Inhibiting plasmid mobility: the effect of isothiocyanates on bacterial conjugation

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Abstract

Bacterial conjugation is the main mechanism for the transfer of multiple antibiotic resistance genes among pathogenic microorganisms. This process could be controlled by compounds that inhibit bacterial conjugation. In this study, the effect of allyl isothiocyanate, L-sulforaphane, benzyl isothiocyanate, phenylethyl isothiocyanate and 4-methoxyphenyl isothiocyanate on the conjugation of broad host range plasmids, which harbor various resistance genes in *Escherichia coli* were investigated; pKM101 (IncN), TP114 ($IncI_2$), pUB307 (IncP) and the low copy number IncW plasmid R7K. Benzyl isothiocyanate (32 mg/L) significantly reduced the conjugal transfer of pKM101, TP114 and pUB307 to 0.3±0.6%, 10.7±3.3% and 6.5±1.0%, respectively. L-sulforaphane (16 mg/L, transfer frequency 21.5±5.1%) and 4-methoxyphenyl isothiocyanate (100 mg/L, transfer frequency 5.2±2.8%) were the only compounds that showed anti-conjugal specificity by actively reducing the transfer of R7K and pUB307, respectively.

Keywords: Isothiocyanates, bacterial conjugation, conjugative plasmids, effector proteins, horizontal gene transfer, plasmid incompatibility groups

1. Introduction

Bacterial conjugation is an adaptive mechanism that allows bacteria to transfer genetic materials, effector proteins and/or toxins from one cell to the other through a conjugative bridge [1, 2]. The genetic materials that are transferred via conjugation usually confer selective advantages to the recipient organism, such as survival, resistance, pathogenicity, infection activities and/or the ability to respond to environmental changes. Conjugation greatly increases bacterial genome plasticity, and has immense clinical relevance as a major route for the spread of multiple antibiotic resistance genes among the microbial community and virulence genes from pathogen to host [2]. It is therefore imperative to find ways to combat conjugation, as a means to decrease the ongoing rise of antibiotic-resistant infections.

Inhibition of bacterial conjugation has not received much research attention because the focus has been on the identification of new classes of antibacterial agents that target processes essential for bacterial growth such as cell wall biosynthesis, the cell membrane, protein synthesis, nucleic acid synthesis and metabolite activity. This traditional approach has produced many therapeutically useful agents so far, but the challenge is that an antibiotic also introduces selective pressure promoting resistant bacteria, and therefore this has led to the current antibiotic resistance crisis. An additional approach of reducing the increasing rate of bacterial antibiotic resistance dissemination and re-sensitizing bacteria to existing antibiotics, would be to target non-essential processes such as conjugation, which are less likely to evoke bacterial resistance. This approach could also have a prophylactic use in cosmeceuticals to reduce plasmid transfer. In addition to bacterial conjugation, other non-essential processes such as plasmid replication [3-5] and plasmid-encoded toxin-antitoxin systems [6, 7] have been exploited with promising potential in antibacterial therapy.

The few efforts directed towards identifying anti-conjugants include small-molecule inhibitors of *Helicobacter pylori cag* VirB11-type ATPase Cagα [8]. The *cag* genes encode for the assembly of the conjugative bridge and injection of the CagA toxin into host cells [8, 9]. In addition, there have been other reports of promising anti-conjugants such as dehydrocrepenynic acid [1], linoleic acid [1], 2-hexadecyanoic acid [10], 2-octadecynoic acid [10], and tanzawaic acids A and B [11]. However, these compounds have stability, toxicity or scarcity issues that need to be addressed. Therefore there is the pressing need to identify safer anti-conjugants to help in the fight against plasmid-mediated transfer and spread of antibiotic resistance and virulence.

In this study, four naturally occurring isothiocyanates (allyl isothiocyanate (**1**), L-sulforaphane (**2**), benzyl isothiocyanate (**3**), phenylethyl isothiocyanate (**4**)) and a synthetic isothiocyanate (4-methoxyphenyl isothiocyanate, **5**) were investigated for their anti-conjugant activity against *E. coli* strains bearing conjugative plasmids with specific antibiotic resistance genes. Isothiocyanates are usually naturally occurring hydrolytic products of glucosinolates, which are commonly found in the *Brassica* vegetables. They are produced when damaged plant tissue releases the glycoprotein enzyme myrosinase, which hydrolyses the *β*-glucosyl moiety of a glucosinolate. This leaves the unstable aglycone, thiohydroxamate-*O*-sulfonate, which rearranges to form an isothiocyanate or other breakdown products [12, 13]. Other isothiocyanates such as 4-methoxyphenyl isothiocyanate and methyl isothiocyanate are synthetically produced and not naturally occurring.

In addition to the anti-conjugant testing, plasmid curing activity and bacterial growth inhibition were also evaluated to help discriminate between true anti-conjugants and substances that reduce conjugation due to elimination of plasmids or function by perturbation

of bacterial growth or physiology. Isothiocyanates possessing the highest anti-conjugant activities were further investigated for cytotoxicity against human dermal fibroblast adult cells (HDFa; C-013-5C).

2. Materials and methods

2.1. Bacterial strains and plasmids

E. coli NCTC 10418 (a susceptible Gram-negative strain), *S. aureus* ATCC 25923 (a susceptible Gram-positive strain), *S. aureus* SA-1199B (a fluoroquinolone-resistant strain, which over-expresses the multidrug-resistant NorA pump) and *S. aureus* XU212 (a tetracycline-resistant strain, which over-expresses the multidrug-resistant TetK pump) were used for the broth dilution assay. Plasmid-containing *E. coli* strains WP2, K12 J53-2 and K12 JD173 were used as donor strains in the plate conjugation and plasmid elimination assays. *E. coli* ER1793 (streptomycin-resistant) and *E. coli* JM109 (nalidixic-resistant) were used as the recipients. Conjugative plasmids used were pKM101 (WP2; incompatibility group N (IncN); ampicillin-resistant), TP114 (K12 J53-2; $IncI_2$; kanamycin-resistant), and R7K (K12 J53-2; IncW; ampicillin-, streptomycin- and spectinomycin-resistant), which were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and conjugative plasmid pUB307 (K12 JD173; IncP; ampicillin-, kanamycin- and tetracycline-resistant) was provided by Prof. Keith Derbyshire, Wadsworth Center, New York Department of Health.

2.2. Broth micro-dilution assay

The antibacterial activity was determined with the broth micro-dilution method as described previously [14], which is a modified version of the procedured described in the British Society for Antimicrobial Chemotherapy (BSAC) guide to sensitivity testing. Bacteria were cultured on nutrient agar slants and incubated at 37°C for 18 hours. A bacterial suspension equivalent to a 0.5 McFarland standard was made from the overnight culture. This was added to Muller-Hinton broth and the test isothiocyanate, which had been serially diluted across a 96-well microtitre plate, to achieve a final inoculum of 0.5×10^5 CFU/mL. Minimum

inhibitory concentrations (MICs) were determined after 18 hours of incubation at 37°C. This was done by visual inspection after the addition of a 1 mg/mL methanolic solution of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) and incubation at 37°C for 20 minutes. This experiment was performed in duplicate in two independent experiments.

2.3. Liquid conjugation assay

The donor cells with plasmids pKM101, TP114 and pUB307 were paired with the recipient ER1793. Plasmid R7K donor cells were paired with the recipient JM109. Research has shown that plasmid carriage by host bacteria is associated with some fitness cost (burden) [15, 16]. This fitness effect of plasmids plays a vital role in their ability to associate with a new bacterial host. As a consequence of this we selected different *E. coli* host, which are known to successfully conjugate [17, 18] and to maintain the study plasmids. The liquid conjugation assay was performed as previously described [19] with slight modifications. Equal volumes (20 μL) of donor and recipient, for which the colony forming units per mL (CFU/mL) had been predetermined (Supplementary Table 1), were introduced into 160 μL of Luria-Bertani broth and the test sample or control. This was incubated at 37°C for 18 hours after which the number of transconjugants and donor cell were determined using antibiotic-containing MacConkey agar plates. A positive control linoleic acid [1] and negative control (donor, recipient and media; without drug or test sample) were included in the experiment. The isothiocyanates were evaluated for anti-conjugant activity at sub-inhibitory concentration (one-quarter of the MIC). Antibiotics were added at the following concentrations for positive identification of donors, recipients and transconjugants (mg/L): amoxicillin (30), streptomycin sulphate (20), nalidixic acid (30), kanamycin sulphate (30). Conjugation frequencies were calculated as the ratio of total number of transconjugants (cfu/mL) to the total number of donor (cfu/mL) and expressed as a percentage relative to the negative control.

This experiment was performed as duplicate in three independent experiments and anti-conjugation activity is reported as the mean ± standard deviation.

2.4. Plasmid elimination assay

This assay was performed as described previously [20] with minor modifications. The *E. coli* donor strains were sub-cultured on appropriate antibiotic-containing MacConkey agar plates to ensure plasmid presence. After incubation of the plates at 37°C for 18 hours, single colonies (2-3) were selected and inoculated into LB. This was incubated for 18 h at 37°C and the colony forming units were determined prior to the assay. Twenty microliters of the overnight culture was then added to a mixture of 180 µL LB and test sample in a 96 well microtitre plate. This was incubated overnight (18 h) at 37°C and subsequently serially diluted, 20 µL was then plated on antibiotic containing MacConkey agar and incubated for 18 h at 37°C. The isothiocyanates were evaluated for plasmid elimination activity at concentrations used in the liquid conjugation assay. Both positive control (promethazine) [21-23] and negative control (mixture without isothiocyanate or control drug) were included in this experiment. Plasmid elimination was calculated using:

$$\text{Plasmid elimination} = \frac{\text{CFU/mL of control} - \text{CFU/mL of test sample}}{\text{CFU/mL of control}} \times 100$$

Antibiotics and concentrations used in MacConkey agar for positive identification of *E. coli* cells harbouring plasmids (mg/L) were: amoxicillin (30), kanamycin sulphate (20 and 30) and nalidixic acid (30). This experiment was performed in duplicate, with three independent experiments.

2.5. Cytotoxicity assay

The isothiocyanates that showed anti-conjugant activity were further assessed for their effect on eukaryotic cell growth. The sulforhodamine B (SRB) colorimetric assay as described

previously [24] was used, with modifications. Human dermal fibroblast, adult cells (HDFa; C-013-5C) were grown in a 75 cm^2 culture flask at 37°C in humidified atmosphere of 5% carbon dioxide using Dulbecco's Modified Eagle's Medium, which was supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, 0.1% gentamicin and amphotericin B. The grown cells were seeded in a 96 well microtitre plated and test samples and media were added. This was then incubated at 37°C in 5% CO_2 for 72 h. Afterwards 50 µL of cold 40% $^{W}/_{V}$ trichloroacetic acid (TCA) solution was added, the plate was placed in the fridge for an hour at 4°C and washed four times with distilled water. The cells were then stained with 0.4% $^{W}/_{V}$ SRB solution and left at room temperature for an hour. Afterwards, the plate was rinsed four times with 1% acetic acid and left overnight (24 h) to dry. Thereafter, 100 µL of 10 mM Tris buffer solution was dispensed into the wells and agitated in an orbital shaker for 5 min, to allow solubilisation of SRB-protein complex. The optical density (OD) was then measured at 510 nm using a microtitre plate reader (Tecan Infinite® M200). The percentage of viable cell was calculated using:

$$\text{Percentage of viable cell} = \frac{\text{OD of test sample-OD of blank}}{\text{OD of negative control-OD of blank}} \times 100$$

This experiment was performed as triplicate in three independent experiments and cytotoxicity has been reported as mean ± standard deviation.

2.6. Statistical analyses

The statistical analyses were carried out using Excel Data Analysis and GraphPad Prism 7. Welch's t-test was used to evaluate the difference between the control conjugal transfer frequency and the test compounds. Results with $p < 0.05$ were considered statistically significant.

3. Results

3.1. The effect of isothiocyanates on the growth of bacteria

To test whether the selected isothiocyanates had growth inhibitory activity against bacterial species and to inform of a suitable concentration for their evaluation in an anti-conjugation assay, the isothiocyanates were tested against susceptible Gram-negative (*E. coli* NCTC 10418) and Gram-positive (*S. aureus* ATCC 25923) standard isolates, and antibiotic effluxing *Staphylococcus aureus* strains (SA-1199B and XU212). Table 1 shows the MIC values for the tested isothiocyanates; their inhibitory activity varied from 16 to 512 mg/L against the evaluated bacteria. Our general observation was that unsurprisingly the isothiocyanates were marginally more active against the Gram-positive than the Gram-negative strains.

3.2. The effect of isothiocyanates on conjugal transfer of plasmids

To investigate whether the selected isothiocyanates have anti-conjugant activity, a range of plasmids belonging to different incompatibility groups (IncN plasmid pKM101, $IncI_2$ plasmid TP114, IncP plasmid pUB307 and IncW plasmid R7K) were employed to test the specificity of conjugation inhibition in *E. coli*. With information (Table 1) about their minimum inhibitory concentration against *E. coli* NCTC 10418 (a susceptible standard strain), the isothiocyanates were tested at a sub-inhibitory concentration, one-quarter of their MICs. Figure 1 shows the effect of the isothiocyanates on the conjugal transfer of the test plasmids. The test isothiocyanates exhibited inhibitory activities ranging from complete reduction in conjugation frequency (0%, considered active), inhibition of conjugation frequency to less than 10% were also considered as active; 10 to 50% were considered moderately active and greater than 50% were considered as inactive.

3.3. Elimination of plasmids from E. coli

To determine whether the observed anti-conjugant activity was not due to the elimination of conjugative plasmids, the donor cells were grown in the presence of the test isothiocyanates, and the plasmid elimination assay was performed. Figure 2 shows the effect of the isothiocyanates on conjugative plasmids. The isothiocyanates exhibited varied plasmid curing activity. Plasmids TP114 and R7K of the incompatibility groups I_2 and P, respectively, were the most eliminated in the donor cells with elimination percentages ranging from 3.0±0.1 to 77.8±8.0. Most of the tested isothiocyanates did not have any plasmid curing effects on pKM101 (IncN), with the exception of allyl isothiocyanate (**1**), which showed a curing effect of 19.4±6.6 %. For pUB307 (IncP), a plasmid curing effect was observed for L-sulforaphane (**2**, 56.7±3.2 %) and phenylethyl isothiocyanate (**4**, 64.8±15.4 %).

*3.4. The effect of increasing concentration of benzyl isothiocyanate (**3**) on conjugal transfer of the plasmids pKM101 (IncN), TP114 (IncI_2) and pUB307 (IncP)*

With benzyl isothiocyanate (**3**) having shown broad range anti-conjugant (conjugal reduction to 0.3±0.6 - 10.7±3.3%, Figure 1) and the least donor plasmid elimination activity (0 - 26.5±5.9%, Figure 2) of all tested compounds, it was further assessed to observe its effect on conjugal transfer with increasing concentration. Generally, there was a gradual increase in anti-conjugal activity against pKM101 and TP114 with increase in concentration from 0.125 to 64 mg/L (Figure 3). This was not the same for plasmid pUB307, there was no significant change in anti-conjugal activity for benzyl isothiocyanate (**3**), and it surprisingly remained active at the low concentrations tested. The observed conjugal transfer of pUB307 in the presence of **3** ranged between 11.3±2.6% and 1.9±2.2% for concentrations of 0.125 and 64 mg/L, respectively.

3.5. *Effect of increasing concentration of 4-methoxyphenyl isothiocyanate (**5**) on conjugal transfer of pUB307*

Among the test isothiocyanates, 4-methoxyphenyl isothiocyanate (**5**) was the most active against IncP plasmid pUB307, with no plasmid curing activity. It was therefore evaluated for the effect of increasing concentration (1-128 mg/L) on the conjugal transfer of plasmid pUB307. The observed activities are shown in Figure 4**.** 4-methoxyphenyl isothiocyanate (**5**) showed a moderate anti-conjugant activity (22.7±1.6%) at the lowest concentration (1 mg/L) and this was steadily maintained up to 32 mg/L, after which there was a sharp increase in conjugal inhibition. Almost complete conjugal inhibition was observed at 128 mg/L.

*3.6. Effect of allyl (**1**) and benzyl (**3**) isothiocyanates on normal growth of human dermal fibroblast, adult cells (HDFa; C-013-5C)*

Allyl (**1**) and benzyl (**3**) isothiocyanates that exhibited active to moderate anti-conjugant activity against all test plasmids were further assessed for cytotoxicity against normal cell growth. This was to determine whether the broad range anti-conjugant activity exhibited by isothiocyanates **1** and **3** were not at cytotoxic concentrations and worth pursuing as potential anti-conjugants for further development. The observed cytotoxic activities are shown in the Figure 5. The IC_{50} for allyl (**1**) and benzyl (**3**) isothiocyanates against HDFa cells was 63.9 mg/L (645 μM) and 30.3 mg/L (203 μM), respectively.

4. Discussion

The discovery of a potent compound that will inhibit the spread of resistance genes and/or resistance mechanisms has clinical relevance, especially in this era of plasmids within species such as *K. pneumoniae* that are carbapenem-resistant. This is highly timely given the lack of treatment options for infections caused by this pathogen. In line with this, selected

isothiocyanates, which are hydrolysis products of glucosinolates commonly found in *Brassica* vegetables, were investigated for the possibility of inhibiting the spread of resistance genes by blocking bacterial conjugation in *E. coli*.

The initial findings from this study showed that allyl isothiocyanate (**1**), L-sulforaphane (**2**), benzyl isothiocyanate (**3**), phenylethyl isothiocyanate (**4**) and 4-methoxyphenyl isothiocyanate (**5**) have some level of antibacterial activity that ranged from 16 to > 512 mg/L against the susceptible *E. coli* NCTC 10418 and *S. aureus* ATCC 23925, and the effluxing multidrug-resistant *S. aureus* strains (SA-1199B and XU212) (Table 1). This corroborates the reported antibacterial activity of the isothiocyanates but due to the variability in the testing methods, bacterial inoculum densities and diversity in susceptibility, it is difficult to compare results [25-31]. The isothiocyanates were found to be less potent in comparison to conventional antibiotics and similar results have been reported by others [25, 27, 31]. Among the tested isothiocyanates, **4** was the most potent against the Gram-positive microbes with MIC values ranging from 16 to 32 mg/L followed by **2** (MIC values ranged from 32 to 64 mg/L), which was also the most potent against Gram-negative *E. coli* NCTC 10418. The antibacterial activity of these isothiocyanates have been explained to be due to their ability to cause physical membrane damage [32, 33], interfere with bacterial redox system, which affects the cell membrane potential [33] or the disruption of major metabolic processes [34, 35].

With the anti-conjugal activity study, broad-range anti-conjugant activity was observed for allyl (**1**) and benzyl isothiocyanate (**3**) at sub-inhibitory concentrations, with **3** being the most potent among the test isothiocyanates (Figure 1). It inhibited the conjugation of plasmids pKM101 (IncN), TP114 (IncI_2) and pUB307 (IncP), and selectively cured plasmid TP114, only. Against plasmids pKM101 and TP114, **3** also reduced conjugal transfer by 97.7±3.3%

and 96.4±4.2%, respectively at 32 mg/L (214.46 μM), and its activity gradually declined with decreasing concentration (Figure 3). This was not the same for pUB307, where **3** continued to show pronounced activity with a 90.8±2.3% reduction in conjugation, even at a low concentration of 0.25 mg/L (1.68 μM). This was interesting, as **3** did not show any plasmid curing activity against this particular plasmid pUB307 and pKM101, ruling out the fact that the observed anti-conjugation may be due to plasmid elimination. Another area of interest was that **3** exhibited broad-range activity; this could mean that **3** either acts on a common target site on the conjugation machinery or that it causes general cell toxicity. However, considering the MIC value (128 mg/L, Table 1) of **3** against the susceptible *E. coli* strain NCTC 10418, the concentrations (≤ 32 mg/L) used for the conjugation assays were at sub-lethal doses and it is less likely to have caused general cell toxicity. With allyl isothiocyanate (**1**), moderate plasmid elimination activity was observed against most of the test plasmids and this may be an indication that its broad-range anti-conjugant activity is due to plasmid curing. The broad-range of activity of **1** and **3** prompted their testing against normal growth of human dermal fibroblast, adult cells (HDFa; C-013-5C). A comparison of the cytotoxic value of **3** against HDFa cells (30.30 mg/L; 203.07 μM) with its anti-conjugant concentration against the test plasmids showed that its IC_{50} level was above the concentrations needed to cause a 50% reduction in conjugal transfer of plasmids; pKM101 (IC_{50} = 2.19 mg/L; 14.68 μM), TP114 (IC_{50} = 1.24 mg/L; 8.31 μM) and pUB307 (IC_{50} = 0.34 mg/L; 2.28 μM) (Figure 5). This suggests that **3** showed anti-conjugant activity at non-toxic concentrations. However, the same cannot be said for compound **1** because, its IC_{50} against HDFa (63.9 mg/L; 644.48 μM) was below the 100 mg/L needed to cause moderate anti-conjugant activity (90-50% reduction) against most of the test plasmids. It is therefore suggested that the concentrations needed to cause a 50% reduction in conjugation is most likely to be closer to the cytotoxic-IC_{50} value.

From this study, specificity of anti-conjugal and plasmid curing activity was observed for 4-methoxyphenyl isothiocyanate (**5**), a synthetic compound. L-sulforaphane (**2**) also exhibited some level of anti-conjugant specificity against the IncW plasmid R7K at 16 mg/L (90.25 μM), but at this same concentration plasmid curing was observed and hence **2** is not a true anti-conjugant (Figure 2). Compound **5**'s anti-conjugant activity at 100 mg/L (605.29 μM) was pronounced for the IncP plasmid pUB307, with a 94.8±2.8% reduction in conjugation, but it showed minimal inhibition or promoted conjugation for the other test plasmids (Figure 1). Its anti-conjugant activity was however concentration-dependent (Figure 4). With the plasmid curing effect, **5** showed elimination of only the IncW plasmid R7K, but it did not have any effect on conjugation of this plasmid. This may give an indication that **5** could have some conjugation promotion factors, and this was observed for pKM101. Conjugation of pKM101 in the presence of **5** exceeded 100% (Figure 1). The anti-conjugation, plasmid curing and pro-conjugation activity exhibited by **5** supports its specificity. This suggests that compound **5** acts on a specific target site, which may not be common to all plasmids. Consequently, it is less likely for resistance to develop against **5** unlike other compounds that target general and essential targets of bacteria, which is the case in many instances of antibiotic resistance [36]. A general observation with the test isothiocyanates is that the presence of oxygen, attached to sulphur or an aromatic carbon conferred some level of anti-conjugal specificity. We therefore hypothesize that the methoxyl substituent on the aromatic ring and the lack of a hydrocarbon chain of **5**, which makes it structurally different from the other test aromatic isothiocyanates, may have contributed to its specificity of activity.

In conclusion, isothiocyanate **3** and **5** were the most promising anti-conjugants identified in this study. Further explorative studies involving structural modification and mechanistic

studies of these isothiocyanates could possibly lead to the identification of a potent anti-conjugant. This will help decrease the spread of multidrug-resistant genes, multidrug resistant bacteria, reduce virulence and help reinstate existing antibiotics.

Declarations

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Supplementary material

Supplementary material relating to this article has been attached.

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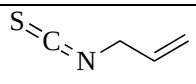
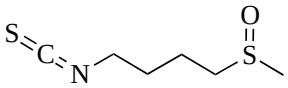
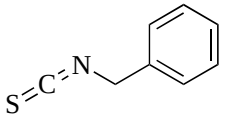
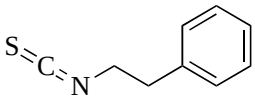
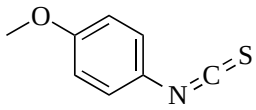
Table 1

Table 1. Minimum inhibitory concentration (MIC) values against *E. coli* and *S. aureus*

Isothiocyanate (sample number)	**Chemical Structure**	**MIC (mg/L)**			
		E. coli NCTC 10418[a]	*S. aureus* ATCC 25923[a]	*S. aureus* SA-1199B[b]	*S. aureus* XU212[c]
Allyl isothiocyanate (**1**)		> 512	512	512	> 512
L-sulforaphane (**2**)		64	64	32	32
Benzyl isothiocyanate (**3**)		128	256	256	512
Phenylethyl isothiocyanate (**4**)		256	16	32	32
4-methoxyphenyl isothiocyanate (**5**)		512	256	128	128
Ciprofloxacin	-	< 0.0625	< 0.0625	-	-
Norfloxacin	-	-	-	32	-
Tetracycline	-	-	-	-	128

[a] susceptible standard strain, [b] fluoroquinolone-resistant strain that over-expresses the NorA efflux pump and [c] tetracycline-resistant strain, which over-expresses the TetK efflux pump.

Figure 1

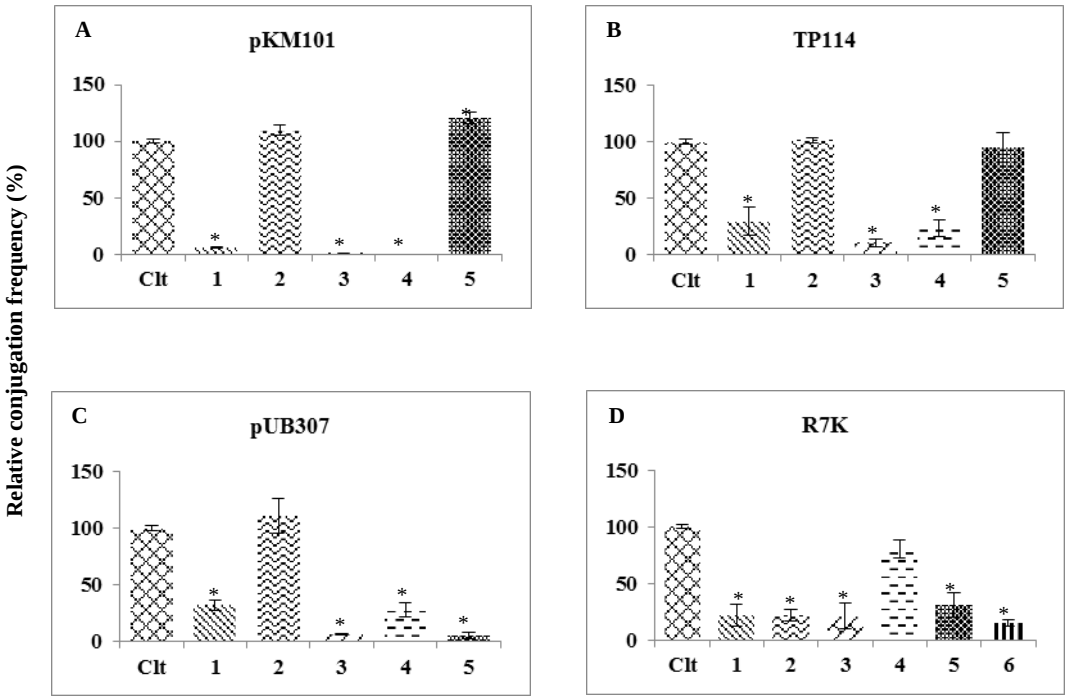


Fig 1. The effect of selected isothiocyanates on the conjugal transfer of: (**A**) IncN plasmid pKM101, (**B**) $IncI_2$ plasmid TP114 (**C**) IncP plasmid pUB307 and (**D**) IncW plasmid R7K, expressed as a percentage relative to a control (**Clt**, without a test compound). The isothiocyanates were tested at sub-inhibitory concentrations: allyl isothiocyanate (**1**, 100 mg/L), L-sulforaphane (**2**, 16 mg/L), benzyl isothiocyanate (**3**, 32 mg/L), phenylethyl isothiocyanate (**4**, 64 mg/L) and 4-methoxyphenyl isothiocyanate (**5**, 100 mg/L). Linoleic acid (**6**), a known anti-conjugant for IncW plasmids was tested at 200 mg/L. Values represent means ± standard deviations of at least three independent experiments measured by the plate conjugation assay. *, $P < 0.05$ (was significantly different from the control).

Figure 2

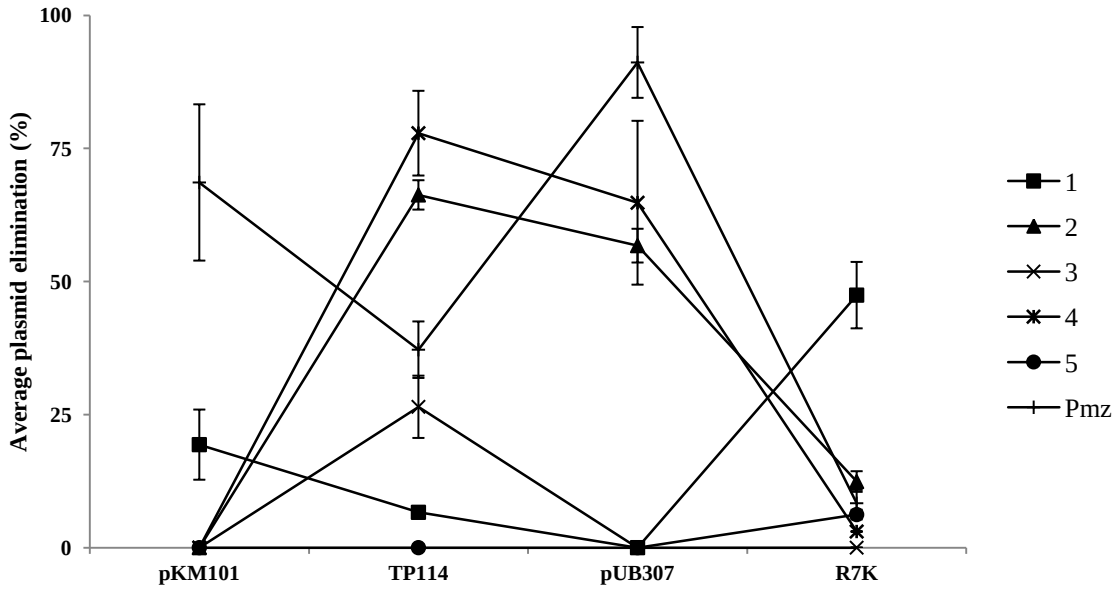


Fig 2. Plasmid elimination activity of the isothiocyanates. The isothiocyanates were tested at concentrations: allyl isothiocyanate (**1**, 100 mg/L), L-sulforaphane (**2**, 16 mg/L), benzyl isothiocyanate (**3**, 32 mg/L), phenylethyl isothiocyanate (**4**, 64 mg/L), 4-methoxyphenyl isothiocyanate (**5**, 100 mg/L) and promethazine (**Pmz**, 16 mg/L).

Figure 3

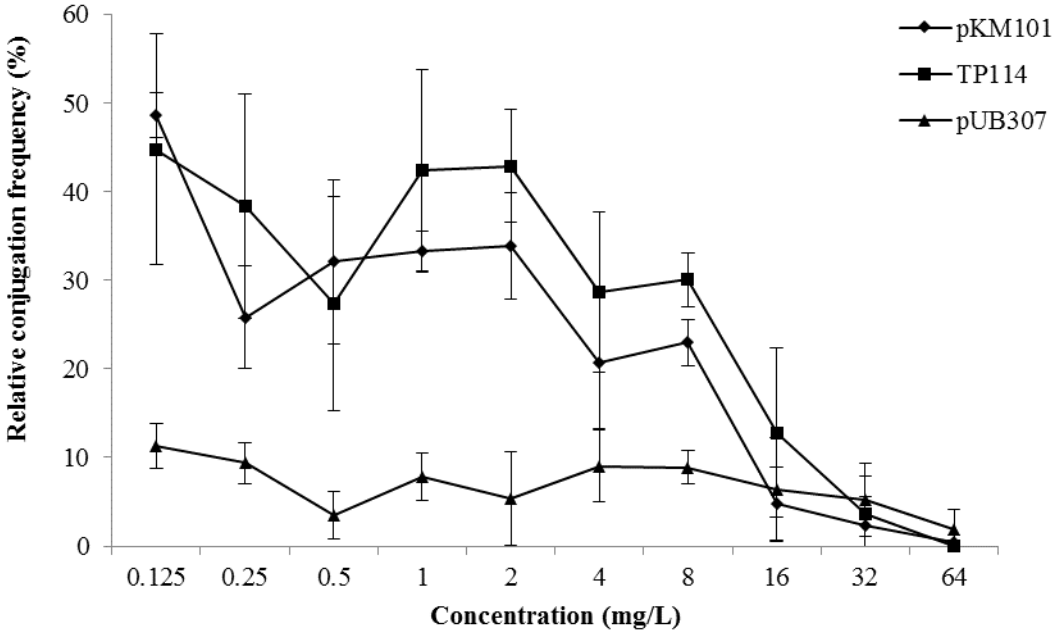


Fig 3. The effect of increasing concentration of benzyl isothiocyanate (**3**) on the conjugal transfer of plasmids pKM101 (IncN), TP114 ($IncI_2$) and pUB307 (IncP) relative to a control, without test sample (100% conjugation frequency). The values represent the mean ± SD of a least three independent experiments measured by plate conjugation assay.

Figure 4

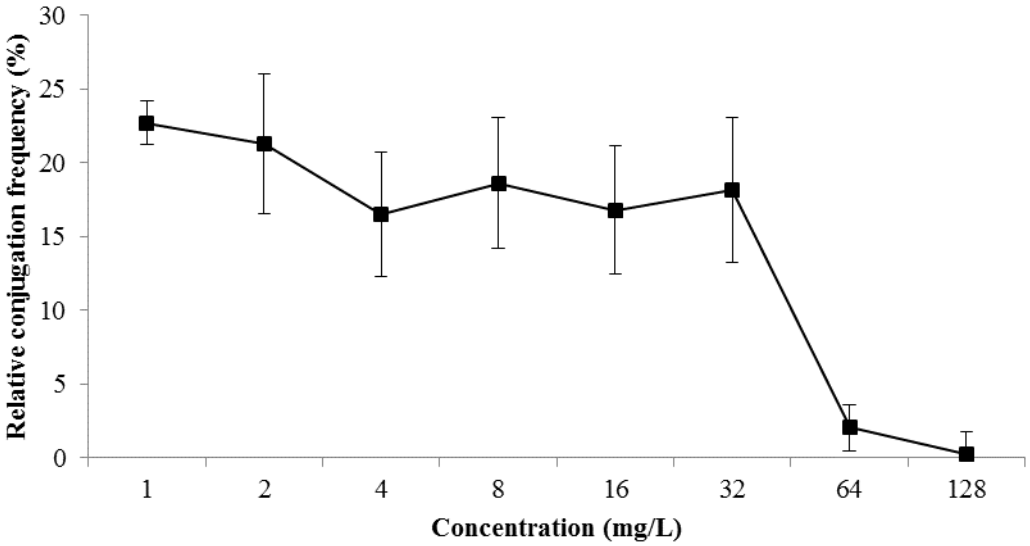


Fig 4. The effect of increasing concentration of 4-methoxyphenyl isothiocyanate (**5**) on the conjugal transfer of IncP plasmid pUB307 relative to a control, without test sample (100% conjugation frequency). The values represent the mean ± SD of a least three independent experiments measured by plate conjugation assay.

Figure 5

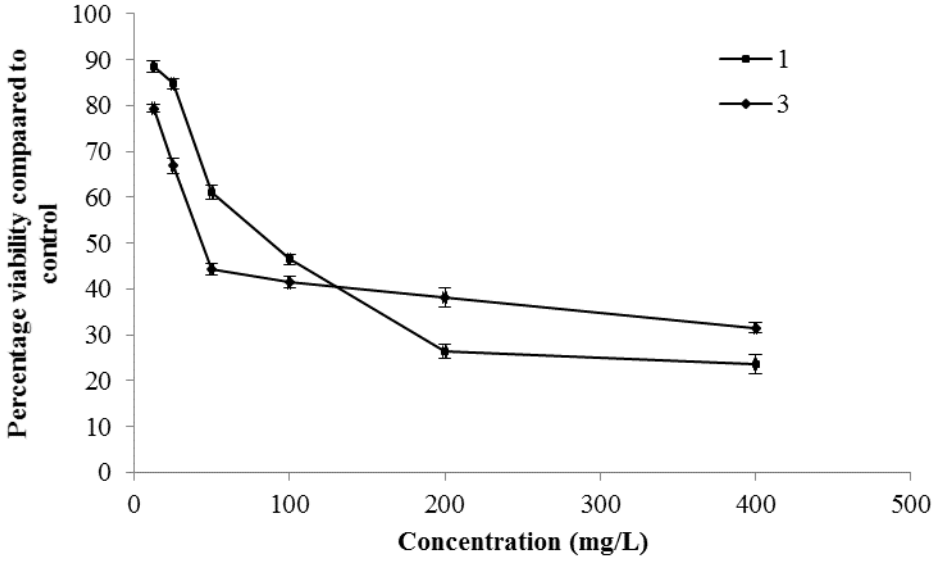


Fig 5. The effect of allyl (**1**) and benzyl (**3**) isothiocyanates on growth HDFa; C-013-5C. The values represent the mean ± SD of a least three independent experiments measured by cytotoxicity assay.

Supplementary data

Click here to download Supplementary data: Supplementary data.doc

Inhibiting plasmid mobility: the effect of isothiocyanates on bacterial conjugation

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Abstract

Bacterial conjugation is the main mechanism for the transfer of multiple antibiotic resistance genes among pathogenic microorganisms. This process could be controlled by compounds that inhibit bacterial conjugation. In this study, the effect of allyl isothiocyanate, L-sulforaphane, benzyl isothiocyanate, phenylethyl isothiocyanate and 4-methoxyphenyl isothiocyanate on the conjugation of broad host range plasmids, which harbor various resistance genes in *Escherichia coli* were investigated; pKM101 (IncN), TP114 ($IncI_2$), pUB307 (IncP) and the low copy number IncW plasmid R7K. Benzyl isothiocyanate (32 mg/L) significantly reduced the conjugal transfer of pKM101, TP114 and pUB307 to 0.3±0.6%, 10.7±3.3% and 6.5±1.0%, respectively. L-sulforaphane (16 mg/L, transfer frequency 21.5±5.1%) and 4-methoxyphenyl isothiocyanate (100 mg/L, transfer frequency 5.2±2.8%) were the only compounds that showed anti-conjugal specificity by actively reducing the transfer of R7K and pUB307, respectively.

Keywords: Isothiocyanates, bacterial conjugation, conjugative plasmids, effector proteins, horizontal gene transfer, plasmid incompatibility groups

1. Introduction

Bacterial conjugation is an adaptive mechanism that allows bacteria to transfer genetic materials, effector proteins and/or toxins from one cell to the other through a conjugative bridge [1, 2]. The genetic materials that are transferred via conjugation usually confer selective advantages to the recipient organism, such as survival, resistance, pathogenicity, infection activities and/or the ability to respond to environmental changes. Conjugation greatly increases bacterial genome plasticity, and has immense clinical relevance as a major route for the spread of multiple antibiotic resistance genes among the microbial community and virulence genes from pathogen to host [2]. It is therefore imperative to find ways to combat conjugation, as a means to decrease the ongoing rise of antibiotic-resistant infections.

Inhibition of bacterial conjugation has not received much research attention because the focus has been on the identification of new classes of antibacterial agents that target processes essential for bacterial growth such as cell wall biosynthesis, the cell membrane, protein synthesis, nucleic acid synthesis and metabolite activity. This traditional approach has produced many therapeutically useful agents so far, but the challenge is that an antibiotic also introduces selective pressure promoting resistant bacteria, and therefore this has led to the current antibiotic resistance crisis. An additional approach of reducing the increasing rate of bacterial antibiotic resistance dissemination and re-sensitizing bacteria to existing antibiotics, would be to target non-essential processes such as conjugation, which are less likely to evoke bacterial resistance. This approach could also have a prophylactic use in cosmeceuticals to reduce plasmid transfer. In addition to bacterial conjugation, other non-essential processes such as plasmid replication [3-5] and plasmid-encoded toxin-antitoxin systems [6, 7] have been exploited with promising potential in antibacterial therapy.

The few efforts directed towards identifying anti-conjugants include small-molecule inhibitors of *Helicobacter pylori cag* VirB11-type ATPase Cagα [8]. The *cag* genes encode for the assembly of the conjugative bridge and injection of the CagA toxin into host cells [8, 9]. In addition, there have been other reports of promising anti-conjugants such as dehydrocrepenynic acid [1], linoleic acid [1], 2-hexadecyanoic acid [10], 2-octadecynoic acid [10], and tanzawaic acids A and B [11]. However, these compounds have stability, toxicity or scarcity issues that need to be addressed. Therefore there is the pressing need to identify safer anti-conjugants to help in the fight against plasmid-mediated transfer and spread of antibiotic resistance and virulence.

In this study, four naturally occurring isothiocyanates (allyl isothiocyanate (**1**), L-sulforaphane (**2**), benzyl isothiocyanate (**3**), phenylethyl isothiocyanate (**4**)) and a synthetic isothiocyanate (4-methoxyphenyl isothiocyanate, **5**) were investigated for their anti-conjugant activity against *E. coli* strains bearing conjugative plasmids with specific antibiotic resistance genes. Isothiocyanates are usually naturally occurring hydrolytic products of glucosinolates, which are commonly found in the *Brassica* vegetables. They are produced when damaged plant tissue releases the glycoprotein enzyme myrosinase, which hydrolyses the *β*-glucosyl moiety of a glucosinolate. This leaves the unstable aglycone, thiohydroxamate-*O*-sulfonate, which rearranges to form an isothiocyanate or other breakdown products [12, 13]. Other isothiocyanates such as 4-methoxyphenyl isothiocyanate and methyl isothiocyanate are synthetically produced and not naturally occurring.

In addition to the anti-conjugant testing, plasmid curing activity and bacterial growth inhibition were also evaluated to help discriminate between true anti-conjugants and substances that reduce conjugation due to elimination of plasmids or function by perturbation

of bacterial growth or physiology. Isothiocyanates possessing the highest anti-conjugant activities were further investigated for cytotoxicity against human dermal fibroblast adult cells (HDFa; C-013-5C).

2. Materials and methods

2.1. Bacterial strains and plasmids

E. coli NCTC 10418 (a susceptible Gram-negative strain), *S. aureus* ATCC 25923 (a susceptible Gram-positive strain), *S. aureus* SA-1199B (a fluoroquinolone-resistant strain, which over-expresses the multidrug-resistant NorA pump) and *S. aureus* XU212 (a tetracycline-resistant strain, which over-expresses the multidrug-resistant TetK pump) were used for the broth dilution assay. Plasmid-containing *E. coli* strains WP2, K12 J53-2 and K12 JD173 were used as donor strains in the plate conjugation and plasmid elimination assays. *E. coli* ER1793 (streptomycin-resistant) and *E. coli* JM109 (nalidixic-resistant) were used as the recipients. Conjugative plasmids used were pKM101 (WP2; incompatibility group N (IncN); ampicillin-resistant), TP114 (K12 J53-2; $IncI_2$; kanamycin-resistant), and R7K (K12 J53-2; IncW; ampicillin-, streptomycin- and spectinomycin-resistant), which were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and conjugative plasmid pUB307 (K12 JD173; IncP; ampicillin-, kanamycin- and tetracycline-resistant) was provided by Prof. Keith Derbyshire, Wadsworth Center, New York Department of Health.

2.2. Broth micro-dilution assay

The antibacterial activity was determined with the broth micro-dilution method as described previously [14], which is a modified version of the procedured described in the British Society for Antimicrobial Chemotherapy (BSAC) guide to sensitivity testing. Bacteria were cultured on nutrient agar slants and incubated at 37°C for 18 hours. A bacterial suspension equivalent to a 0.5 McFarland standard was made from the overnight culture. This was added to Muller-Hinton broth and the test isothiocyanate, which had been serially diluted across a 96-well microtitre plate, to achieve a final inoculum of 0.5×10^5 CFU/mL. Minimum

inhibitory concentrations (MICs) were determined after 18 hours of incubation at 37°C. This was done by visual inspection after the addition of a 1 mg/mL methanolic solution of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) and incubation at 37°C for 20 minutes. This experiment was performed in duplicate in two independent experiments.

2.3. Liquid conjugation assay

The donor cells with plasmids pKM101, TP114 and pUB307 were paired with the recipient ER1793. Plasmid R7K donor cells were paired with the recipient JM109. Research has shown that plasmid carriage by host bacteria is associated with some fitness cost (burden) [15, 16]. This fitness effect of plasmids plays a vital role in their ability to associate with a new bacterial host. As a consequence of this we selected different *E. coli* host, which are known to successfully conjugate [17, 18] and to maintain the study plasmids. The liquid conjugation assay was performed as previously described [19] with slight modifications. Equal volumes (20 μL) of donor and recipient, for which the colony forming units per mL (CFU/mL) had been predetermined (Supplementary Table 1), were introduced into 160 μL of Luria-Bertani broth and the test sample or control. This was incubated at 37°C for 18 hours after which the number of transconjugants and donor cell were determined using antibiotic-containing MacConkey agar plates. A positive control linoleic acid [1] and negative control (donor, recipient and media; without drug or test sample) were included in the experiment. The isothiocyanates were evaluated for anti-conjugant activity at sub-inhibitory concentration (one-quarter of the MIC). Antibiotics were added at the following concentrations for positive identification of donors, recipients and transconjugants (mg/L): amoxicillin (30), streptomycin sulphate (20), nalidixic acid (30), kanamycin sulphate (30). Conjugation frequencies were calculated as the ratio of total number of transconjugants (cfu/mL) to the total number of donor (cfu/mL) and expressed as a percentage relative to the negative control.

This experiment was performed as duplicate in three independent experiments and anti-conjugation activity is reported as the mean ± standard deviation.

2.4. Plasmid elimination assay

This assay was performed as described previously [20] with minor modifications. The *E. coli* donor strains were sub-cultured on appropriate antibiotic-containing MacConkey agar plates to ensure plasmid presence. After incubation of the plates at 37°C for 18 hours, single colonies (2-3) were selected and inoculated into LB. This was incubated for 18 h at 37°C and the colony forming units were determined prior to the assay. Twenty microliters of the overnight culture was then added to a mixture of 180 µL LB and test sample in a 96 well microtitre plate. This was incubated overnight (18 h) at 37°C and subsequently serially diluted, 20 µL was then plated on antibiotic containing MacConkey agar and incubated for 18 h at 37°C. The isothiocyanates were evaluated for plasmid elimination activity at concentrations used in the liquid conjugation assay. Both positive control (promethazine) [21-23] and negative control (mixture without isothiocyanate or control drug) were included in this experiment. Plasmid elimination was calculated using:

$$\text{Plasmid elimination} = \frac{\text{CFU/mL of control} - \text{CFU/mL of test sample}}{\text{CFU/mL of control}} \times 100$$

Antibiotics and concentrations used in MacConkey agar for positive identification of *E. coli* cells harbouring plasmids (mg/L) were: amoxicillin (30), kanamycin sulphate (20 and 30) and nalidixic acid (30). This experiment was performed in duplicate, with three independent experiments.

2.5. Cytotoxicity assay

The isothiocyanates that showed anti-conjugant activity were further assessed for their effect on eukaryotic cell growth. The sulforhodamine B (SRB) colorimetric assay as described

previously [24] was used, with modifications. Human dermal fibroblast, adult cells (HDFa; C-013-5C) were grown in a 75 cm^2 culture flask at 37°C in humidified atmosphere of 5% carbon dioxide using Dulbecco's Modified Eagle's Medium, which was supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, 0.1% gentamicin and amphotericin B. The grown cells were seeded in a 96 well microtitre plated and test samples and media were added. This was then incubated at 37°C in 5% CO_2 for 72 h. Afterwards 50 µL of cold 40% $^{W}/_{V}$ trichloroacetic acid (TCA) solution was added, the plate was placed in the fridge for an hour at 4°C and washed four times with distilled water. The cells were then stained with 0.4% $^{W}/_{V}$ SRB solution and left at room temperature for an hour. Afterwards, the plate was rinsed four times with 1% acetic acid and left overnight (24 h) to dry. Thereafter, 100 µL of 10 mM Tris buffer solution was dispensed into the wells and agitated in an orbital shaker for 5 min, to allow solubilisation of SRB-protein complex. The optical density (OD) was then measured at 510 nm using a microtitre plate reader (Tecan Infinite® M200). The percentage of viable cell was calculated using:

$$\text{Percentage of viable cell} = \frac{\text{OD of test sample-OD of blank}}{\text{OD of negative control-OD of blank}} \times 100$$

This experiment was performed as triplicate in three independent experiments and cytotoxicity has been reported as mean ± standard deviation.

2.6. Statistical analyses

The statistical analyses were carried out using Excel Data Analysis and GraphPad Prism 7. Welch's t-test was used to evaluate the difference between the control conjugal transfer frequency and the test compounds. Results with $p < 0.05$ were considered statistically significant.

3. Results

3.1. The effect of isothiocyanates on the growth of bacteria

To test whether the selected isothiocyanates had growth inhibitory activity against bacterial species and to inform of a suitable concentration for their evaluation in an anti-conjugation assay, the isothiocyanates were tested against susceptible Gram-negative (*E. coli* NCTC 10418) and Gram-positive (*S. aureus* ATCC 25923) standard isolates, and antibiotic effluxing *Staphylococcus aureus* strains (SA-1199B and XU212). Table 1 shows the MIC values for the tested isothiocyanates; their inhibitory activity varied from 16 to 512 mg/L against the evaluated bacteria. Our general observation was that unsurprisingly the isothiocyanates were marginally more active against the Gram-positive than the Gram-negative strains.

3.2. The effect of isothiocyanates on conjugal transfer of plasmids

To investigate whether the selected isothiocyanates have anti-conjugant activity, a range of plasmids belonging to different incompatibility groups (IncN plasmid pKM101, $IncI_2$ plasmid TP114, IncP plasmid pUB307 and IncW plasmid R7K) were employed to test the specificity of conjugation inhibition in *E. coli*. With information (Table 1) about their minimum inhibitory concentration against *E. coli* NCTC 10418 (a susceptible standard strain), the isothiocyanates were tested at a sub-inhibitory concentration, one-quarter of their MICs. Figure 1 shows the effect of the isothiocyanates on the conjugal transfer of the test plasmids. The test isothiocyanates exhibited inhibitory activities ranging from complete reduction in conjugation frequency (0%, considered active), inhibition of conjugation frequency to less than 10% were also considered as active; 10 to 50% were considered moderately active and greater than 50% were considered as inactive.

3.3. Elimination of plasmids from E. coli

To determine whether the observed anti-conjugant activity was not due to the elimination of conjugative plasmids, the donor cells were grown in the presence of the test isothiocyanates, and the plasmid elimination assay was performed. Figure 2 shows the effect of the isothiocyanates on conjugative plasmids. The isothiocyanates exhibited varied plasmid curing activity. Plasmids TP114 and R7K of the incompatibility groups I_2 and P, respectively, were the most eliminated in the donor cells with elimination percentages ranging from 3.0±0.1 to 77.8±8.0. Most of the tested isothiocyanates did not have any plasmid curing effects on pKM101 (IncN), with the exception of allyl isothiocyanate (**1**), which showed a curing effect of 19.4±6.6 %. For pUB307 (IncP), a plasmid curing effect was observed for L-sulforaphane (**2**, 56.7±3.2 %) and phenylethyl isothiocyanate (**4**, 64.8±15.4 %).

*3.4. The effect of increasing concentration of benzyl isothiocyanate (**3**) on conjugal transfer of the plasmids pKM101 (IncN), TP114 (IncI_2) and pUB307 (IncP)*

With benzyl isothiocyanate (**3**) having shown broad range anti-conjugant (conjugal reduction to 0.3±0.6 - 10.7±3.3%, Figure 1) and the least donor plasmid elimination activity (0 - 26.5±5.9%, Figure 2) of all tested compounds, it was further assessed to observe its effect on conjugal transfer with increasing concentration. Generally, there was a gradual increase in anti-conjugal activity against pKM101 and TP114 with increase in concentration from 0.125 to 64 mg/L (Figure 3). This was not the same for plasmid pUB307, there was no significant change in anti-conjugal activity for benzyl isothiocyanate (**3**), and it surprisingly remained active at the low concentrations tested. The observed conjugal transfer of pUB307 in the presence of **3** ranged between 11.3±2.6% and 1.9±2.2% for concentrations of 0.125 and 64 mg/L, respectively.

3.5. *Effect of increasing concentration of 4-methoxyphenyl isothiocyanate (**5**) on conjugal transfer of pUB307*

Among the test isothiocyanates, 4-methoxyphenyl isothiocyanate (**5**) was the most active against IncP plasmid pUB307, with no plasmid curing activity. It was therefore evaluated for the effect of increasing concentration (1-128 mg/L) on the conjugal transfer of plasmid pUB307. The observed activities are shown in Figure 4**.** 4-methoxyphenyl isothiocyanate (**5**) showed a moderate anti-conjugant activity (22.7±1.6%) at the lowest concentration (1 mg/L) and this was steadily maintained up to 32 mg/L, after which there was a sharp increase in conjugal inhibition. Almost complete conjugal inhibition was observed at 128 mg/L.

*3.6. Effect of allyl (**1**) and benzyl (**3**) isothiocyanates on normal growth of human dermal fibroblast, adult cells (HDFa; C-013-5C)*

Allyl (**1**) and benzyl (**3**) isothiocyanates that exhibited active to moderate anti-conjugant activity against all test plasmids were further assessed for cytotoxicity against normal cell growth. This was to determine whether the broad range anti-conjugant activity exhibited by isothiocyanates **1** and **3** were not at cytotoxic concentrations and worth pursuing as potential anti-conjugants for further development. The observed cytotoxic activities are shown in the Figure 5. The IC_{50} for allyl (**1**) and benzyl (**3**) isothiocyanates against HDFa cells was 63.9 mg/L (645 μM) and 30.3 mg/L (203 μM), respectively.

4. Discussion

The discovery of a potent compound that will inhibit the spread of resistance genes and/or resistance mechanisms has clinical relevance, especially in this era of plasmids within species such as *K. pneumoniae* that are carbapenem-resistant. This is highly timely given the lack of treatment options for infections caused by this pathogen. In line with this, selected

isothiocyanates, which are hydrolysis products of glucosinolates commonly found in *Brassica* vegetables, were investigated for the possibility of inhibiting the spread of resistance genes by blocking bacterial conjugation in *E. coli*.

The initial findings from this study showed that allyl isothiocyanate (**1**), L-sulforaphane (**2**), benzyl isothiocyanate (**3**), phenylethyl isothiocyanate (**4**) and 4-methoxyphenyl isothiocyanate (**5**) have some level of antibacterial activity that ranged from 16 to > 512 mg/L against the susceptible *E. coli* NCTC 10418 and *S. aureus* ATCC 23925, and the effluxing multidrug-resistant *S. aureus* strains (SA-1199B and XU212) (Table 1). This corroborates the reported antibacterial activity of the isothiocyanates but due to the variability in the testing methods, bacterial inoculum densities and diversity in susceptibility, it is difficult to compare results [25-31]. The isothiocyanates were found to be less potent in comparison to conventional antibiotics and similar results have been reported by others [25, 27, 31]. Among the tested isothiocyanates, **4** was the most potent against the Gram-positive microbes with MIC values ranging from 16 to 32 mg/L followed by **2** (MIC values ranged from 32 to 64 mg/L), which was also the most potent against Gram-negative *E. coli* NCTC 10418. The antibacterial activity of these isothiocyanates have been explained to be due to their ability to cause physical membrane damage [32, 33], interfere with bacterial redox system, which affects the cell membrane potential [33] or the disruption of major metabolic processes [34, 35].

With the anti-conjugal activity study, broad-range anti-conjugant activity was observed for allyl (**1**) and benzyl isothiocyanate (**3**) at sub-inhibitory concentrations, with **3** being the most potent among the test isothiocyanates (Figure 1). It inhibited the conjugation of plasmids pKM101 (IncN), TP114 (IncI_2) and pUB307 (IncP), and selectively cured plasmid TP114, only. Against plasmids pKM101 and TP114, **3** also reduced conjugal transfer by 97.7±3.3%

and 96.4±4.2%, respectively at 32 mg/L (214.46 μM), and its activity gradually declined with decreasing concentration (Figure 3). This was not the same for pUB307, where **3** continued to show pronounced activity with a 90.8±2.3% reduction in conjugation, even at a low concentration of 0.25 mg/L (1.68 μM). This was interesting, as **3** did not show any plasmid curing activity against this particular plasmid pUB307 and pKM101, ruling out the fact that the observed anti-conjugation may be due to plasmid elimination. Another area of interest was that **3** exhibited broad-range activity; this could mean that **3** either acts on a common target site on the conjugation machinery or that it causes general cell toxicity. However, considering the MIC value (128 mg/L, Table 1) of **3** against the susceptible *E. coli* strain NCTC 10418, the concentrations (≤ 32 mg/L) used for the conjugation assays were at sub-lethal doses and it is less likely to have caused general cell toxicity. With allyl isothiocyanate (**1**), moderate plasmid elimination activity was observed against most of the test plasmids and this may be an indication that its broad-range anti-conjugant activity is due to plasmid curing. The broad-range of activity of **1** and **3** prompted their testing against normal growth of human dermal fibroblast, adult cells (HDFa; C-013-5C). A comparison of the cytotoxic value of **3** against HDFa cells (30.30 mg/L; 203.07 μM) with its anti-conjugant concentration against the test plasmids showed that its IC_{50} level was above the concentrations needed to cause a 50% reduction in conjugal transfer of plasmids; pKM101 (IC_{50} = 2.19 mg/L; 14.68 μM), TP114 (IC_{50} = 1.24 mg/L; 8.31 μM) and pUB307 (IC_{50} = 0.34 mg/L; 2.28 μM) (Figure 5). This suggests that **3** showed anti-conjugant activity at non-toxic concentrations. However, the same cannot be said for compound **1** because, its IC_{50} against HDFa (63.9 mg/L; 644.48 μM) was below the 100 mg/L needed to cause moderate anti-conjugant activity (90-50% reduction) against most of the test plasmids. It is therefore suggested that the concentrations needed to cause a 50% reduction in conjugation is most likely to be closer to the cytotoxic-IC_{50} value.

From this study, specificity of anti-conjugal and plasmid curing activity was observed for 4-methoxyphenyl isothiocyanate (**5**), a synthetic compound. L-sulforaphane (**2**) also exhibited some level of anti-conjugant specificity against the IncW plasmid R7K at 16 mg/L (90.25 μM), but at this same concentration plasmid curing was observed and hence **2** is not a true anti-conjugant (Figure 2). Compound **5**'s anti-conjugant activity at 100 mg/L (605.29 μM) was pronounced for the IncP plasmid pUB307, with a 94.8±2.8% reduction in conjugation, but it showed minimal inhibition or promoted conjugation for the other test plasmids (Figure 1). Its anti-conjugant activity was however concentration-dependent (Figure 4). With the plasmid curing effect, **5** showed elimination of only the IncW plasmid R7K, but it did not have any effect on conjugation of this plasmid. This may give an indication that **5** could have some conjugation promotion factors, and this was observed for pKM101. Conjugation of pKM101 in the presence of **5** exceeded 100% (Figure 1). The anti-conjugation, plasmid curing and pro-conjugation activity exhibited by **5** supports its specificity. This suggests that compound **5** acts on a specific target site, which may not be common to all plasmids. Consequently, it is less likely for resistance to develop against **5** unlike other compounds that target general and essential targets of bacteria, which is the case in many instances of antibiotic resistance [36]. A general observation with the test isothiocyanates is that the presence of oxygen, attached to sulphur or an aromatic carbon conferred some level of anti-conjugal specificity. We therefore hypothesize that the methoxyl substituent on the aromatic ring and the lack of a hydrocarbon chain of **5**, which makes it structurally different from the other test aromatic isothiocyanates, may have contributed to its specificity of activity.

In conclusion, isothiocyanate **3** and **5** were the most promising anti-conjugants identified in this study. Further explorative studies involving structural modification and mechanistic

studies of these isothiocyanates could possibly lead to the identification of a potent anti-conjugant. This will help decrease the spread of multidrug-resistant genes, multidrug resistant bacteria, reduce virulence and help reinstate existing antibiotics.

Declarations

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Supplementary material

Supplementary material relating to this article has been attached.

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