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Biocide tolerance in foodborne pathogens

Key external stakeholders:

Food industry, biocide producers, regulatory authorities

Practical implications for stakeholders:

The outcome of this project is a greater understanding of how foodborne pathogens including *E. coli* O157 and *Salmonella* spp. respond to the presence of biocidal agents, with a particular emphasis on triclosan.

- A panel of verocytotoxigenic *E.coli* (VTEC) and *Salmonella* isolates were found to have minimum inhibitory concentrations (MIC) less than the recommended working concentrations of a number of commercial biocide formulations, although some possessed an MIC of greater than 50% of the working concentration of some agents. This highlights the importance of strict adherence to manufacturer guidelines and appropriate training of personnel.
- Mutants with an enhanced tolerance to triclosan were readily obtained for both *Salmonella* and VTEC. In the case of *Salmonella* corresponding alterations to the strains' antibiotic profiles were observed, illustrating an additional public health risk.
- A spectroscopic method was developed for the detection of quaternary ammonium compounds on stainless steel surfaces, allowing for the detection of residue build up which may constitute a risk for pathogen exposure to sub lethal concentrations of such agents. This would increase the likelihood of resistance developing.

Main results:

A bank of foodborne pathogen isolates were tested against commercial biocide formulations. Although all isolates had an MIC below the recommended working concentration for all the biocide formulations tested a concern is that for some isolate-biocide combinations the MIC was 50% of the working concentration. Such a concentration may easily occur in real world situations, either due to over dilution, handler error or high organic load. Through this study the transcriptomic and proteomic response of triclosan tolerant *E. coli* O157 and *Salmonella* mutants in comparison with their reference strains were characterised in detail, identifying key responses for each pathogen. Subsequent phenotypic studies showed key changes which may contribute to enhanced pathogen persistence. A spectroscopic method was developed for measuring the potential buildup of biocidal agents on industrial surfaces.

Opportunity / Benefit:

The findings of this project provide a detailed analysis of the response of two key foodborne pathogens to sub lethal exposure to biocides commonly used in the farm to fork chain and how these responses may contribute to pathogen persistence in the food chain. The project findings underline the key importance of utilising biocidal agents as directed. Furthermore, the spectroscopic method developed and validated as part of this project is readily transferable to industry for the measurement of the buildup of biocide residues on industrial surfaces.

Collaborating Institutions:

University College Dublin

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1. Project background:

Biocides are deployed at all stages of the farm-to-fork continuum to eliminate or reduce pathogenic microorganisms and thus decrease the likelihood of human or animal exposure to such infectious agents. However, the emergence of biocide resistant bacteria has been reported. In contrast to their increased usage there has not been a corresponding increase in the understanding of the microbial response to biocides. It is vital that this is investigated as it is unknown if the development of resistance has an impact on bacterial response to stresses such as those encountered in food processing. It may enhance virulence of strains or may contribute to subsequent resistance to antibiotics. Such events may thus contribute to the enhanced persistence and dissemination of pathogenic strains in the food chain and pose an increased risk to consumer health. In an effort to fill these knowledge gaps the objectives of this project were (i) to establish the biocide resistance in a panel of key Gram-negative pathogens, namely *Salmonella* and verocytotoxigenic *E. coli*, (ii) to compare the response of biocide resistant strains with that of parent strains to stresses such as those encountered in food processing, (iii) to provide an understanding of biocide resistance using genomic, proteomic and metabolomic technologies and (iv) to determine if biocide resistance impacts on virulence characteristics and gene transfer. Such information will be crucial so that guidance can be provided to the food industry regarding appropriate biocide usage so as to minimise the development of biocide resistance.

2. Questions addressed by the project:

- Do a bank of verocytotoxigenic *E. coli* and *Salmonella* isolates have a minimum inhibitory concentration greater than the working concentration of eight biocide formulations used in the Irish food industry?

- Is there a correlation between biocide and antibiotic resistance?

- What are the genomic, proteomic and phenotypic differences between biocide susceptible and biocide tolerant foodborne pathogens?

- Does biocide tolerance impact on other stress responses in foodborne pathogens?

- In dry cleaning processes can biocide residues build up on surfaces?

3. The experimental studies:

A multidisciplinary approach was required to address the research questions posed. A high throughput microtitre plate based screening strategy was developed to determine the MIC and minimum bacteriocidal concentration (MBC) of a large panel of VTEC and *Salmonella* strains for eight commercial biocide formulations used by the Irish food industry. Concurrently, antibiotic resistance profiles were also generated for all strains. Whole genome microarray studies were used to determine the transcriptomic changes in mutants with increased tolerance to triclosan, with the studies validated using real time quantitative PCR. This work was correlated with a proteomic study done using two dimensional fluorescence difference gel electrophoresis. Phenotype microarrays were utilised to identify metabolomics changes in triclosan tolerant mutants. Attachment and invasion studies were undertaken using cell culture assays and biofilm studies were undertaken using agar based and microtitre plate assays. Finally, a spectroscopic assay was developed for the rapid measurement of quaternary ammonium compounds on stainless steel surfaces.

4. Main results:

The results obtained in this project are described in detail in the publications listed below and further manuscripts currently in review and therefore a brief overview is provided here. The first task of this project focused on the testing of a large panel of VTEC and *Salmonella* isolates for potential resistance to eight commercial biocide formulations which were selected in consultation with Irish food industry representatives. In all cases the MICs were found to be less than the recommended working concentration. Of concern is that in some cases the MIC was over 50% of the recommended working concentration. Initially mutants were

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selected with increased tolerance to commercial biocides but the resistance phenotype was not stable. Therefore a subsequent panel of stable mutants with increased tolerance to triclosan, benzylkonium chloride or chlorhexidine was selected. For all mutants the MIC for commercial biocide formulations was not altered. For the *E. coli* isolates the antibiotic resistance profiles were also not altered but for the some of the *Salmonella* mutants an increase in antibiotic resistance was observed.

The biocide tolerant mutants were compared to their reference strains in a number of different ways. They were compared for their tolerance to common food processing stresses, namely heat and low pH, but in general no significant differences were observed. Transcriptomic studies using whole genome microarrays were undertaken to compare the gene expression of the strains upon sublethal exposure to the biocide. A wealth of information was obtained in these studies, one example being the upregulation of the flagellar assembly pathway in the triclosan tolerant *E. coli* O157 isolate with 33 of 38 genes being highly upregulated. Transmission electron microscopy indicated the presence of extended flagella in this strain. Flagella are connected with biofilm formation and motility which may contribute to strain persistence and virulence. A proteomic study was undertaken using fluorescent 2D DIGE which corroborated the results obtained in the transcriptomic study. It also showed the upregulation of general metabolism proteins and outer membrane proteins in the biocide tolerant mutants. Efflux pump inhibitor studies also showed the clear involvement of efflux pumps in tolerance to the biocides tested. Triclosan targets FabI, a protein involved in fatty acid biosynthesis but the work undertaken in this project clearly indicates that high level triclosan resistance involves a number of different cellular mechanisms.

In a parallel study a spectroscopic method was developed and validated for the measurement of quaternary ammonium compound residues on stainless steel surfaces. This is particularly relevant in dry cleaning regimes. The study showed that these residues could remain stable on stainless steel surfaces for at least six days.

5. Opportunity/Benefit:

This project has provided a much greater insight into foodborne pathogen response to sub lethal exposure to biocidal agents commonly used in commercial formulations. It provides an in depth understanding of the key pathways affected by exposure to low levels of biocide and therefore how this may impact on a number of factors including persistence, antibiotic resistance and resistance to other food processing stresses. Such knowledge is of benefit to food industry managers, regulators and policymakers considering the utilisation of such agents.

The spectroscopic method developed as part of this project would be useful to the quality control sector of food processing factories. The instrument required for residues monitoring, a spectrophotometer, is widely available in industrial plants which makes this technique beneficial to the food industry.

6. Dissemination:

Dissemination of the results of this project was achieved through a number of different routes. A stakeholder focused conference highlighting the results of the project was held on November 10th 2012 at the Teagasc Food Research Centre Ashtown. Discussions regarding the results of this project have taken place with industry collaborators and with the relevant sections of the Department of Agriculture, Food and the Marine and the Food Safety Authority of Ireland. Furthermore, a number of peer reviewed papers have been published as a result of this project and are listed below. A number of other manuscripts are currently in review prior to publication. Both oral (14) and poster (10) presentations have been given regarding various aspects of the project at national and international conferences and workshops throughout the lifetime of the project. Two PhD theses have been submitted and an MSc is currently being finalised.

Main publications:

Sheridan Á, Lenahan M., Condell O., Bonilla-Santiago R., Sergeant K., Renaut J., Duffy G., Fanning S., Nally J. E. and C.M. Burgess. (2013) Proteomic and phenotypic analysis of verocytotoxigenic *Escherichia coli* (VTEC) with tolerance to triclosan. *Journal of Proteomics* 80: 78-90

Sheridan À., M. Lenahan, G. Duffy, S. Fanning and C.M. Burgess (2012). The potential of biocide tolerance in *Escherichia coli* and its impact on the response to food processing stresses. *Food Control*, 26:98-106.

Condell O., C. Iversen, S. Cooney, K. Power, C. Walsh, CM Burgess and S. Fanning (2012). Efficacy of biocides used in the modern food industry to control *Salmonella* - links between biocide tolerance and resistance to clinically relevant antimicrobial compounds. *Applied and Environmental Microbiology* 78(9):3087-97

Condell O, Sheridan Á, Power KA, Bonilla-Santiago R, Sergeant K, Renaut J, Burgess C, Fanning S, Nally

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JE. (2012) Comparative proteomic analysis of *Salmonella* tolerance to the biocide active agent triclosan. *Journal of Proteomics* 75(14):4505-19.

Popular publications:

Burgess C. (2009). Biocide resistance: a food safety challenge? The Ashtown Food Innovator, issue 6.

Lenahan M., Á. Sheridan, G. Duffy, S. Fanning, O. Condell, C.M. Burgess, (2011). Microbial tolerance to biocides. TResearch, Volume 6: Number 2, 22-23.

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