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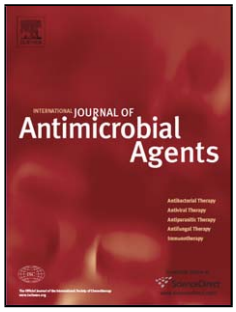

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Prevalence of decreased susceptibility to triclosan in *Salmonella enterica* isolates from animals and humans and association with multiple drug resistance

Justin L. Copitch, Rebekah N. Whitehead, Mark A. Webber *

Antimicrobial Agents Research Group, School of Immunity and Infection, University of Birmingham, Birmingham B15 2TT, UK

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* Corresponding author. Tel.: +44 121 414 2859.

E-mail address: m.a.webber@bham.ac.uk (M.A. Webber).

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ABSTRACT

Previous laboratory studies have implicated triclosan as a possible selective force driving resistance to multiple antibiotics and have identified a number of triclosan resistance mechanisms in *Salmonella enterica*. The aim of this work was to determine the prevalence of decreased susceptibility to triclosan in a panel of human and animal isolates of *S. enterica* and to identify the mechanisms of triclosan resistance in these strains. Over 400 animal and human isolates of non-typhoidal *Salmonella* were screened for decreased susceptibility to triclosan and a panel of antibiotics. The prevalence of decreased susceptibility to triclosan was ca. 4%. Of the isolates with decreased triclosan susceptibility, 56% were multidrug-resistant (MDR) compared with 12% of triclosan-sensitive isolates. MDR and triclosan-resistant strains showed increased efflux activity compared with strains with reduced susceptibility to triclosan alone. No high-level triclosan resistance was seen in this panel of isolates. A reservoir of strains with low-level decreased triclosan susceptibility is present in animals and humans. These isolates are MDR as a result of generic mechanisms of antimicrobial resistance and do not carry specific mutations within *fabI*.

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1. Introduction

Non-typhoidal *Salmonella* (NTS) are a major cause of enteric illness both in humans and animals [1]. NTS usually cause a self-limiting mild illness but can have serious sequelae. The primary route of NTS infection in humans is via ingestion of contaminated food, in particular poultry and eggs [2]. Antibiotic-resistant *Salmonella* strains resistant to multiple therapeutic drugs are becoming increasingly common, and infection with a multidrug-resistant (MDR) strain of *Salmonella* significantly increases the risk of morbidity and mortality [3].

Triclosan is a broad-spectrum antimicrobial bisphenol biocide [4]. It is used in a wide array of domestic products including deodorants, soaps, kitchen utensils and toothpastes [4]. When first introduced, the mechanism of action of triclosan was unknown and was considered to be non-specific, acting to disrupt bacterial membranes [5]. In the early 1990s, triclosan-resistant *Staphylococcus aureus* were described, and several later studies with *Escherichia coli*, *Pseudomonas aeruginosa*, *Mycobacterium smegmatis*, *Mycobacterium tuberculosis* and *S. aureus* showed that triclosan does in fact have a defined bacterial target, the FabI enzyme, essential for fatty acid synthesis [6]. Several mechanisms of resistance to triclosan have been demonstrated in different bacteria. These include mutations in or upregulation of *fabI*, derepression of efflux pumps, carriage of alternative enoyl-acyl reductases, production of degradative enzymes and alteration of fatty acid metabolism pathways [7,8].

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Of particular concern has been the demonstration of upregulation of efflux pumps in response to biocide exposure, including triclosan, a consequence of which is an increased degree of resistance to multiple drugs, albeit at a relatively low level [9].

In this study, we sought to identify human and animal isolates of *Salmonella enterica* with decreased susceptibility to triclosan, to investigate whether these isolates were MDR and to analyse the mechanisms of triclosan resistance in isolates with reduced susceptibility.

2. Materials and methods

2.1. Bacterial strains

Over 400 isolates (60 human isolates and 368 animal isolates) of *S. enterica* (comprising 40 serovars) from animals or humans collected over the last 20 years were screened for resistance to triclosan. All bacterial strains were stored at –20 °C on Protect™ beads.

2.2. Identification of triclosan-resistant Salmonella isolates

All strains were inoculated onto LB agar plates containing 0.12 mg/L triclosan, a concentration that equates to two times the minimum inhibitory concentration (MIC) of wild-type strains [10]. Strains were also grown on a control agar plate without triclosan as well as an XLD agar plate to presumptively identify strains as *Salmonella*. All strains that were unable to grow on these plates were discarded.

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2.3. Susceptibility testing

MICs of triclosan and a panel of antibiotics, including ciprofloxacin, chloramphenicol, tetracycline, ampicillin, kanamycin and nalidixic acid, required to inhibit growth of isolates identified in the triclosan breakpoint testing were determined at least five times using the agar dilution method according to the guidelines of the British Society for Antimicrobial Chemotherapy (BSAC) [11]. Interpretive breakpoints for ampicillin were according to BSAC [11]; the following breakpoints were used for ciprofloxacin (0.25 mg/L), tetracycline (8 mg/L), chloramphenicol (8 mg/L), kanamycin (8 mg/L) and nalidixic acid (>32 mg/L) for which BSAC do not suggest breakpoints against *Salmonella*. *Salmonella enterica* serovar Typhimurium SL1344 was used as a wild-type control for susceptibility testing. Multidrug resistance was defined as resistance to three or more separate drug classes. All strains where susceptibility testing confirmed triclosan resistance levels to be higher than that in SL1344 were retained for further characterisation.

2.4. Genotyping of fabI

Polymerase chain reaction (PCR) was used to amplify *fabI* from strains of interest using specific primers (AAC GTC ACC TGC CGG AGA TA and TGG ATT ATC CTG GCG TAT GC). The resulting amplimers were sequenced at the Functional Genomics Laboratory at the University of Birmingham (Birmingham, UK). Sequences were analysed and compared with the *fabI* sequences from *S.* Typhimurium SL1344, LT2 and DT104 and from *E. coli* K-

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12, CFT073, 101-1, 536 and 042 strains using the programme ClustalW2, and mutations resulting in amino acid changes were recorded. The consequences of substitutions within FabI were predicted using the SWISS-MODEL programme.

2.5. Transduction of mutations

Where mutations resulting in amino acid changes were found in *fabI*, P22 phage transduction was used to transfer the mutant *fabI* allele into SL1344 to determine the role of these mutations in resistance to triclosan [12]. Transductants were recovered on LB agar plates containing 0.25 mg/L triclosan. The MIC of triclosan required to inhibit these transductants was determined and *fabI* was sequenced to identify any specific target site mutations.

2.6. Efflux activity

To determine the level of efflux activity of strains with decreased triclosan sensitivity, intracellular accumulation of the fluorescent dye Hoechst 33342 was measured as previously described [13]. Hoechst 33342 intercalates with DNA and fluoresces, allowing intracellular accumulation to be identified. It is also a substrate of the major multidrug efflux system AcrAB-TolC. Accumulation was measured using a FLUOstar OPTIMA (BMG Labtech, Offenburg, Germany) in the presence and absence of the efflux pump inhibitor phenyl-arginine β-naphthylamide (PAβN) added to cultures to a final concentration of 100 mg/L (the MIC of this compound is >400 mg/L). Mean

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accumulation values were calculated from eight biological replicates and standard deviations were calculated. Student's *t*-test was used to assess the significance of differences between strains.

2.7. Expression of acrB

Expression of *acrB* by isolates with an active efflux phenotype was determined by reverse transcriptase PCR. RNA was prepared from duplicate biological cultures (grown to an optical density at 600 nm of ca. 0.7). Five millilitres of culture was harvested and RNA was prepared using the SV40 Total RNA Isolation Kit (Promega, Southampton, UK) according to the manufacturer's recommendations. Quantities of RNA were determined using a NanoDrop (Fisher Scientific, Loughborough, UK). Two micrograms of RNA was reverse-transcribed into cDNA using Superscript III (Invitrogen, Paisley, UK) and two replicates were made for each RNA preparation giving four samples from each strain. Specific primers were used to amplify 16S rRNA as a control (cDNA diluted 1:1000) and *acrB* (undiluted cDNA used as template). Amplimers (5 μL) were separated on 1% agarose gels and band intensities were determined using the GeneTools program (Syngene, Cambridge, UK). Average intensities of 16S rRNA and *acrB* were calculated for each strain, and expression of *acrB* was normalised using data from 16S rRNA.

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3. Results

3.1. Prevalence of triclosan resistance

Of the 428 strains of *Salmonella* screened, 111 showed growth on plates containing 0.12 mg/L triclosan and appeared to be characteristic of *Salmonella* on XLD agar. Of these 111 strains, 16 were found to consistently have a higher MIC of triclosan than SL1344 and were selected for further characterisation. The level of resistance to triclosan in these 16 strains was low and 15/16 isolates were inhibited by 0.25 mg/L or 0.5 mg/L triclosan; one isolate (L298) demonstrated a higher level of triclosan resistance and required 4 mg/L of triclosan for inhibition (Table 1). Of these 16 strains, 9 (56%) were MDR. Full MIC profiles were also determined for 33 isolates from the same panel that did not show increased resistance to triclosan, of which only 4 (12%) were MDR. The prevalence of multidrug resistance amongst triclosan-resistant strains suggests an association between multidrug resistance and triclosan resistance and was statistically significant according to the χ^2 test ($P < 0.05$).

3.2. Genotyping of fabI and significance of mutations

When sequenced, five strains with decreased triclosan susceptibility were found to carry missense mutations within *fabI*. Strains L308, L406, L488, L494 and L501 all possessed the same change of valine to isoleucine at codon 115. All of these strains had a low triclosan MIC (0.25 mg/L or 0.5 mg/L). No other missense mutations were found in any strain. Codon 115 is at the edge of one of the two active site regions of FabI (codons 95–115 and 190–210),

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suggesting that substitutions here may be relevant to function. To determine whether this novel substitution has a role in triclosan resistance, a variety of approaches were used. Bioinformatic analysis of the *S.* Typhimurium LT2 FabI sequence using the SIFT algorithm predicted that this substitution would be well tolerated by the protein. In agreement with this, the mutant FabI allele was modelled by SWISS-MODEL, which predicted a secondary structure virtually indistinguishable to the wild-type protein (data not shown). Furthermore, alignment of FabI amino acid sequences from four strains of *Salmonella* (*S.* Typhimurium LT2, *S.* Typhimurium DT104, *S.* Typhimurium SL1344 and *S.* Enteritidis PT4) and five strains of *E. coli* (CFT073, 536, 101-1, 042 and K-12) using ClustalW2 revealed this substitution to be present in two of the five *Salmonella* FabI sequences and all the *E. coli* sequences. Susceptibility of wild-type strains of both species to triclosan is very similar and it would appear (in conjunction with transduction results; see below) that this substitution at codon 115 does not contribute to increased triclosan resistance.

3.3. Transduction of triclosan resistance

Strain L488, which demonstrated decreased susceptibility to triclosan and contained the Val115Ile mutation, was used as a donor to transduce the mutation(s) conferring decreased triclosan susceptibility into SL1344 using P22 phage. Transductants were successfully recovered on agar plates containing 0.25 mg/L triclosan. Genotyping of FabI from these transductants revealed a wild-type sequence, demonstrating that the substitution at codon 115 of the parent did not confer the decreased triclosan susceptibility of this

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isolate as it was not transferred even though the triclosan resistance phenotype was.

3.4. Efflux activity of triclosan mutants

Accumulation of the fluorescent intercalating dye Hoechst 33342, which is a substrate of the AcrAB-TolC efflux system, was analysed for all isolates with decreased susceptibility to triclosan (Fig. 1). Strains L308, L325, L424, L616 and L631 all accumulated <50% as much Hoechst 33342 as SL1344, and strains L292, L298, L306, L494 and L501 accumulated <25%. Six isolates (L292, L308, L325, L424, L616 and L631) showed a greater than four-fold increase in accumulation of Hoechst 33342 when an efflux pump inhibitor was added, indicating that the low levels of dye accumulation in these strains is likely to be a result of increased expression of efflux pumps. Expression of *acrB* was increased in all the isolates with decreased triclosan sensitivity compared with that of SL1344 (Fig. 2). This correlated well with the Hoechst accumulation data and demonstrates that the isolates with decreased triclosan susceptibility are efflux mutants that overexpress *acrB*.

4. Discussion

Recently we have described the multifactorial basis of resistance to triclosan in *Salmonella* and have shown that very high-level resistance to triclosan is relatively easy to select in laboratory conditions. In this study, we wanted to know whether similar phenotypes are seen in real world isolates from animals and man and whether decreased susceptibility to triclosan is associated with

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multidrug resistance. Of the 428 isolates of *Salmonella* that were screened for decreased susceptibility to triclosan, ca. 4% demonstrated decreased susceptibility to triclosan. Analysis of these isolates showed that the level of resistance to triclosan was generally low (triclosan MIC range 0.25–4 mg/L), suggesting that different mechanisms of resistance are present in different isolates. Decreased susceptibility to triclosan was seen in eight separate serovars, indicating no association with a particular serotype or clone. These findings are similar to a previous report by Randall et al. [14] that found only one strain of *Salmonella* with a triclosan MIC > 0.5 mg/L from a panel of 388 isolates. Studies of triclosan susceptibility in oral commensal flora have found little or no evidence of triclosan resistance. Another large study of bacteria isolated from hands in a community setting found no statistically significant correlation between susceptibility to triclosan and antibiotics, although there was an association between strains with higher triclosan MICs and antibiotic resistance [15]. Whilst in agreement with previous studies in that the incidence and level of triclosan resistance in these isolates is low, of note in this study was the marked association between decreased triclosan susceptibility and multidrug resistance. Significantly higher rates of multidrug resistance were seen amongst isolates with reduced susceptibility to triclosan (56%) than among triclosan-sensitive isolates (12%).

One of the main aims of this study was to determine the mechanisms of triclosan resistance prevalent in isolates and to identify whether the diverse laboratory-selected phenotypes observed previously were present in field isolates [10]. No significant contribution of *fabI* mutations to triclosan

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susceptibility was seen in any isolate. The only substitution observed in these isolates was predicted by bioinformatics to have little effect on FabI and was not transferred when resistance was transduced from a strain carrying the substitution to a wild-type strain. It therefore seems unlikely that FabI contributes to the low-level resistance seen in this panel of isolates. Whilst studies with laboratory mutants commonly identify mutants resistant to triclosan with alterations in FabI, it may be that in a competitive environment alterations to FabI may carry a significant fitness cost prohibitive to their selection and expansion. The main mechanism of resistance observed was efflux. The majority of isolates with decreased resistance to triclosan were also MDR and demonstrated greater efflux activity and *acrB* expression than control strains. The absence of 'triclosan-specific' mechanisms of resistance as seen in our previous studies with laboratory-selected mutants with very high levels of triclosan resistance may reflect a fitness cost of these mutations or the lack of high-level triclosan selective pressure in the environment. Whether the derepression of MDR efflux systems seen in these isolates is a consequence of triclosan exposure in the environment or other substrates is not possible to determine. However, the increasing proliferation of triclosan in the environment is a concern as the data presented here suggest that MDR efflux pump derepression is an effective mechanism allowing survival at relatively low levels of triclosan. Therefore, low levels of triclosan in the environment may promote selection of multidrug resistance via efflux pump derepression, which is a phenotype of clinical relevance. The use of triclosan should be restricted to applications where it is likely to be beneficial, and the proliferation of its use in domestic products is questionable.

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Competing interests

None declared.

Ethical approval

Not required.

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Fig. 1. Accumulation of Hoechst 33342 by isolates with decreased triclosan susceptibility after 5 min. Open bars indicate accumulation without efflux pump inhibitor and filled bars indicate accumulation in the presence of the efflux pump inhibitor phenyl-arginine β-naphthylamide (PAβN). * Indicates isolates that isolates accumulate significantly less Hoechst 33342 than SL1344. MDR, multidrug-resistant.

Fig. 2. Expression of *acrB* by isolates with decreased triclosan susceptibility. Results are from one experiment and are representative of repeat experiments. Error bars indicate standard deviation from mean expression.

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Table 1

Antimicrobial susceptibility profiles for triclosan-resistant *Salmonella* strains and controls [a]

Strain	Serovar	Source	Year isolated	MIC (mg/L)						
				TRI	CIP	TET	AMP	CHL	KAN	NAL
SL1344	Typhimurium	Wild-type control		0.12	0.015	2	2	4	2	4
L702	Typhimurium	Triclosan-resistant control		64	0.25	4	4	8	16	>128
L10	Typhimurium	Human	1990	0.5	0.06	4	4	8	16	8
L292	Saintpaul	Poultry	1992	0.5	0.5	4	>32	8	4	>128
L298	Anatum	Poultry	1992	4	0.5	2	1	4	32	>128
L306	Typhimurium	Poultry	1993	0.25	0.25	2	2	8	32	>128
L308	Ohio	Poultry	1993	0.5	0.5	4	4	16	16	>128
L325	Typhimurium	Poultry	1993	0.5	1	>32	>32	>32	16	>128
L361	Typhimurium	Poultry	2000	0.5	0.06	4	1	>32	16	>128
L406	Senftenberg	Poultry	2000	0.25	0.5	2	2	8	32	>128
L424	Typhimurium	Poultry	2000	0.5	0.12	8	16	>32	16	16
L476	Senftenberg	Poultry	2000	0.25	0.5	2	1	4	32	>128
L488	Newport	Poultry	2000	0.25	0.5	2	1	16	32	>128
L494	Indiana	Poultry	2000	0.25	0.5	2	1	4	32	>128
L501	Typhimurium	Poultry	2000	0.25	0.25	2	1	4	32	>128

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L616	Typhimurium	Poultry	2001	0.5	0.12	8	4	16	16	32
L631	Typhimurium	Poultry	2001	0.5	0.12	8	4	16	16	32
L798	Group B	Human	2002	0.25	0.12	>32	>32	16	16	16

MIC, minimum inhibitory concentration.

[a] Drug abbreviations and breakpoints used: TRI, triclosan (0.25 mg/L); CIP, ciprofloxacin (0.25 mg/L); TET, tetracycline (8 mg/L); AMP, ampicillin (16 mg/L); CHL, chloramphenicol (8 mg/L); KAN, kanamycin (8 mg/L); NAL, nalidixic acid (>32 mg/L).

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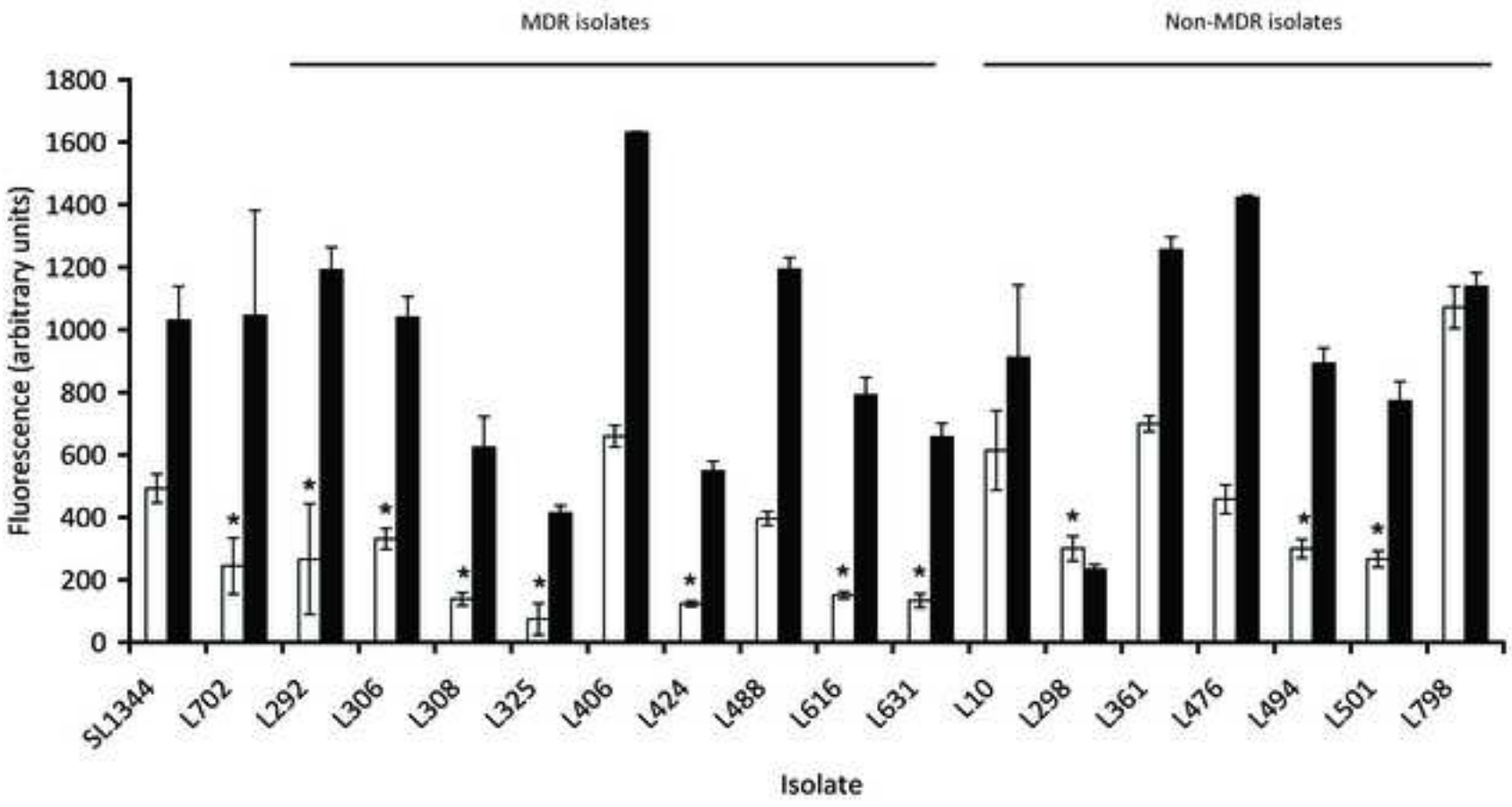
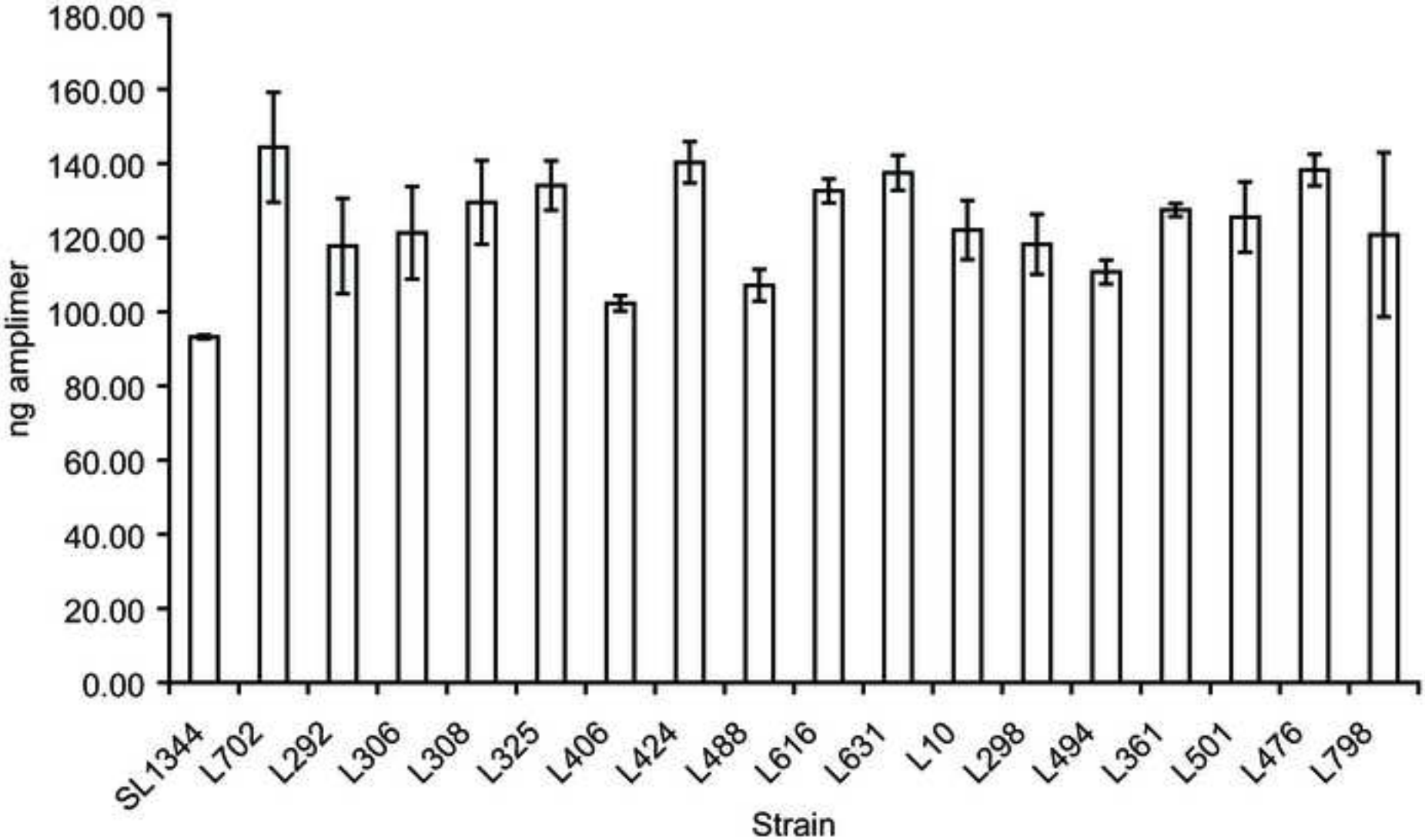
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