

High-Throughput Determination of Bacterial Growth Kinetics using a FLUOstar OPTIMA

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- Growth rates of biocide resistant mutants of salmonella and their parent strains were determined and compared
- Growth curves were monitored in absorbance mode automatically over 24 h using the FLUOstar OPTIMA
- Biocide exposure selects for strains with increased tolerance to biocides and there is no obvious fitness cost

Introduction

Salmonella enterica serovar Typhimurium are amongst the leading causes of gastrointestinal disease in the U.K. Non typhoidal salmonella were responsible for over 12,500 cases of enteritis in the U.K in 2004 [HPA] and over 65,000 across Europe, causing significant morbidity and mortality.¹ The main route of human infection is via the food chain, with poultry representing a major reservoir for salmonella. Multiple antibiotic resistance (MAR) in salmonella has been increasing and over 30% of isolates of *S. Typhimurium* reported to the Enter-net surveillance network this year were MAR.¹

Recent regulations preventing the use of antibiotics for prophylactic treatment of animals have been introduced in an attempt to reduce the emergence of antibiotic resistance in the food chain. This has necessitated an increase in the use of bio-security measures to maintain standards of animal health. These measures include application of biocides to reduce microbial contamination of animal houses. Efflux is one mechanism that can confer MAR, efflux pumps are membrane proteins that actively export a wide range of toxic substrates including antibiotics, dyes and biocides to the cells external environment, thereby preventing accumulation of toxic agents and mediating resistance to these agents as a result.²

The wide spectrum of substrates recognized by efflux systems has prompted concern that exposure of a bacterium to one substrate could select for over-expression of an efflux system and consequent resistance to all other substrates.³ As biocides are substrates, it is possible that exposure of a bacterium to a biocide could result in selection of an efflux mutant which has reduced susceptibility to antibiotics, which it has not previously encountered. In this study, mutants of salmonella resistant to biocides were selected. The growth rates of biocide resistant mutants were determined and compared to parent strains and the effect of the addition of biocides during growth were determined.

Materials and Methods

Selection of biocide resistant mutants

Previously, a panel of *Salmonella* Typhimurium was used to select biocide resistant mutants. SL1344 was used as a reference control strain, L108 is a derivative of SL1344 lacking the TolC porin and L358 is a multiply antibiotic resistant DT104 isolate. L378 is a ciprofloxacin resistant isolate from poultry and L357 is a representative DT104 isolate. The biocides AQAS (a quaternary ammonium compound),

Superkill (a mix of aldehydes), Farm Fluid S (a mixture of tar oils and phenolics) and Virkon S (peroxygen generating compound) were used to select for tolerant mutants by incorporation in agar and overnight incubation after inoculation with each strain.

Analysis of growth kinetics of biocide tolerant mutants

The rate of growth in Luria-bertani broth for each biocide tolerant mutant selected was determined over 24 h at 37°C using a FLUOstar OPTIMA (figure 1, BMG LABTECH, U.K.).



Fig. 1: FLUOstar OPTIMA multimode plate reader from BMG LABTECH

100 µL of sterile Luria-bertani broth was dispensed into clear sterile 96-well microplates and inoculated with overnight culture of each strain to give a final inoculum of 4%. Readings were taken every ten minutes of absorbance of each well (scanned at 600 nm) in the microplates over the 24 h time period and results recorded automatically. Each strain was analysed in triplicate wells on at least three separate occasions to give nine data sets for analysis. Additionally, each strain was challenged with either 0.5 X, or the MIC (minimum inhibitory concentration) of the selective biocide for the parent after two hours growth (mid logarithmic growth phase) in order to determine whether the inhibitory ability of each biocide was reduced in mutants, respective to parent strains.

Data were analysed using Excel (Microsoft, USA) to calculate means and standard deviations for each strain. Differences between strains were analysed for statistical significance using the Student's t-test.

Results and Discussion

None of the biocide selected mutants were compromised in their ability to grow in Luria-bertani broth relative to their parents in the absence of biocide. Mutants derived from L358 (S2, S22, S23) after exposure to Superkill grew significantly better than L358 in the absence of biocide. The majority of biocide selected mutants were more resistant to the addition of biocides to the media than their respective parent strains, including those strains for which the MIC of the selective biocide had remained unchanged when compared to the parent (table 1). AQAS selected mutant A27 and A26 were able to grow significantly ($p > 0.01$) better upon both the addition of 0.5 X and the MIC of L378 and L108, respectively, to the media (figures 2 and 3).

Table 1: Parent strains are in bold and mutants are listed below their respective parents. Mutants with increased resistance to biocide compared to their parents are indicated in red.

| Strain and Genotype | Selective agent | MIC (%) | | | |
|-------------------------------------|-----------------|------------|--------------|------------------|--------------|
| | | Virkon | Superkill | AQAS | FFS |
| L357 (DT104 'A') | | 0.4 | 0.025 | 0.1 | 0.2 |
| F1 | FFS (1X MIC) | | | | 0.2 |
| F2 | FFS (1X MIC) | | | | 0.2 |
| L378 (CipR VLA52) | | 0.4 | 0.025 | 1.6 | 0.2 |
| A27 | | | | >3.2 | |
| L108 (tolC::aph from SL1344) | | 0.4 | 0.006 | >0.003 | 0.025 |
| A26 | AQAS (2X MIC) | | | 0.12 | |
| L358 (MDR DT104) | | 0.4 | 0.025 | 1.6 | 0.2 |
| S2 | S'kill (1X MIC) | | 0.025 | | |
| S22 | S'kill (2X MIC) | | 0.025 | | |
| S23 | S'kill (2X MIC) | | 0.025 | | |
| SL1344 | | 0.4 | 0.025 | 0.1 | 0.2 |
| V6 | V'kon (2X MIC) | 0.8 | | | |
| V7 | V'kon (2X MIC) | 0.8 | | | |

Superkill selected mutants S2, S22 and S23 all grew significantly ($p > 0.01$) faster than their parent, L358 in biocide free broth and all grew significantly ($p > 0.01$) better than L358 in the presence of both 0.012 and 0.025% Superkill (figure 4). Virkon selected mutants V6 and V7 both grew significantly ($p > 0.01$) better than L354 when challenged with 0.5 X or the MIC of Virkon for L354 (figure 5). No significant differences were observed between Farm Fluid S selected mutants F1 and F2 and their parent L357 in biocide free broth or when exposed to 0.5 X the MIC of Farm Fluid S. However both mutants grew significantly ($p > 0.05$) better when challenged with the MIC of Farm Fluid S (0.1% and 0.2% final concentration) as shown in figure 6.

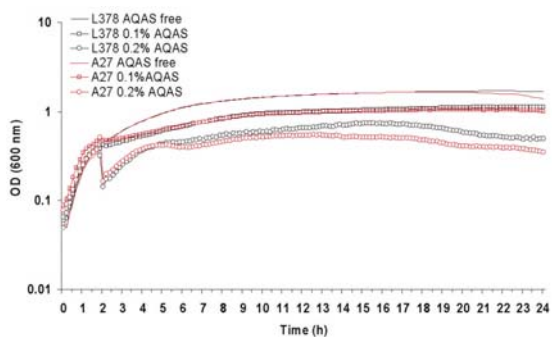


Fig. 2: Growth of AQAS selected mutant A27 and parent L378 was monitored in LB broth at 37°C after inoculation with 4% vol/vol of overnight culture. AQAS (0.1% and 0.2% final conc.) was added to cultures at 2 h.

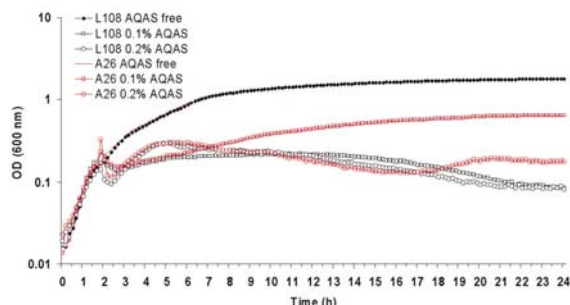


Fig. 3: Growth of AQAS selected mutant A26 and parent L108 was monitored in LB broth at 37°C after inoculation with 4% vol/vol of overnight culture. AQAS (0.1% and 0.2% final conc.) was added to cultures at 2 h.

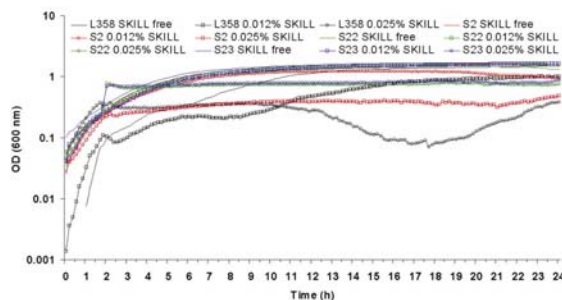


Fig. 4: Growth of Superkill selected mutants S2, S22, S23 and parent L358 was monitored in LB broth at 37°C after inoculation with 4% vol/vol of overnight culture. Superkill (0.012% and 0.025% final conc.) was added to cultures at 2 h.

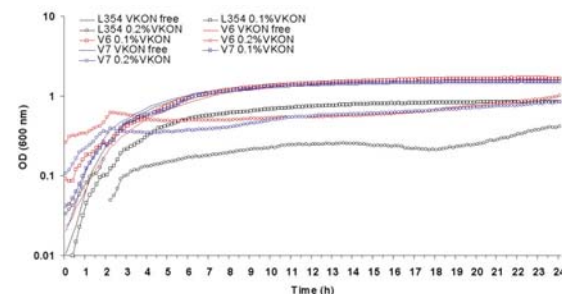


Fig. 5: Growth of Virkon selected mutants V6, V7 and parent L354 was monitored in LB broth at 37°C after inoculation with 4% vol/vol of overnight culture. Virkon (0.1% and 0.2% final conc.) was added to cultures at 2 h.

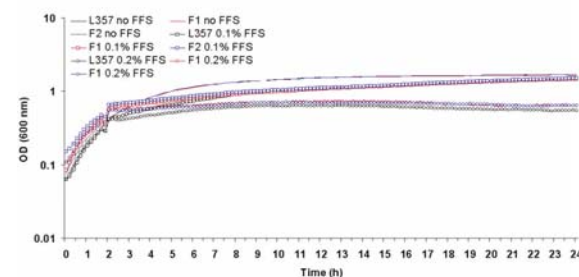


Fig. 6: Growth of Farm Fluid selected mutants F1, F2 and parent L357 was monitored in LB broth at 37°C after inoculation with 4% vol/vol of overnight culture. Farm Fluid S (0.1% and 0.2% final conc.) was added to cultures at 2 h.

Conclusion

This data clearly indicates that biocide exposure selects for strains with increased tolerance to biocides at sub-MIC concentrations and at the MIC and that there is no obvious fitness cost in these strains when compared to their parents in biocide free broth. Further work is in progress to analyse the mechanisms of resistance present in these mutant strains and to determine the incidence of cross resistance to antibiotics.

References

1. Enter-net: <http://www.hpa.org.uk/hpa/inter/enter-net>
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3. Gilbert P, McBain AJ. *Clin Microbiol Rev.* 2003 16(2):189-208.

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