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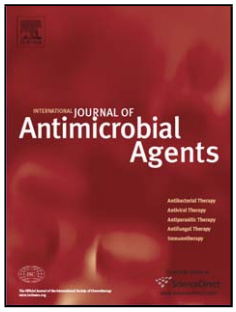

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Efflux pumps may play a role in tigecycline resistance in *Burkholderia* species

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ABSTRACT

The purpose of this study was to investigate the role of multidrug resistance efflux pumps in relation to decreased susceptibility to tigecycline in clinical isolates of *Burkholderia cepacia* complex (BCC). The role of efflux pumps was analysed using the efflux pump inhibitor (EPI) MC-207,110. Minimum inhibitory concentrations (MICs) were determined for each strain against tigecycline alone and in the presence of 64 mg/L MC-207,110. The effect of efflux pump inhibition on the susceptibility of BCC isolates to tigecycline was assessed by a checkerboard titration assay. Ala-Nap uptake assay was performed to determine efflux pump activity in different strains. The checkerboard titration assay showed that the MIC decreased with increasing concentrations of EPI. MICs for tigecycline in the clinical isolates ranged between 8 mg/L and 32 mg/L, whereas in the presence of MC-207,110, MICs decreased significantly (range <0.125–1.0 mg/L; 16 to >256 times reduction). Efflux pump activity was shown to be greatest in strains with the highest MIC and vice versa. In conclusion, BCC possess efflux pumps that influence their resistance to tigecycline. Use of an inhibitor of these pumps restored sensitivity to the antibiotic. Therefore, a combination of tigecycline and EPI to augment its efficacy may present an attractive therapeutic option.

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1. Introduction

Gram-negative microorganisms such as *Pseudomonas aeruginosa* and *Burkholderia cepacia* are clinically important pathogens of the cystic fibrosis (CF) lung. A hallmark of these infections is failure to eradicate the organism because of high-level multiple drug resistance [1], which is known to emerge during treatment [2]. Tigecycline, a novel expanded broad-spectrum glycylcycline antibiotic, has been shown to be active against a broad range of Gram-negative, Gram-positive, anaerobic and atypical bacteria [3]. However, its activity against *P. aeruginosa* is poor, with the microorganism's inherent resistance attributed to the activity of broad-substrate efflux pumps [4]. The activity of tigecycline against *B. cepacia* complex (BCC) has also been reported to be poor [5], but the mechanisms of resistance are poorly defined.

Drug efflux pump expression is a common resistance mechanism leading to high levels of drug resistance as a result of apparent synergism with the atypically impermeable outer membrane, thus limiting influx of antimicrobial agents. However, the action of these pumps may be blocked using efflux pump inhibitors (EPIs), compounds that bind within the specific substrate pocket and inhibit efflux pump activity [6]. MC-207,110 is the first identified broad-spectrum EPI that effectively inhibits all clinically relevant efflux pumps in Gram-negative bacteria [7,8].

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The aim of this study was to determine whether tigecycline resistance in BCC is medicated by efflux pumps and, if so, whether EPIs can be used to augment the efficacy of the glycylcycline molecule.

2. Materials and methods

2.1. Bacterial strains and media

Burkholderia strains used in this study (Table 1) were isolated from CF patients at the Royal Hospital for Sick Children (Yorkhill Division), Glasgow, UK. All strains were maintained and grown on Luria broth or agar at 37 °C. Mueller–Hinton broth was used for all susceptibility testing assays.

2.2. Susceptibility testing

Tigecycline white powder was kindly provided by Wyeth Pharmaceuticals (Princeton, NJ) and the EPI L-Phe-L-Arg-β-naphthylamide (MC-207,110) was purchased from Sigma-Aldrich (Gillingham, UK). These were prepared freshly at working concentrations in ddH_2O when required. The minimum inhibitory concentration (MIC) of tigecycline was determined by standard Clinical and Laboratory Standard Institute (CLSI) broth microdilution methodology in 96-well microtitre plates. A checkerboard assay was employed to show the effect of different concentrations of MC-207,110 on the MIC of tigecycline, as described previously by Lomovskaya et al. [9]. This was undertaken using five strains encompassing the different genomovars (given in parenthesis), including K56-2

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(III), YHBCC1 (II), YHBCC13 (IV), YHBCC14 (V) and YHBG1. Tigecycline was tested at a range from 0.06 mg/L to 64 mg/L in combination with MC-207,110 at a range of 8 mg/L to 512 mg/L. Viability was assessed using an Alamar Blue® colorimetric assay as per the manufacturer's instructions (Invitrogen, Paisley, UK). Following these initial experiments, a defined concentration of MC-207,110 was selected (64 mg/L) for subsequent microdilution testing on the entire panel of strains. This was based upon data obtained from the checkerboard assay, which showed no antimicrobial activity at the concentrations tested.

2.3. Ala-Nap uptake assay

Efflux pump activity was assessed using an Ala-Nap fluorescent assay as previously described [9]. Ala-Nap is enzymatically cleaved inside cells to produce highly fluorescent β-naphthylamine, thus the higher the fluorescence the lower the efflux pump activity. Selected isolates were washed and standardised to 5×10^5 cells in buffer solution [K_2HPO_4 (50 mM), $MgSO_4$ (1 mM) and glucose (0.4%)] at pH 7.0 and dispensed into black, flat-bottomed microtitre plate (Costar® 3603; Corning-Costar Corp., Corning, NY). The reaction was initiated by the addition of Ala-Nap at a final concentration of 128 mg/L. Fluorescence was quantified at 30-s intervals for 1 h at 37 °C using a fluorescent plate reader (FLUOstar OPTIMA; BMG LABTECH, Aylesbury, UK) with an excitation wavelength of 320 nm and an emission wavelength of 460 nm.

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3. Results

The sensitivity of 17 different strains of BCC and 3 *Burkholderia gladioli* to tigecycline was evaluated by broth microdilution according to CLSI methodology. The MIC_{50} and MIC_{90} (MIC for 50% and 90% of the isolates, respectively) for BCC were 32 mg/L (range 8–32 mg/L) (Table 1). *Burkholderia gladioli* strains were more sensitive (range 4–8 mg/L). For EPI studies, the MIC value of MC-207,110 was determined to be >512 mg/L for all the strains tested. To assess the effect of inhibition of efflux pumps upon the susceptibility of BCC, a standard checkerboard titration assay was performed on five strains. An inverse relationship between tigecycline and MC-207,110 concentration was observed, i.e. the MIC of tigecycline decreased with an increase in the concentration of MC-207,110 (Fig. 1), indicating that MC-207,110 at a concentration of 64 mg/L exhibited a 50% potentiating effect on a decrease in the MIC. This concentration exhibited no detrimental effect on bacterial viability when assessed using an Alamar Blue® assay. This concentration was therefore used for all subsequent experiments using standard CLSI broth microdilution methods. The MICs of tigecycline in the presence of MC-207,110 were determined for each BCC strain and it was found that the MIC_{50} and MIC_{90} values were reduced to 0.5 mg/L and 1 mg/L, respectively, with a range of <0.125 mg/L to 1 mg/L (Table 1). The level of MIC reduction ranged from 16 to >256 times for both BCC and *B. gladioli*. Subsequent analysis of β-naphthylamine production by three strains demonstrated differential efflux activity (Fig. 2). The rate of cleavage of Ala-Nap was highest in YHBG3 (MIC = 8 mg/L; MIC reduction >64) compared with

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YHBCC4 (MIC = 16 mg/L; MIC reduction = 32) and YHBCC12 (MIC = 32 mg/L; MIC reduction >256), i.e. higher MICs correlated with greater efflux.

4. Discussion

The increasing prevalence of resistant bacteria demands that a renewed effort be made to seek new and alternative antibiotics effective against drug-resistant pathogens. Tigecycline is a novel antibiotic developed to overcome widespread bacterial resistance to tetracycline and minocycline. It is effective against many Gram-positive and Gram-negative microorganisms but has little activity against the non-fermenting CF pathogens *P. aeruginosa* and *Burkholderia* spp. The mechanism of resistance has been clearly demonstrated for *P. aeruginosa*, but in BCC it remains to be determined [4,10]. The data presented here indicate a role for efflux pumps.

The effect of efflux pumps was studied by inhibiting those systems using a broad-spectrum EPI MC-207,110, a substrate of efflux pumps. Previous studies have demonstrated MC-207,110 to be a potential inhibitor of efflux pumps in Gram-negative bacteria such as *P. aeruginosa* as well as potentiating the activities of levofloxacin and chloramphenicol [7,9,11]. However, according to Chan et al. [12], MC-207,110 did not have any potentiating effect on the efflux of erythromycin or streptomycin by the BpeAB-OprM pump in *Burkholderia pseudomallei*. It can be concluded from these studies that MC-207,110 works as an active competitor of some, but not all, antibacterial drugs. Efflux pumps were

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shown to be the mechanism of tigecycline resistance in *Acinetobacter baumannii*, which was shown to be reversible with MC-207,110 [13]. Moreover, a study by Hirakata et al. [14] reported that in addition to its role as an inhibitor of efflux pump activity, it was also capable of reducing the invasiveness of *P. aeruginosa* PA01.

In this study, we report that MC-207,110 augmented the effect of tigecycline in a concentration-dependent manner in members of the BCC, demonstrating that these microorganisms may utilise efflux pumps as a resistance mechanism. As the concentration of EPI increased, the MIC to tigecycline was reduced, indicating the concentration-dependent competition for the extrusion of tigecycline through active efflux. The potency (MIC_{90} = 32 mg/L) of tigecycline in the absence of MC-207,110 was similar to the results published by Milatovic et al. [5], but in the presence of MC-207,110 the MICs decreased significantly (MIC_{90} = 1 mg/L). Direct assessment of the efflux activity demonstrated a correlation between efflux activity and high MIC values, and conversely the lower MICs of *B. gladioli* were correlated with low efflux pump activity (Fig. 2). Therefore, this supports the hypothesis that members of the BCC utilise efflux pumps for resistance to antibiotics.

In conclusion, CF pathogens of the BCC possess efflux pump proteins that have evolved to become integral to their natural physiological function and that also confer inherent resistance to tigecycline. However, these pumps can be

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inactivated using competitive inhibitors to restore the activity of tigecycline. Whether EPIs can be used for combination therapy with tigecycline in CF patients remains to be seen. Tigecycline is derived from a tetracycline that has been shown to have the capacity to modulate inflammatory processes. Using EPIs in combination with tigecycline in CF patients may provide the opportunity for an antibiotic with unrestricted broad-spectrum antimicrobial activity whilst also suppressing inflammation of the CF lung. Further studies are ongoing to investigate tigecycline further in this respect.

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Competing interests

None declared.

Ethical approval

Not required.

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Fig. 1. Schematic representation of a checkerboard assay of *Burkholderia cepacia* ATCC 17765. Minimum inhibitory concentrations (MICs) of tigecycline alone [0.06–64 mg/L (row 1)], the efflux pump inhibitor (EPI) MC-207,110 alone [8–512 mg/L (column 1)] and tigecycline plus MC-207,110 in combination were determined. Open circles (○) represent no growth within the wells and filled circles (●) represent growth within the well. MC-207,110 exhibits no antimicrobial activity and, as its concentration increases in combination with tigecycline, the inhibition of *Burkholderia* growth increases. Similar results were observed for four other strains tested.

Fig. 2. Ala-Nap (128 mg/L) uptake of selected strains YHBG3, YHBCC4 and YHBCC12. A linear increase in fluorescence as a function of time is illustrated owing to intracellular hydrolysis of Ala-Nap. Greater fluorescence was quantified in the case of YHBG3 (*Burkholderia gladioli*), with an MIC of 8 mg/L, indicating low efflux activity. In the case of YHBCC4 (MIC = 16 mg/L) and YHBCC12 (MIC = 32 mg/L), less fluorescence was quantified owing to high efflux activity.

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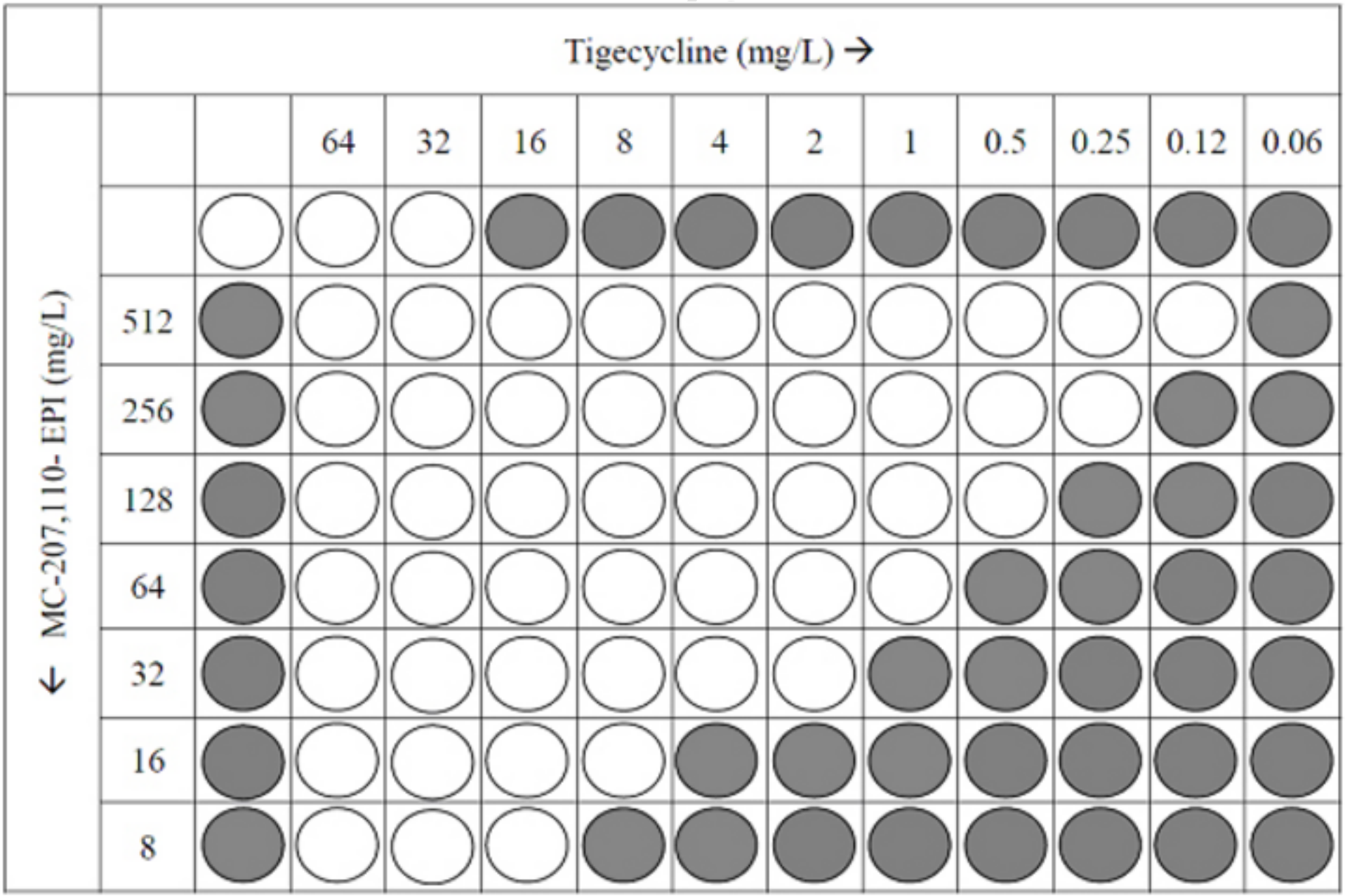
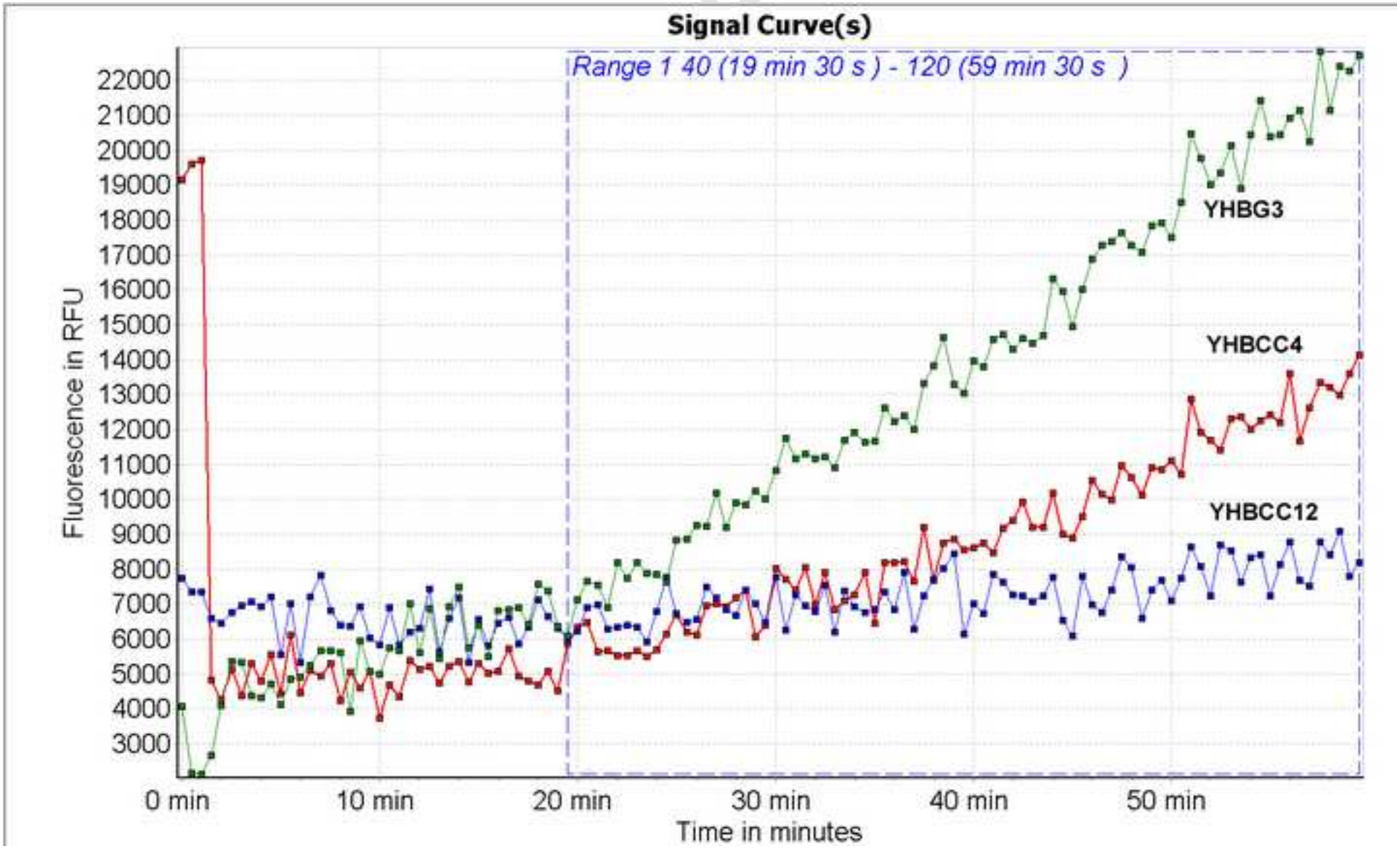
Table 1

Minimum inhibitory concentrations (MICs) of tigecycline in the presence and absence of the efflux pump inhibitor MC-207,110 (64 mg/L)

Strain	Tigecycline MIC (mg/L)		Fold change
	Tigecycline alone	Tigecycline plus MC-207,110	
Burkholderia cepacia complex (genomovar)			
K56-2 (III)	32	0.5	64
ATCC 17765 (III)	32	1	32
YHBCC1 (II)	32	1	32
YHBCC2 (II)	32	1	32
YHBCC3 (II)	32	0.5	64
YHBCC4 (II)	16	0.5	32
YHBCC5 (III)	16	0.25	64
YHBCC6 (III)	32	1	32
YHBCC7 (III)	32	1	32
YHBCC8 (III)	32	1	32
YHBCC9 (III)	16	0.25	64
YHBCC10 (III)	32	1	32
YHBCC11 (III)	16	0.5	32
YHBCC12 (III)	32	<0.125	>256
YHBCC13 (IV)	8	0.5	16
YHBCC14 (V)	16	0.25	64
YHBCC15 (V)	16	1	16
Burkholderia gladioli			
YHBG1	8	0.25	32
YHBG2	4	0.25	16
YHBG3	8	<0.125	>64

Edited Figure 1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