to the ET12 epidemic clone but *B. cenocepacia* J2315 lacks O antigen (Ortega et al. 2005). In another experiment, both compounds were tested on the mutant strain *B. cenocepacia* RSF34, in which a modification of the LPS core increases susceptibility to different antimicrobial peptides (Loutet et al. 2006). No difference in growth inhibition was observed in the presence or absence of BNA4- $(RXR)_4XB$ or BNA4-$(RFF)_3RXB$ (Table 2). Taken together these results indicate that the antisense compounds show robust inhibition of expression of ETF *in vitro* but failed to inhibit growth *in vivo* despite the modifications to confer stability and ability to penetrate bacterial cells. The most probable causes for the lack of activity *in cellulo* are that the peptide conjugates did not reach the cytoplasm of *B. cenocepacia* or they failed to exert the inhibitory activity once inside the cells. The penetrating peptide $(RFF)_3RXB$ used in this study has been previously conjugated to a phosphorodiamidate morpholino oligomer (PMO) designed to target the *acpP* gene. This peptide-conjugated PMO was effective in inhibiting *B. cenocepacia* J2315 (Greenberg et al. 2010). While the reasons for the disagreement with previous results are not known, one possible scenario is that the penetrating properties of $(RFF)_3RXB$ are compromised when the peptide is conjugated to a BNA/DNA mixmer. Further studies modifying the chemical nature of the oligomers as well as the utilization of other cell penetrating peptides may clarify the causes of the lack of activity of the compounds used in this work and provide with active compounds capable of inhibition of growth of *B. cenocepacia*. These studies will also permit to start defining peptides and analogs that are appropriate for each particular bacterial species.

In conclusion, we demonstrate that an electron transfer flavoprotein (ETF) of *B. cenocepacia*, previously shown to be essential *in vitro*, is also essential for survival in the *C. elegans* model of infection. Depletion of ETF does not increase formation of persister cells, and its mRNA can be successfully inhibited *in vitro* by antisense technology. Thus, ETF holds promise as a new antibacterial target. We also describe that the *C. elegans* infection model performed in liquid medium allows modulation of bacterial essential gene expression *in vivo* when essential genes are under the control of the rhamnose-inducible promoter. This

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assay can be applied to study the effect of any essential gene inhibition during infection conditions. Confirmation of the essentiality of ETF in other animal models of infection is necessary to validate ETF as an antibacterial target.

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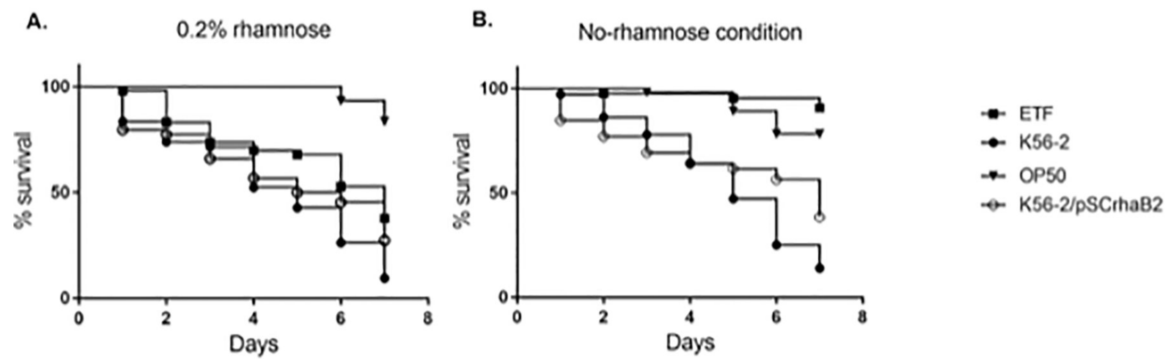

Figure 1.

Liquid killing assays. *C. elegans* was allowed to feed for 16 hours on *B. cenocepacia* K56–2, *E. coli* OP50 (non-pathogenic control), or the *B. cenocepacia CGetf* mutant (ETF) in the presence of 0.2% rhamnose to mimic WT levels of *etf* expression. Twenty to 30 worms were added to 96-wells plates containing 100 μl of liquid killing medium with (A) or without (B) the presence of 0.2% rhamnose. Four wells were inoculated per condition. The number of worms was counted every 24 hours for 7 days and plotted as survival curves using GraphPad Prism. K56–2 carrying the plasmid pSCrhaB2, which confers resistant to trimethoprim, was included as a control.

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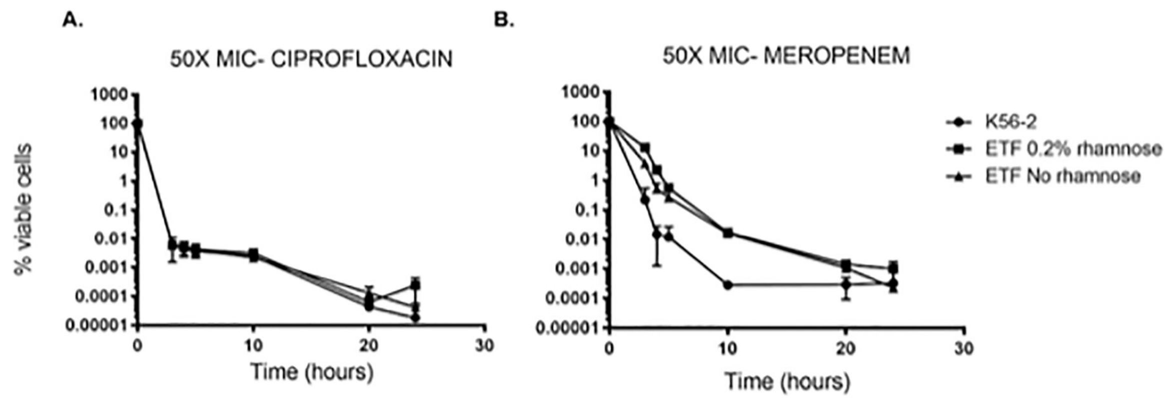

Figure 2.

Persister cells formation of the *CGetf* mutant strain. The percentage of viable cells (y-axis) was calculated after treatment with 100 μg/mL ciprofloxacin (A) or 800 μg/mL meropenem (B) of exponentially growing cells of, wild type K56–2 (black circles) and CG*etf* (ETF) in the presence (black squares) or absence (black triangles) of 0.2% rhamnose. X-axis indicates time in hours. Time 0 indicates the initial % of viable cells before treatment. An aliquot of each culture was taken at 3, 4, 5, 20 and 24 hours' post addition of antibiotic, washed with LB and plated for colony counting. The graphs include standard deviation (SD) of three biological replicates.

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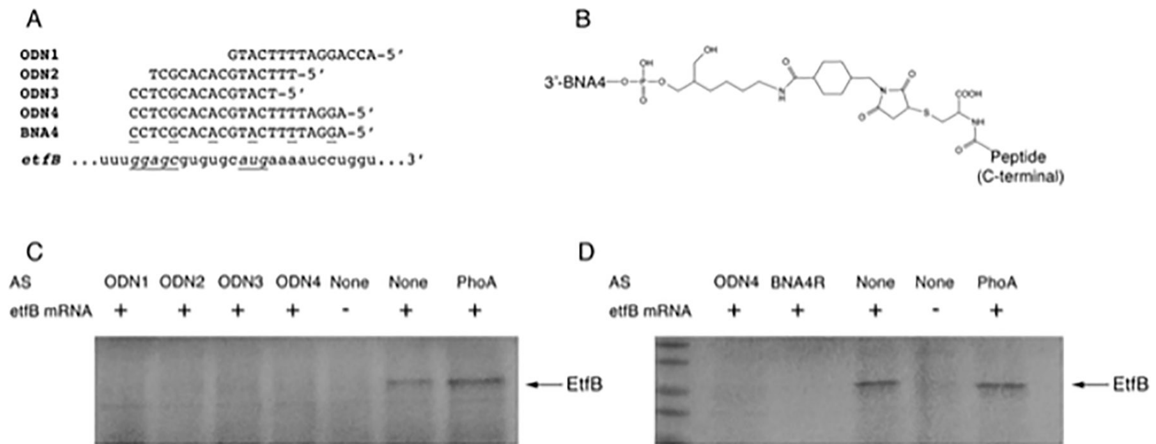

Figure 3.

Inhibition of *etf* expression by antisense oligodeoxynucleotides (ODNs). A) Sequences of the ODNs, BNA, and mRNA target regions used in this study. Upper case, lower case, and underlined letters represent deoxyribonucleotide, ribonucleotide, and BNA residues, respectively. The ribosome binding site and start codon are shown as underlined italics. B) Chemical structure of the BNA4 linked to penetrating peptides $(RXR)_4XB$ or (RFF)3RXB. C and D) *In vitro* inhibition of translation of mRNA *etfB* by ODNs using the *E. coli* S30 Cell Free Extract System. Additions to the reaction mixtures are shown on top. AS, antisense ODN added. PhoA, antisense targeting the region encompassing the start codon of the*phoA* gene. The arrow shows the location of the EtfB protein. BNA4R, BNA4-$(RXR)_4XB$ conjugate. ODNs were purchased from Integrated DNA Technologies, Inc. (Coralville, Iowa). $(RXR)_4XB$-Cys-SMCC-C6 amino-2',4'-BNA^{NC}-DNA (R, arginine; X, 6-aminohexanoic acid; B, β-alanine) and (RFF)3RXB-Cys-SMCC-C6 amino-2'–4'-BNA^{NC}-DNA (BNA4-$(RXR)_4XB$ and BNA4-$(RFF)_3RXB$, respectively) were purchased from Bio-Synthesis Inc., Lewisville, Texas.

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Table 1.

Bacterial strains and plasmids used in this study.

Strains	Relevant phenotype	Reference
E. coli OP50	Nonpathogenic strain for feeding *C. elegans*	*Caenorhabditis* Genetic Center (CGC), University of Minnesota, Minneapolis, USA
B. cenocepacia J2315	(LMG18863), ET12 linage, CF isolate	(Mahenthiralingam et al. 2002)
B. cenocepacia K56–2		
B. cenocepacia RSF34	K56–2, *hld*A, LPS defective	
B. cenocepacia CGetf	K56–2; Tp^{r*}, rhamnose-dependent *etfBA* expression	(Bloodworth et al. 2015)
Plasmid		
pSCrhaB2	oripBBRl *rhaR rhaS PrhaB* Tp^{r*} mob^{+}	(Cardona and Valvano 2005)

Tp^{r}, trimethoprim resistant.

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Table 2.

Minimum Inhibitory Concentrations (MICs) of peptide conjugated BNA4 of different *B. cenocepacia* strains.

Strain	BNA4-$(RXR)_4$XB	BNA4-$(RFF)_3$RXB	Trimethoprim*	Polymyxin B**
B. cenocepacia J2315	>20 μM	>20 μM	8 μg/mL	>512 μg/mL
B. cenocepacia K56–2	>20 μM	>20 μM	8 μg/mL	>512 μg/mL
B. cenocepacia RSF34	>20 μM	>20 μM	8 μg/mL	8 μg/mL

*Trimethoprim is known to inhibit *B. cenocepacia* growth and it was included as a control,

**Polymyxin B was used to test the sensitivity of strain RSF34 to antimicrobial peptides.