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Effect of subinhibitory concentrations of four commonly used biocides on the conjugative transfer of Tn*916* in *Bacillus subtilis*

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Objectives: Large amounts of biocides are used to reduce and control bacterial growth in the healthcare sector, food production and agriculture. This work explores the effect of subinhibitory concentrations of four commonly used biocides (ethanol, hydrogen peroxide, chlorhexidine digluconate and sodium hypochlorite) on the conjugative transposition of the mobile genetic element Tn*916*.

Methods: Conjugation assays were carried out between *Bacillus subtilis* strains. The donor containing Tn*916* was pre-exposed to subinhibitory concentrations of each biocide for a defined length of time, which was determined by an analysis of the transcriptional response of the promoter upstream of *tet*(M) using β-glucuronidase reporter assays.

Results: Ethanol significantly ($P=0.01$) increased the transfer of Tn*916* by 5-fold, whereas hydrogen peroxide, chlorhexidine digluconate and sodium hypochlorite did not significantly affect the transfer frequency.

Conclusions: These results suggest that exposure to subinhibitory concentrations of ethanol may induce the transfer of Tn*916*-like elements and any resistance genes they contain.

Keywords: ethanol, hydrogen peroxide, chlorhexidine digluconate, sodium hypochlorite, resistance gene transfer, Tn*916*

Introduction

Biocides are chemical compounds capable of inactivating microorganisms.[1] These are used for disinfection, antisepsis and preservation to inhibit or reduce bacterial loads in various settings, such as healthcare, agriculture and the food industry.[1,2] In Denmark, the yearly consumption of biocides has been estimated to comprise up to 5000 tonnes,[2] compared with <160 tonnes of antimicrobial agents for therapy.[3] Despite the widespread use of these compounds, our knowledge about their mode of action, especially at subinhibitory concentrations, and the microbial response to exposure is relatively limited.[4,5] The working concentrations of disinfectants and antiseptics are generally much higher than the lethal dose; however, the efficacy of a biocide can be significantly reduced due to the presence of organic matter (e.g. blood, serum, pus and food debris), overdilution or insufficient contact time with microorganisms. Also, the presence of residual concentrations might result in bacterial exposure to subinhibitory concentrations.[6]

During recent years, it has been suggested that the use of biocides might lead to increased resistance to antimicrobial agents used for treatment of humans and animals, through increasing mutation rates in bacteria or increasing horizontal gene transfer;[7,8] however, evidence for such an increase is currently lacking.[9] Previous studies have shown that stress in bacteria caused by subinhibitory concentrations of antibiotics can promote the transfer of antibiotic resistance and virulence genes.[10–13] Plasmid conjugation or transduction of resistance determinants in *Staphylococcus aureus* has been found to be either not affected or reduced by subinhibitory concentrations of a number of biocides, including povidone-iodine, chlorhexidine and different quaternary ammonium compounds.[14,15] However, one compound, cetrimide, was found to cause a pronounced increase in the transduction efficiency.[14] Effects of biocides on the transfer of other resistance determinants and in other species have, however, not been studied.

Mobile genetic elements, such as conjugative transposons, are important vectors in the dissemination of antibiotic resistance determinants. Tn*916* is a conjugative transposon and the prototype of a large family of related elements. They have an extremely broad host range, including >30 bacterial genera, and have been found in both pathogenic and commensal bacteria.[16] Most of

these elements contain the tetracycline resistance gene *tet*(M), but some members of this family also confer resistance to other antimicrobial agents, e.g. macrolides, kanamycin, mercury and cetrimonium bromide. Furthermore, Tn*916*-like elements have also been found to contain non-conjugative transposons (e.g. Tn*917*), which contain additional antibiotic resistance genes.[16,17] Transcription of *tet*(M) in Tn*916* leads to the transcription of downstream genes involved in recombination and conjugation of the element. Transcription of *tet*(M) is regulated by a tetracycline-dependent transcriptional attenuation mechanism reliant on the levels of charged tRNA molecules within the cell.[18,19] It has subsequently been suggested that any stress that the cell encounters (other than exposure to tetracycline) that results in the build-up of charged tRNAs is also likely to cause an increase in the transcription of *tet*(M) and downstream genes and possibly an increase in transfer.[16]

The aim of this study was to test this hypothesis by investigating the effect of subinhibitory concentrations of four commonly used biocides (ethanol, hydrogen peroxide, chlorhexidine digluconate and sodium hypochlorite) on the conjugative transposition of Tn*916* between *Bacillus subtilis* strains. *B. subtilis* was used as it has suitable genetic tools available, is genetically easy to manipulate and is a model organisms for the analysis of Tn*916* biology.

Materials and methods

Chemicals and reagents

Chloramphenicol, fusidic acid sodium salt, rifampicin, streptomycin sulphate salt and tetracycline hydrochloride were purchased from Sigma. Tetracycline discs (30 μg) were from Oxoid. Brain heart infusion (BHI) agar and broth were obtained from either Oxoid or Difco and BBL™ Mueller–Hinton II broth (MH II) was from Becton, Dickinson and Company. Pre-made tryptone soya agar plates with 5% sheep blood (blood agar plates) were purchased from Oxoid. The biocides included in this study were chlorhexidine digluconate [20% (w/v)] from Alfa Aesar, hydrogen peroxide (30% solution) from Fluka, sodium hypochlorite (10%–15% available chlorine) from Sigma and absolute ethanol from either BDH Prolabo or Kemetyl AB. 4-Nitrophenyl β-D-glucuronide was obtained from Sigma.

Bacterial isolates

All bacterial isolates and plasmids included in this study are listed in Table 1.

Construction of *B. subtilis gusA* reporter strain

In order to determine the optimal time of exposure to subinhibitory concentrations of biocides and tetracycline (positive control compound) prior to filter mating, a reporter construct was generated in *B. subtilis* as follows. A 450 bp fragment of Tn*916*, which includes the *tet*(M) promoter, *orf12* and the terminator sequences was amplified by PCR using the primers P*tet*(M) For (5′-GGCGGCGGGTACCCAAAGCAACGCAGGTATCTC-3′) and P*tet*(M) Rev (5′-GGCGGCGGAATTCGTGATTTTCCTCCAT-3′). The restriction sites KpnI and EcoRI were included in the primers (underlined). Next, the cwp2 promoter fragment was removed from the pUC19-based pCBR026[22] and replaced with the KpnI-EcoRI-digested *tet*(M) promoter amplicon, resulting in pUC19-P*tet*(M)-*gusA*. The fused P*tet*(M)-*gusA* was excised from pUC19-P*tet*(M)-*gusA* on a KpnI-BamHI fragment and directionally cloned into the corresponding sites of pHCMC05, resulting in the *B. subtilis* reporter construct pHCMC05-P*tet*(M)-*gusA*. The construct was then transformed into *B. subtilis* BS34A[21] using a previously described protocol.[23] BS34A contains a wild-type copy of Tn*916* providing resistance to tetracycline. BS34A was used as the reporter strain and the donor strain in all of the transfer studies.

Determination of biocide MICs by the broth microdilution method

The MIC of each of the four biocides was determined as recommended by the CLSI guidelines.[24] Isolates were grown overnight (ON) on blood agar or BHI agar plates at 37°C. Colonies were resuspended in 0.9% NaCl to a turbidity equivalent to that of a 0.5 McFarland standard or an OD_{600} between 0.08 and 0.13, and then 100-fold diluted in MH II. Biocide working solutions were prepared in MH II just before 2-fold dilution series were made in 96-well round-bottomed microtitre plates (Nunc) (50 μL per well). Then, 50 μL of the cell suspension was transferred to the microtitre plate and incubated for 16–20 h at 37°C under aerobic conditions. *Escherichia coli* ATCC 25922 or *S. aureus* ATCC 29213 were included as control strains to test reproducibility of the susceptibility testing procedure. The MIC value was defined as the lowest concentration of the compounds giving rise to

Table 1. Bacterial isolates and plasmids included in this study

	Relevant properties	Reference or source
B. subtilis		
CU2189	recipient strain	[20]
BSCU2189RF	RIF- and FUS-resistant derivative of CU2189	this study
BS34A	*B. subtilis*::Tn*916* (CU2189×FM12A); TET^R, contains a single copy of Tn*916*	[21]
BS34ASTR	STR-resistant derivative of BS34A	this study
BS34A::pHCMC05-P*tet*(M)-*gusA*	BS34A including plasmid pHCMC05 containing a P*tet*(M)-*gusA* construct and CHL^R marker	this study
Plasmids		
pCBR026	pUC19 containing the *gusA* reporter gene under the control of the cwp2 promoter from *Clostridium difficile*	[22]
pUC19-P*tet*(M)-*gusA*	pCBR026 with the cwp2 promoter replaced by the *tet*(M) promoter upstream of *gusA*	this study
pHCMC05	*E. coli*/*B. subtilis* shuttle vector	Bacillus Genetic Stock Centre, USA
pHCMC05-P*tet*(M)-*gusA*	pHCMC05 containing the *tet*(M) promoter upstream of *gusA*	this study

RIF, rifampicin; FUS, fusidic acid; TET, tetracycline; STR, streptomycin; CHL^R, chloramphenicol resistant; TET^R, tetracycline resistant.

no visible growth. MIC determinations were done in duplicate and as a minimum repeated twice.

Effect of biocides on β-glucuronidase enzyme activity

Culture preparation for β-glucuronidase enzyme assay

B. subtilis BS34A::pHCMC05-P*tet*(M)-*gusA* was grown ON at 37°C on BHI agar plates supplemented with 10 mg/L chloramphenicol to select for the reporter plasmid construct. Cells were inoculated in 20 mL of BHI (without chloramphenicol) and grown ON at 37°C with rotary shaking (200 rpm). ON cultures were diluted to an OD_{600} of ∼0.1 in 500 mL Erlenmeyer flasks containing a final volume of 100 mL of BHI broth and incubated at 37°C with shaking. After 2 h of growth, tetracycline (10 mg/L) or subinhibitory concentrations (0.25 × MIC) of ethanol (40 000 mg/L), hydrogen peroxide (20 mg/L), chlorhexidine digluconate (0.5 mg/L) or sodium hypochlorite (1250 mg/L) were added to the cultures. The OD_{600} was measured and 5 mL samples were collected before (1.5 and 2.0 h of growth) and after addition of the compounds (0.5, 1.0, 1.5 and 2.0 h of exposure). Cells were harvested by centrifugation (3000 **g**, 4°C, 10 min) and pellets were stored at −80°C. Cells from each of the 2.0 h samples were plated on BHI and BHI supplemented with 10 mg/L chloramphenicol agar to determine the stability of pHCMC05-P*tet*(M)-*gusA*.

Measurement of β-glucuronidase enzyme activity

The β-glucuronidase activity was measured as previously described[25] with some modifications. Cell pellets were thawed at room temperature and resuspended in 800 μL of Z-buffer (60 mM $Na_2HPO_4{\cdot}7H_2O$/40 mM $NaH_2PO_4{\cdot}H_2O$/10 mM KCl/1 mM $MgSO_4{\cdot}7H_2O$/50 mM 2-mercaptoethanol) adjusted to pH 7.0. An aliquot of 8 μL of toluene was added and the mixture was transferred to a new tube with ∼250 μL of unwashed glass beads (150–212 μm in diameter) (Sigma) and treated in a RiboLyser (Hybaid) at a speed of 6.5 for 2× 25 s, with a 1 min pause between the two runs. Lysates were cooled on ice for 2 min and glass beads were removed by centrifugation (3000 **g**, 4°C, 3 min). Four hundred microlitres of the supernatant was transferred to a fresh tube and 400 μL of Z-buffer was added. Samples were incubated at 37°C for 5 min and the enzyme reactions were started by adding 160 μL of 6 mM 4-nitrophenyl β-D-glucuronide. The enzymatic reaction was stopped after incubation at 37°C for 5 min with 400 μL of a 1 M disodium carbonate solution and cell debris was removed by centrifugation (3000 **g**, 25°C, 10 min). Finally, the OD_{405} values of the supernatants were measured and the specific enzyme activities were calculated using the following equation: $(A_{405}\times1000)/[OD_{600}\times t\ (\text{min})\times1.25\times\text{volume (mL)}]$.[25] Experiments were performed on three separate occasions, except for exposure to tetracycline, which was repeated six times.

Measurement of the effect of biocides on the conjugative transposition of Tn916

A derivative of *B. subtilis* BS34A, selected for resistance to streptomycin (BS34ASTR), was used as the donor strain and a derivative of *B. subtilis* CU2189, selected for resistance to rifampicin and fusidic acid (BSCU2189RF), was used as a recipient strain. Conjugation by filter mating was performed as previously described[26] with some modifications. *B. subtilis* BS34ASTR and BSCU2189RF were grown ON at 37°C on blood agar plates including a tetracycline disc on the donor plate for selection of Tn*916*. Colonies from ON plates were inoculated in 10 mL of BHI broth and incubated ON (without antimicrobial agents) at 37°C with shaking (150 rpm). ON cultures were diluted in pre-warmed BHI broth to an OD_{600} of ∼0.1 in 500 mL Erlenmeyer flasks to a final volume of 100 mL. Cultures were grown with shaking (200 rpm) until donor cultures reached an OD_{600} of between 0.5 and 0.6. Then, subinhibitory concentrations (0.25 × MIC) of ethanol (20 000 mg/L), hydrogen peroxide (10 mg/L), chlorhexidine digluconate (0.5 mg/L), sodium hypochlorite (1250 mg/L) and tetracycline (10 mg/L) were added to separate donor cultures and these were further grown for 2.0, 1.5, 0.5, 1.5 and 1.0 h, respectively. The length of exposure time of each compound was equal to the length of that expected to have the greatest effect on transcription from the promoter upstream of *tet*(M) based on the β-glucuronidase enzyme assay. Within each repetition, one culture where no compound was added served as the donor control. Then, donor and recipient cells were harvested (6000 **g**, 5 min, 4°C) and resuspended in BHI broth to an OD_{600} of ∼0.5 and 5.0, respectively, and mixed in a 1:1 volume, resulting in an output recipient:donor ratio of ∼1:1. An aliquot of 500 μL of each mixture was transferred to a sterile filter (0.45 μm, white gridded, 47 mm; Millipore) placed on a BHI agar plate. The cell mixtures were left to absorb into the filter for 0.5 h and then incubated at 37°C for 17.5 h. After incubation, mating filters were transferred to 10 mL of 0.9% NaCl and resuspended by vortex mixing. The numbers of donors and recipients were determined by counting on BHI agar supplemented with 10 mg/L tetracycline or 12.5 mg/L rifampicin and 5 mg/L fusidic acid, respectively, after 24 h of incubation at 37°C. Transconjugants were selected on BHI agar plates containing 10 mg/L tetracycline, 12.5 mg/L rifampicin and 5 mg/L fusidic acid and counted after 48 h of incubation at 37°C. At least 10 transconjugants from each transfer experiment were verified by subculturing on transconjugant plates twice and once on BHI agar plates supplemented with 100 mg/L streptomycin, on which only donor cells can grow. Transconjugants were also screened for the presence of *tet*(M) by PCR using primers *tet*(M)-1 (5′-GTTAAATAGTGTTCTTGGAG-3′) and *tet*(M)-2 (5′-CTAAGATATGGCTCTAACAA-3′).[27] Conjugation experiments were repeated five times.

The input recipient–donor ratio and the stability of Tn*916* in the control and exposed cultures were estimated in two of the conjugation experiments by plating donor pre-mating cultures on BHI agar plates both with and without the addition of 10 mg/L tetracycline and recipient pre-mating cultures on antibiotic-free BHI agar plates.

Data analysis

β-Glucuronidase enzyme activity

Measures of the specific β-glucuronidase enzyme activities in exposed cultures (prior to and after addition of biocides) were standardized to the corresponding control sample as the percentage difference in β-glucuronidase enzyme activity. The transcriptional effect of biocides on enzyme activity was estimated as the difference in the standardized enzyme activity after addition of the compound (0.5, 1.0, 1.5 and 2.0 h samples) relative to the enzyme activity before addition (0 h sample). The enzyme activity before addition was estimated as the average of the two samples collected before addition of the biocides.

Conjugative transposition of Tn916

The transfer frequencies of Tn*916* were calculated as (transconjugants per mL)/(output donor cells per mL). The significance of changes in the transfer frequencies between control and treated conjugations was statistically tested using the paired, two-sided, Student's *t*-test, where a pair represents the transfer frequency of the control and the treated conjugations within an experimental repetition. The normality of the differences in the transfer frequencies between the control and exposed conjugations were visually assessed using QQ plots.

Results

The MIC values for *B. subtilis* strains BS34A::pHCMC05-P*tet*(M)-*gusA* and BS34ASTR of each of the biocides are shown in Table S1 (available as Supplementary data at *JAC* Online) together with

the corresponding subinhibitory concentrations (0.25 × MIC) used in the reporter assays and the Tn*916* conjugation experiment.

Determination of the optimum time of exposure to biocides prior to filter-mating experiments

Transcription from the promoter upstream of *tet*(M) was estimated by cloning it upstream of a promoterless β-glucuronidase (*gusA*) reporter construct in *B. subtilis*. The effect of each biocide and tetracycline on the β-glucuronidase enzyme activity is shown in Figure 1. The greatest deviations in GusA activity from the normalized value were chosen for the times for pre-exposure to the biocides prior to filter mating. The stability of the reporter construct pHCMC05-P*tet*(M)-*gusA* during all of the experiments was found to be similar (an average of 74%–85%) at the end of the experiment, apart from the experiment where cells were challenged with tetracycline. In this experiment, the average stability was 64% (Figure S1, available as Supplementary data at *JAC* Online).

Effect of biocides on the conjugative transposition of Tn916

The conjugative transfer of Tn*916* was studied in *B. subtilis* where donors were pre-grown separately in ethanol, hydrogen peroxide, chlorhexidine digluconate, sodium hypochlorite and tetracycline for 2.0, 1.5, 0.5, 1.5 and 1.0 h, respectively, prior to filter mating. The results for the effects of biocides and tetracycline on the conjugative transposition of Tn*916* are presented in Table 2 (full data are provided in Table S2, available as Supplementary data at *JAC* Online). Tetracycline and ethanol significantly ($P=0.01$) enhanced the transfer of Tn*916*, corresponding to an average increase of 12- and 5-fold, respectively. Hydrogen peroxide, chlorhexidine digluconate and sodium hypochlorite did not significantly affect the transfer frequency of Tn*916*.

The stability of Tn*916* in the donor cells was assessed in pre-mating cultures and was not found to be significantly different between the exposed and the control cultures (Table S3, available as Supplementary data at *JAC* Online). Some variation in the output recipient–donor ratio occurred between matings with pre-growth of donors in tetracycline and ethanol and the corresponding controls. When the output recipient–donor ratios were compared with the transfer frequencies, the results did not suggest that differences in transfer were due to variations in this ratio. Furthermore, the input recipient–donor ratio was determined for two experiments. In both cases, differences in transfer frequencies were not found to correlate with variations in the input recipient–donor ratio, since this ratio for the treated matings was within the range of the controls (Figure S2, available as Supplementary data at *JAC* Online).

Discussion

The effects of subinhibitory concentrations of ethanol, hydrogen peroxide, chlorhexidine digluconate, sodium hypochlorite and tetracycline on the conjugal transfer of Tn*916* between *B. subtilis* strains were analysed. The MIC values of the four biocides for *B. subtilis* BS34A were comparable to the MIC values found for other Gram-positive bacteria.[28–33] The subinhibitory concentration of each biocide used in this study was set to one-quarter of the MIC.

In order to determine the optimal time of pre-exposure to the various biocides prior to filter mating, we determined the GusA activity of a plasmid-based *gusA* gene under the control of the Tn*916* promoter upstream of *tet*(M). The greatest difference for increase in GusA activity occurred at 2 h after exposure for ethanol, 1.5 h for hydrogen peroxide, 0.5 h for chlorhexidine digluconate and 1.5 h for sodium hypochlorite. Although this is a relatively crude assessment of the transcriptional activity of the *tet*(M) promoter in response to biocide exposure, it provided valuable data on which to base the design of the conjugation experiments. The variability of the data is likely due to the fact that we added the biocides after 2 h of growth rather than at an identical OD of the culture; therefore, there may have been slight differences in the cellular

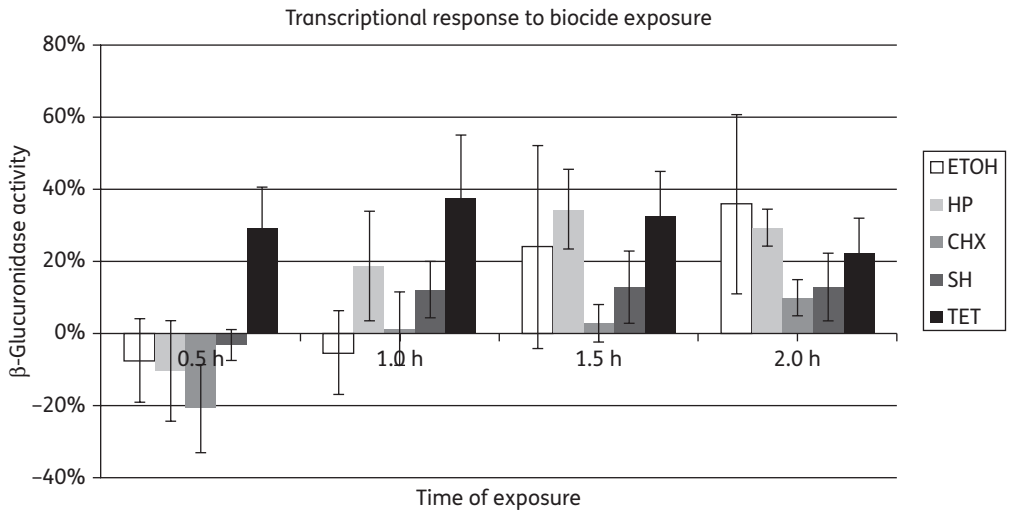

Figure 1. Average effect of ethanol (ETOH; 40 000 mg/L), hydrogen peroxide (HP; 20 mg/L), chlorhexidine digluconate (CHX; 0.5 mg/L), sodium hypochlorite (SH; 1250 mg/L) and tetracycline (TET; 10 mg/L) on β-glucuronidase enzyme activity. Data illustrate changes in enzyme activity after addition of compounds (0.5–2.0 h) relative to that before addition (0.0 h). Experiments investigating the effect of TET were repeated six times, whereas the effect of biocides was repeated three times. Error bars represent ± SD.

Table 2. Effect of the presence of ethanol, hydrogen peroxide, chlorhexidine digluconate, sodium hypochlorite and tetracycline in the pre-growth medium on the conjugative transfer of Tn*916* between *B. subtilis* strains

	Output cells (cfu/mL)							
	control			exposure to biocide				
Biocide	D	R/D	TF	D	R/D	TF	TF ratio (exposed/control)	*P* value
ETOH 20000 mg/L	$2.8\times10^{9}\pm1.4\times10^{9}$	1.2 ± 0.5	$6.5\times10^{-10}\pm3.7\times10^{-10}$	$1.5\times10^{9}\pm3.3\times10^{8}$	3.2 ± 1.0	$2.3\times10^{-9}\pm7.2\times10^{-10}$	4.7 ± 3.3	0.01
HP 10 mg/L	$2.6\times10^{9}\pm5.7\times10^{8}$	1.2 ± 0.2	$9.0\times10^{-10}\pm6.2\times10^{-10}$	$2.3\times10^{9}\pm9.7\times10^{8}$	2.0 ± 1.5	$2.4\times10^{-9}\pm1.4\times10^{-9}$	4.0 ± 4.0	0.11
CHX 0.5 mg/L	$2.1\times10^{9}\pm7.1\times10^{8}$	1.7 ± 0.5	$9.4\times10^{-10}\pm7.0\times10^{-10}$	$2.4\times10^{9}\pm7.1\times10^{8}$	1.9 ± 0.9	$7.0\times10^{-10}\pm6.4\times10^{-10}$	0.7 ± 0.2	0.09
SH 1250 mg/L[a]	$2.6\times10^{9}\pm5.7\times10^{8}$	1.2 ± 0.2	$9.0\times10^{-10}\pm6.2\times10^{-10}$	$3.2\times10^{9}\pm1.1\times10^{9}$	1.1 ± 0.3	$1.7\times10^{-9}\pm1.2\times10^{-9}$	3.0 ± 3.3	0.32
TET 10 mg/L	$2.9\times10^{9}\pm6.0\times10^{8}$	1.3 ± 0.5	$1.4\times10^{-9}\pm1.1\times10^{-9}$	$5.8\times10^{8}\pm2.9\times10^{8}$	11.7 ± 8.2	$1.1\times10^{-8}\pm5.9\times10^{-9}$	11.8 ± 6.9	0.01

R, recipient; D, donor; TF, average transfer frequency; ETOH, ethanol; HP, hydrogen peroxide; CHX, chlorhexidine digluconate; SH, sodium hypochlorite; TET, tetracycline.
Transfer frequencies were calculated as (transconjugants per mL)/(output donor cells per mL) and the *P* value was determined using the paired Student's *t*-test.
[a]SH solution containing 14% available chlorine.

response to the biocide. As a control, the experiment was carried out with inhibitory concentrations of tetracycline and the maximum response was observed at 1 h following exposure, which agrees with the proposed mechanism for the transcriptional regulation of Tn*916* and previous transcriptional analysis.[18]

Pre-exposing donors to tetracycline resulted in an average 12-fold increase in the transfer frequency of Tn*916*, which is in good agreement with the tetracycline-induced Tn*916* transfer study reported by Showsh and Andrews[13] between *B. subtilis* and *Bacillus thuringiensis* subsp. *israelensis* (19-fold increase calculated as transconjugants per output recipients). In our study, exposure of donors to ethanol also significantly increased the transfer of Tn*916* by 5-fold.

In contrast to the specific action of tetracycline and antimicrobial agents in general, biocides have multiple target sites within the cell, but knowledge about the exact mechanism of action is limited, especially at subinhibitory concentrations, where only a few target sites might be involved.[4,5] Therefore, the specific mechanisms of action of subinhibitory concentrations of biocides on cells are difficult to elucidate. However, one hypothesis that is currently being tested is that the multiple, non-specific actions of the biocides slow down protein synthesis and lead to an increase in the pool of charged tRNAs within the cell; the rate-determining step in the derepression of transcriptional attenuation proposed for Tn*916*.[18] Since the regulatory region of Tn*916* is conserved in nearly all of the Tn*916*-like elements,[16] ethanol might not only induce the spread of Tn*916*, but also that of many other members of the Tn*916*-like family and their resistance genes.

An ethanol-induced increase in the horizontal transfer of Tn*916* is a possible concern from both a clinical and a social perspective. Ethanol is primarily used for hard-surface disinfection and skin antisepsis in hospitals, but is also widely used for disinfection in the food industry, day care institutions and for preservation purposes.[1,2,34] In addition, vast quantities of ethanol are consumed in alcoholic beverages every day by many of the world's population.

In summary, these results show that subinhibitory concentrations of ethanol significantly increase the transfer frequency of Tn*916*. Current investigations are focused on determining the exact molecular mechanisms for this increase in transfer frequency, with the obvious focus being on the effect on transcriptional attenuation upstream of *tet*(M).

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Transparency declarations

None to declare.

Supplementary data

Tables S1 to S3 and Figures S1 and S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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