Sunday 169

Adenine Methylation and Antimicrobial Resistance

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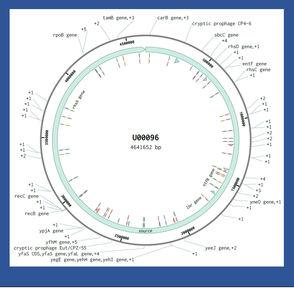
Abstract

Development of antimicrobial resistance (AMR) continues to be a global concern. In order to minimise the impact of such it is imperative to gather as much data as possible on the mechanisms and key pathways involved.
The aim of this project was to investigate the role of adenine methylation in the development of resistance to triclosan and associated antibiotic cross-resistance.

Triclosan resistance was induced in *E. coli* through repeated exposure to sub-inhibitory concentrations. Pacbio SMRT sequencing was used to identify methylated bases in both wild type and resistance bacteria. Modified bases within genes were identified through genome annotation, and differentially methylated genes collated.

Conclusions

E. coli rapidly became resistant to triclosan and exhibited increased resistance to multiple antibiotics.
There were no statistically significant differences in global methylation.
A number of genes associated with a range of functions were seen to be differentially methylated between the wild type and resistant cultures.



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INTRODUCTION

Triclosan is a widely used antimicrobial; it has been associated with resistance development and development of antibiotic cross-resistance, following the discovery of its competitive inhibition of enoyl reductase, which is involved in fatty acid biosynthesis[1]. A number of publications[2,3] have reported the development of antibiotic resistance following exposure to sub-inhibitory concentrations of triclosan. While it is thought that the mechanisms of resistance may be acquired through efflux, the mechanisms of this acquired resistance have yet to be fully confirmed.

In order to maximise survival, clonal bacterial populations exhibit cell-cell variation. While it has been generally assumed that mutation is the cause of such variation, it is becoming increasingly apparent that mechanisms such as DNA methylation can also result in such[4, 5]. DNA methylation has been shown to affect a range of processes including: regulation of secretion systems in *P. aeruginosa* and enteroaggressive *E. coli*, phase regulation in uropathogenic *E. coli*, virulence and pathogenesis in a range of bacteria including *Y. pestis* and *P. multocida*[6]. It has been suggested that DNA methylation may also impact resistance development[5].

Recent advances in single molecule real time (SMRT) sequencing have revolutionised the amount and type of data that can be obtained from a genome. In addition to determining the base at any given position, it is now possible to obtain data relating to the rate of base incorporation. This kinetic information presents characteristic patterns in response to the presence of DNA base modifications, including methylation, and thus presents a tractable approach to global methylation analysis in multiple species[7]. Limited studies have exploited this new technology and it is yet to be applied to the study of AMR.

METHODS

Sub-inhibitory concentrations of triclosan were determined using a broth microdilution assay. Resistance was induced by growing wild type *E. coli* K12 cultures in the presence of sub-inhibitory concentrations of triclosan until growth characteristics matched those grown without triclosan. Antibiotic resistance was determined using a disk diffusion method (M13 antibiogram rings, Mast UK).

DNA was extracted (Purelink, Invitrogen) and quality and quantity was assessed using agarose gel electrophoresis and microvolume spectrophotometry. Samples were found to be acceptable for sequencing with 260/230 values between 2-2.2 and 260/280 values of approx. 1.8.
PacBio SMRT sequencing was carried out by The Genome Analysis Centre (Norwich, UK).

Motif analysis was carried out using Motif Finder (PacBio).
Genomes were annotated using SeqMap and methylated bases aligned to genes using IntersectBed.
Enrichment analysis was carried out using Panther.

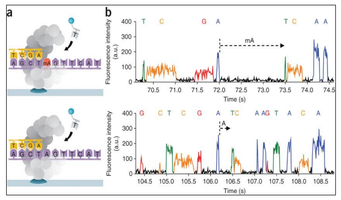

Figure 1. SMRT sequencing [8].

RESULTS

MIC Determination

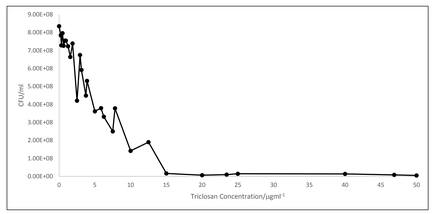

Figure 2. MIC determination.

MIC was determined to be 15µg/ml.
A sub-inhibitory concentration of 1µg/ml was selected for mutation induction.

RESULTS

Growth Characteristics

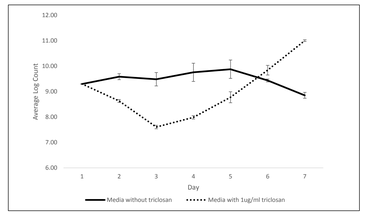

Figure 3. Resistance induction through exposure to sub-inhibitory concentrations of triclosan.

E. coli K12 cultures were grown in the presence of triclosan until growth levels matched those of these grown without. It took an average of 7 days for the strain to become resistant

Antibiotic Cross Resistance

No significant differences were observed between wild type and triclosan resistant *E. coli* in terms of their antibiotic resistance profiles.

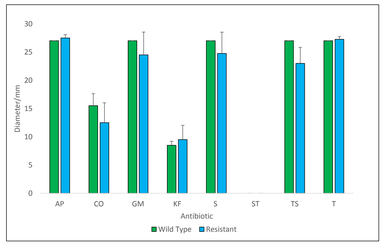

Figure 4. Antibiotic disk diffusion diameters, susceptible and resistant *E. coli.*

Methylated Motifs

Table 1. Methylated motifs.

Motif	Mtase	Susceptible	Resistant
AACNNNNNNGTGC	M.EcoK	587	586
ATATAGCA	Unknown	36	94
GATC	Dam	37802	38329
GCACNNNNNNGTT	M.EcoK	587	588

There was no significant difference between the levels of global methylation between resistant and susceptible strains. Red sites denote the methylated base

Enrichment Analysis

There was no enrichment found when methylation was decreased.
When there was no change in methylation, both GATC and non-GATC sites had enrichment of genes associated with catalytic activity and hydrolase activity. The enrichment of oxidoreductase and transferase activity for GATC sites compared to transporter activity for non-GATC suggests different regulatory roles for the different methyltransferases.

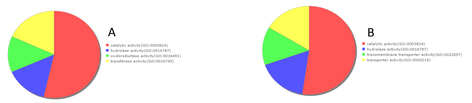

Figure 5. Molecular function over-representation. A: No change in methylation for GATC sites. B: No change in methylation for non-GATC sites.

RESULTS

Enrichment Analysis

Where there was an increase or no change in methylation between resistant and susceptible strains there was enrichment of genes for proteins localised to the membrane for both GATC and non-GATC sites. There were a variety of molecular functions associated with an increase in methylation, including transmembrane transporter activity associated with both GATC and non-GATC sites and binding for non-GATC sites.

Figure 5. Molecular function over-representation. A: Increased methylation for GATC sites. B: Increased methylation for non-GATC sites.

Table 2. Most highly differentially methylated genes.

GATC				Non-GATC			
Gene	Difference	Gene	Difference	Gene	Difference	Gene	Difference
plsB	2	mdtP	-2	evgS	6	bglX	-2

Discussion

It was found that both the Dam and M.EcoK methylome was largely unaltered by triclosan exposure. There was more variation associated with the ATATAGCA motif which may relate to a non-canonical Dam site [9].

There was enrichment of genes associated with the membrane when there was increased or decreased methylation, regardless of the site. Molecular function enrichment analysis showed no enrichment when methylation was decreased and no significant differences when there was no change in methylation. However, there were differences between GATC and non-GATC sites, with enrichment in transmembrane transporter activity and transporter activity when methylation increased at non-GATC sites. This may be a reflection of the role of this type of molecule in resistance development.

The genes plsB, mdtP, evgS and bglX showed the highest levels of differential methylation.

MdtP is an outer membrane factor family component of the MdtNOP multidrug efflux pump which is involved in sulphur drug resistance [10]. It has two GATC sites hemimethylated in resistant strains compared to fully methylated in susceptible strains. Neither site is in the vicinity of the promoter region, however there is growing evidence that transcription factor targets can occur within genes and that non-canonical binding sites can have hidden functionality [11], highlighting a potential impact of this hemimethylation on gene expression. Gene activation by hemimethylation may be more common than repression, this effect may be supported here via the decrease in susceptibility to the sulphonamide cotrimoxazole (TS) seen in resistant strains.

EvgS encodes a sensor kinase for acid and drug resistance. As a component of the EvgAS signal transduction system it is involved in the transcriptional regulation of acrAB, tolC, emrKY, mdtEF, and mdfA [12]. There are a number of sites hemimethylated in the resistant strain that are not methylated in susceptible strains. All 6 are located on the negative strand, 4 within the first 800bp of the gene. There is a crp and a narL binding site in the vicinity of 2 hemimethylated sites and 2 are in the vicinity of promoter elements, with the remaining sites towards the middle of the gene. It may be that hemimethylation also causes an increase in expression here which would lead into the development of resistance via the regulation of the efflux pumps. Given the susceptible strains show no methylation at these sites, the effect may be mediated through the relief of repression.

PlsB is involved in phospholipid metabolism and has been linked to persister cell formation in E. *coli* [13]. There are 2 fully methylated sites towards the end of the gene. If we consider the potential functionality of within-gene transcription factor targets and that hemimethylation is related to an increase in gene expression, the full methylation of these sites may be related a decrease in the expression of this gene and a move away from a subpopulation of persisters.

BglX encodes a glucosidase, these enzymes have not been linked to antibiotic resistance to date.

Ongoing work is focused on assessing the functional effect of the differential methylation.

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