VIABILITY OF ENGINEERED BIOCATALYSTS IN BIOTRANSFORMATION

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Abstract

This project aims to exploit engineered biofilms as biocatalysts in the biotransformations of enantiomerically pure compounds for fine chemical and pharmaceutical industry. It aims for conditions to be designed which would improve reactions and formation of the engineered biofilms.

Tsoligkas et al. (2012) has previously engineered a biofilm to act as a biocatalyst using tryptophan synthase, TrpBA produced from plasmid pSTB7 to catalyse the biotransformation of haloindoles to L-halotryptophans. To build on this work, biofilm formation and how the cells react to the biotransformation were investigated through flow cytometry and analysis of colony forming units (CFU).

For biofilms to be formed from Escherichia coli (E. Coli) K-12, it was found that the plasmid pT7-csgD had to be present or the strain required an ompR234 point mutation to allow production of curli for extracellular polymeric substances to form a biofilm. This demonstrates the importance of CsgD as a regulator for formation, as without an increase in cellular concentration E. coli cells failed to attach to glass surfaces.

From planktonic data it is apparent that carrying out the biotransformation with 5-chloroindole has a toxic effect on metabolically active E. coli PHL644 pSTB7. The source of this toxicity is not clear, it may be due to the products of the reaction, the chloroindole being metabolised or incorporated into the cellular proteins.

Efflux data indicates that cells are incubated with fluoroindole have decreased efflux, an advantage for biotransformation.

Acknowledgments

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Chapter 1. Introduction

1.1. Overview

Bacteria do not live primarily as planktonic cells but as biofilms; sessile communities with a complex 3D structure. Bacteria can switch between these different lifestyles (Danese et al. 2000, Ogasawara et al. 2010). This study hopes to take advantage of this biofilm state to perform biocatalysis reactions for biotransformations of enantiomerically pure compounds for the fine chemical and pharmaceutical industry. The reaction under investigation is the use of enzyme tryptophan synthase (TrpBA) to catalyse the conversion of haloindoles to L-halotryptophans. This biocatalysis has previously been done through using whole cells or by immobilising the TrpBA enzyme. It is hoped to use a biofilm to provide immobilised cells, in a self produced matrix, where the bacteria could be engineered to perform the reactions required. Biofilms are highly robust due to their self-immobilised nature and this is the root of their enhanced resistance and persistence in adverse environmental conditions and to antibiotics, making them of interest to industry (Barnhart and Chapman 2006, Rosche et al. 2009, Zhang et al. 2007). As a biofilm produces its own matrix that provides protection to the cells so that they can survive for longer and are capable of catalysing reactions for longer periods. Thus biofilm-catalysed biotransformations are potentially advantageous when compared to planktonic microbes in batch or fed-batch reactors or immobilised enzymes since immobilisation is intricate and there are no universal ways to perform it (Rosche et al. 2009).

1.2. Biofilm Structure and Function

The biofilm is a distinct lifestyle for bacteria which provides adaption to the environment (Jackson et al. 2001). For example, some Gram negative bacteria change from a planktonic to biofilm mode in a nutrient rich medium, but return to a planktonic lifestyle when nutrients are depleted to search for more (Adnan et al. 2010). Biofilms consist of a community of bacteria in an extracellular matrix which consists of extracellular polymeric substances (EPS) that form a complex three dimensional (3D) architecture which has water channels and pillars that promote transfer of nutrients to individual bacterial, aiding survival (Jackson et al. 2001, Danese et al. 2000, Barnhart and Chapman 2006). Many species of microbes can form biofilms and their self-secreted EPS matrix allows protection so they can grow on many surfaces and environments persistently. They also contain cells at different growth phases, which allows for persistence (Rosche et al. 2009).

Biofilms can be formed by many bacteria on different surfaces and solid-liquid or liquid-gas interfaces and often occur in infections. Biofilms are highly robust due to their self-immobilised nature and lower metabolic rate and this is the root of their enhanced resistance and persistence in adverse environmental conditions and to antibiotics, making them of interest both medically (Costerton et al. 1999) and to industry (Barnhart and Chapman 2006, Rosche et al. 2009, Zhang et al. 2007).

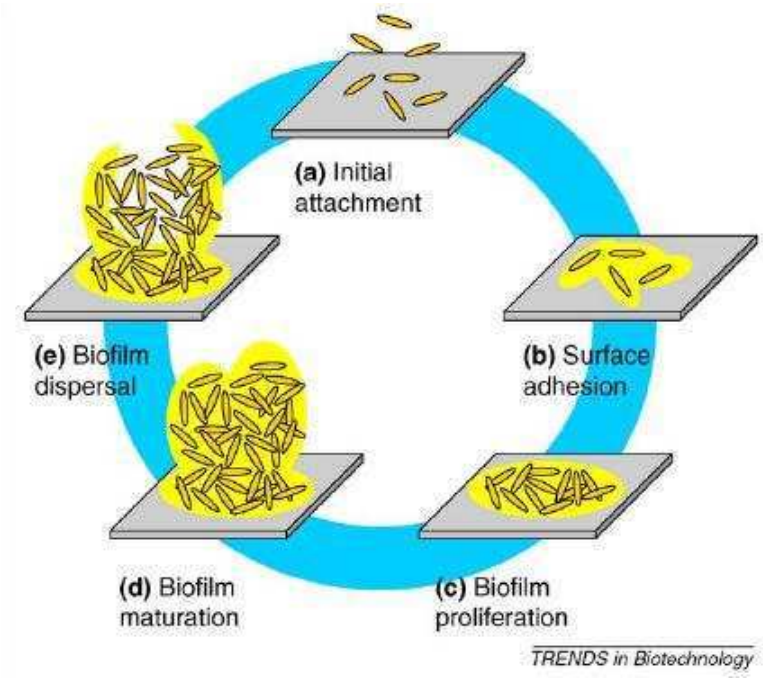


Figure 1.1: The Cyclical Nature of a Biofilm
The different stages of biofilm formation, attachment, maturation and dispersal (Rosche et al. 2009)

Biofilms are made by bacteria experiencing distinct environmental and physical cues (Ito et al. 2009). When bacteria switch between planktonic and biofilm lifestyles, cells undergo a transition resulting in a marked difference in characteristics and behaviour including cell morphology, physiology and metabolism. This results in genes being expressed from different loci, normally for extracellular factors that promote surface colonisation and cell-cell contacts (Ogasawara et al. 2010, Barnhart and Chapman 2006). Due to the diverse nature of the biofilm and its varying response to the environment, there are many highly regulated processes taking places, such as formation and dispersal (Wood et al. 2011). Many genes are differentially expressed in biofilms including those involved in metabolism to signal transport (Zhang et al. 2007).

1.3. Stages of biofilm development

Biofilm growth is a multistage process (Figure 1) determined by many cellular, environmental and surface factors. A biofilm is normally formed at an air-liquid interface or on a submerged surface where cells can attach through appendages and EPS components, fimbriae, flagellar, polysaccharides, proteins, lipids and DNA This formation can be dependent on quorum sensing (QS), a type of cell-cell communication (Rosche et al.. 2009, Wood et al.. 2011). Biofilms are more normally formed more readily by motile bacteria, but non motile E. coli

do form biofilms given enough time (Danese et al. 2000). In this work we are mostly concentrating on the initial stages of biofilm development.

Biofilm development is a cyclical process (Figure 1.) and occurs as follows: Initial cell attachment to a surface through motility (a), followed by surface adhesion (b), permanent attachment through monolayer formation and biofilm proliferation (c). Maturation then occurs through migration of clusters of cells to form multilayer microcolonies and the production of EPS to develop a heterogeneous and complex 3D structure (d). Finally there is dispersal in response to different cues (Rosche et al. 2009, Wood et al. 2011).

Different stages of biofilm development have different expression of genes particularly those involved in the synthesis of amino acids and membrane transporter proteins (May and Okabe 2011). Differential expression occurs in different media also, such as the fim gene cluster being required for biofilm formation in rich LB media. This results in biofilms developing distinct phenotypes depending on their growth medium (Danese et al. 2000).

Biofilms have a complex 3D architecture with channels and pores. The formation of this structure is dependent upon adhesion, aggregation and community expansion factors (Rosche et al. 2009, Adnan et al. 2010). EPS is extracellular polymeric substance which is made up of many components and is secreted into the matrix surrounding the bacteria in a biofilm. Atomic Force Microscopy, which measures intermolecular forces, has been used to measure adhesion forces in biofilms. In Gram positive bacterial biofilms the EPS is highly packed with large adhesion forces. Conversely, Gram negative bacterial biofilms have less tightly-packed EPS and there are lower adhesion forces (Tsoligkas et al. 2012).

1.4. Biofilm determinants and their regulation

1.4.1. Attachment

Different factors and interactions are required for biofilm formation to occur as many cell surface structures are required. In E. coli, attachment and adherence of biofilms normally requires flagella, type I pili and curli fimbriae. For attachment to take place flagellar mediated motility is required to create primary cell surface contacts then type I pili are used to stabilise these (May and Okabe 2011, Jackson et al. 2001, Danese et al..

2000). Motility through flagella allow for cell surface spread and abiotic surface attachment resulting in permanent attachment (Zhang et al. 2007).

1.4.2. Maturation

When a biofilm reaches maturation cell motility would become a hindrance so flagellar gene expression is decreased (Jackson et al. 2001). In maturation, distinct layers and structures develop, these have characteristic metabolic pathways. This may be due to diffusion limits creating a concentration gradient resulting in differences in solute uptake and utilisation giving variability in the state of different cells (Rosche et al. 2009). Mature biofilms are thought to be brought about due to cell surface structures, type I fimbriae, curli, exopolysaccharide polymers, etc. However not all these structures are always required for biofilm formation and maturation (May and Okabe 2011).

1.4.3. Dispersal

Active dispersal of a biofilm is normally initiated by the bacteria, whereas passive dispersal occurs through the action of external forces. Biofilm dispersal can occur under favourable conditions for expansion, as well as unfavourable conditions, such as lack of nutrients. Some biofilm dispersal is believed to be cell cycle dependent and dispersal based on motility is a result of intracellular cyclic-di-Guanylyl Monophosphate (c-di-GMP) concentrations. Other ways of activating dispersal are through degrading or repressing adhesion compounds in the EPS, degrading the growth substrate of the biofilm, lysing a sub-population of cells, inducing motility, addition of an extracellular surfactant, modifying fimbrial adhesion or increasing cell division at the outside of the biofilm (Jackson et al.. 2001, Ma et al.. 2011).

1.4.4. Curli

The cells ability to form curli is an important part of biofilm formation which is tied in with its low temperature dependent production (Barnhart and Chapman 2006, Zhang et al. 2007). Curli is a protein component of the extracellular matrix (ECM) and is a bacterial amyloid that promotes adherence (Barnhart and Chapman 2006).

Curli proteins are produced as soon as cells attach to a surface at initial attachment and are required for cell-cell interactions and virulence (Ogasawara et al. 2010) and are also required for a biofilm to form a 3D structure (Zhang et al. 2007). Later in biofilm development curli expression is turned off to allow further biofilm

maturation to occur (Barnhart and Chapman 2006). Curli production is also linked to bacterial cellulose synthesis (another component of EPS), this leads to the formation of an ECM with tight cell-cell and cell-surface interactions (Figure 2 Brombacher et al.. 2006).

In E. coli the operons for curli are csgBAC and csgDEFG which code for six proteins. The csgBAC operon encodes CsgA a structural subunit, and CsgB, a nucleator protein which are made of repeat motifs and share 30% sequence homology. CsgC has no known role in curli synthesis and no transcript has been found from its gene. Both proteins are required for curli assembly through a nucleation precipitation pathway. The absence of CsgB results in monomers of CsgA being secreted as CsgA is normally excreted from bacteria and nucleated into fibres by CsgB (Barnhart and Chapman 2006). The csgDEFG operon encodes four accessory proteins: CsgD a positive transcription regulator to csgB; CsgG, an outer membrane lipoprotein that could form a channel which mediates the secretion of CsgA and CsgB; CsgE, a periplasmic protein; and CsgF, another periplasmic protein that interacts with CsgG (Barnhart and Chapman 2006). CsgD is an important regulator during ECM production as it regulates curli synthesis and indirectly effects cellulose production through adrA.

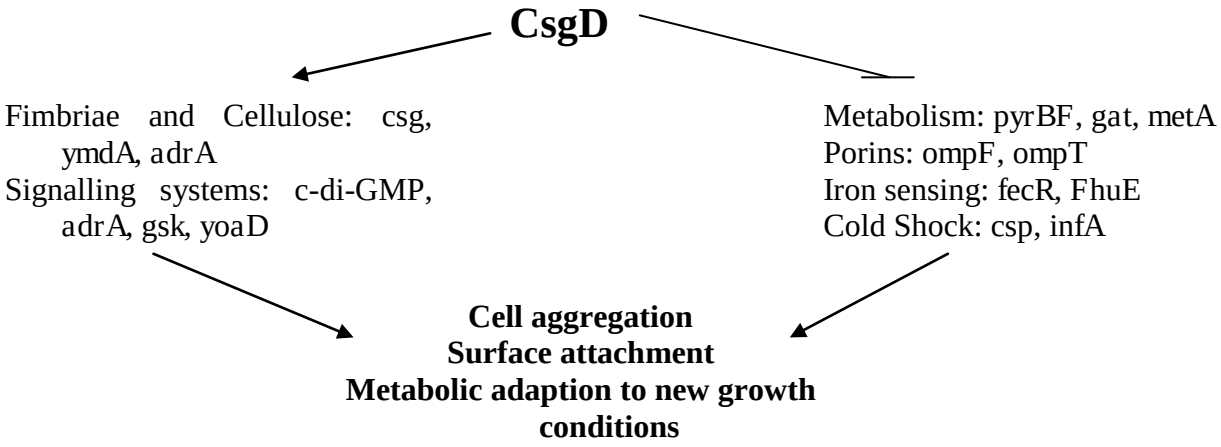


Figure 1.2: How CsgD transcription affects cells

Curli expression is dependent on physical and environmental cues and has a complicated regulation of the csgDEFG and csgBAC operons. Osmolarity affects the csgDEFG operon (via the EnvZ-OmpR two component regulatory system), controlling curli production. Temperature dependent regulation of curli occurs after CsgD transcription has occurred (Brombacher et al. 2006).

The curli operons' expression is environment dependent and is at its maximum when the growth media has a very low salt concentration, limited nutrients and is microaerophillic (low in oxygen). CsgD is known to positively regulate the csgBA operon. RpoS is a stationary phase sigma factor which is responsible for curli expression in stationary phase. Cooperative regulation occurs when there is interaction between RpoS and CRL which binds to the csgBA promoter. MlrA is activated by RpoS and it, in turn is a positive transcription regulator of csgD (Barnhart and Chapman 2006).

1.4.5. CsgD Formation

CsgD is a LuxR family transcription regulator associated with the cytoplasmic membrane that activates the expression of csg operons and has binding sites in csgB, adrA and the pepD promoters. CsgD has low expression in exponential phase but higher expression in stationary phase (Ogasawara et al. 2010, Brombacher et al. 2006). The csgDEFG operon has two promoters which show features of RpoD dependence, and its transcription is also dependent on RpoS. When CsgD is expressed there is an increase in the expression of RpoS (Ogasawara et al. 2010).

Studies have suggested that CsgD is under control of several stress regulation proteins: OmpR, CpxR, Crl, CRP, H-NS, IHF and RcsB. Also, a phosphorelay network from PhoQP, that senses Mg^{2+}, to RstBA, that senses low pH, is involved in the regulation of the csgD promoter. The complete regulation of CsgD synthesis is still poorly defined. Ten transcription factors and two sigma factors have been shown to regulate the csgD promoter; there is direct binding to the promoter by CpxR, H-NS, IHF, OmpR and RstA. OmpR and IHF are known to be positive regulation factors and CpxR and H-NS are negative. The increase of expression of csgD in stationary phase may be due to the positive influence of IHF. When the cell enters stationary phase production of RstA, OmpR and CpxR are increased (Ogasawara et al. 2010).

The binding affinity for the csgD promoter is highest for IHF and CpxR followed by OmpR, H-NS and RstA. These five transcription factors all bind within the same region between -61 and -43 base pairs (relative to the transcription start point) and IHF, CpxR and HN-S also bind at an upstream region, -188 to -159. So these transcription factors could compete to bind to the promoter. Ogasawara et al. (2010) also found that there is cooperation between some factors; between CpxR with H-NS, OmpR with IHF, and RstA with IHF. During

stationary phase IHF has an increased concentration whereas H-NS has a steady concentration (Ogasawara et al. 2010).

CpxA is a sensor kinase and CpxR is a response regulator, which responds to envelope stress and the misfolding of periplasmic proteins. Both CpxA and CpxR negatively regulate csgD. The Rcs pathway is required for biofilm formation and responds to outer membrane stress and also negatively regulates csgD (Barnhart and Chapman 2006).

CsgD activates transcription of the csgBAC operon encoding the structural subunits of curli and adrA, a positive effector of cellulose biosynthesis. CsgD has been found to control modulation of cellulose production through activation of yoaD which encodes a c-di-GMP esterase. CsgD also represses biofilm formation and cell-cell aggregation through repressing fecR which is a regulatory protein that responds to iron and cspA, another regulatory protein, which responds to cold shock. CsgD suppresses glycine autotrophy and stimulates serine hydroxymethyl transferase activity by glyA. This produces more glycine, which aids curli synthesis as curli contains more glycine than other proteins (Ogasawara et al. 2010, Brombacher et al. 2006).

1.4.6. OmpR regulation of curli

The ompR234 allele is a mutated version of ompR where leucine is substituted for arginine at ompR234. This substitution increases the transcription of ompR and its dependent genes such as csgD. OmpR234 increases transcription from the csgD promoter and increased levels of CsgD can increase curli and cellulose biosynthesis and biofilm formation in E. coli by constitutively activating curli gene expression (Barnhart and Chapman 2006). EnvZ is a sensor kinase that detects extracellular osmolarity; EnvZ activates OmpR via photophostransfer, which then activates genes involved in diverse responses to osmolarity. OmpR is involved in curli expression and thus biofilm formation through positively regulating csgD (Barnhart and Chapman 2006).

csgD is also a known target of the small regulatory RNAs OmrA/B which interacts with csgD mRNA. When OmrA and OmrB are overexpressed they inhibit at an upstream start site and decrease CsgD levels which in turn decreases both curli and cellulose production (Holmqvist et al. 2010).

Regulation of N-acetylglucosamine-6-phosphate (GlcNAc-6P) also has an effect on curli production: deletion of nagA, a GlcNAc-6P deacetylase gene, results in a decrease in the activity of curli specific promoters and thus a drop in curli production. Disruption of nagC, which encodes the nag operon regulator, also results in a drop of curli production. Also when there is an increase in GlcNAC-6P levels, there is a downregulation of curli production (Ogasawara et al. 2010).

1.4.7. Flagella

When flagella of E. coli are turned off in the switch from planktonic growth to biofilm formation, it allows for cell-cell adhesion to occur. FlhDC is the primary transcription regulator in the control of flagellar gene activation and synthesis. It forms heterotetramers ($FlhD_2C_2$) (Ogasawara et al. 2010).

1.4.8. Antigen 43

Antigen 43 (Ag43) is encoded by the agn43 or flu gene and is an outer membrane protein that is a self recognising adhesin which increases biofilm formation in minimal medium and influences autoaggregation of E. coli. Ag43 has been found to facilitate cell-cell interactions and cell-surface contacts; cells deficient in Ag43 are deficient in forming biofilms (Danese et al. 2000, Zhang et al. 2007).

Deletion of OxyR increases the ability of E. coli to form biofilms. OxyR is activated during periods of oxidative stress and regulates ag43 under stress. OxyR/Dam mediate phase variable transcriptional regulation of ag43 (Danese et al. 2000).

1.4.9. Polymeric Components of ECM

Osmotic stress is a factor which induces the production of EPS, capsular colanic acid and poly-N-acetyl glucosamine and activation of cell-cell adhesion during biofilm development and maturation. When EPS synthesis is promoted there is a formation of a thick mature biofilm (May et al. 2009).

Colanic acid is an exopolysaccharide important in the in the maturation stage of biofilm development. It is required with curli and/or type I pili for the 3D architecture to form in the biofilm (Jackson et al. 2001). The Rcs phosphorelay positively controls expression of the cps operon which encodes proteins for the production and secretion of EPS colanic acid (Ferrieres et al. 2009).

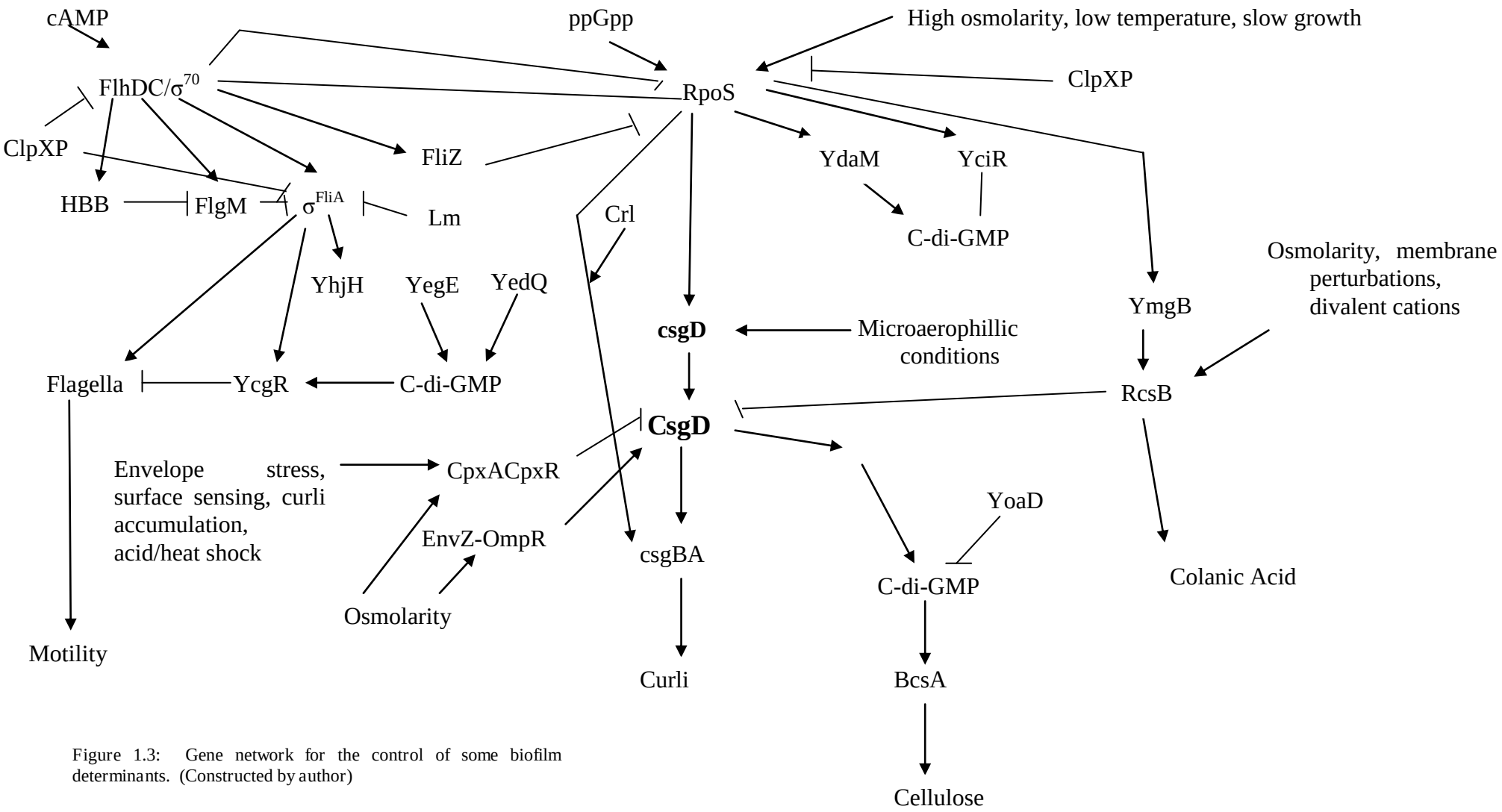


Figure 1.3: Gene network for the control of some biofilm determinants. (Constructed by author)

Extracellular DNA (eDNA) is another component of the EPS, it is important for initial attachment and for maturation of biofilms to a complex 3D structure (Wood et al. 2011).

Poly-N-acetylglucosamine (PNAG) is an EPS component which aids biofilm formation. It increases the EPS production and is dependent on pgaA gene function. When the YddV protein is overexpressed there is a ten times increase in the expression of pgaA. YddV appears to directly regulate pgaABCD. PNAG can be degraded by Dispersin B (Tagliabue et al. 2010).

NhaR responds to Na^+ ions and regulates transcription initiation of pgaABCD. However pgaABCD expression is mainly regulated post transcriptionally through the RNA binding of CsrA resulting in negative control. CsrA binds to an untranslated region (UTR) to block translation and promote degradation of the RNA. YddV positively regulates pgaABCD but not through CsrA or by mRNA stabilisation (Tagliabue et al. 2010).

YdeH is a protein which affects the production of PNAG through the stabilisation of PgaD (Tagliabue et al. 2010).

1.5. Global regulators of biofilm formation

1.5.1. RpoS

RpoS is a sigma factor that activates the transcription of genes required for the stationary phase, during stress and within a biofilm. Sigma factors are small proteins which allow for RNA polymerase to bind to specific gene promoters. When under stress, cells induce alternate sigma factors. RpoS is a master regulator of several stress responses. When E. coli is growing under normal conditions RpoS is present at low concentrations and is readily degraded by proteases. When stressed, E. coli generates more RpoS which causes a reduction in growth rate enabling the bacteria to survive. RpoS expression is controlled in a very complex manner at the levels of transcription, translation, proteolysis and allosteric activation (Jackson et al. 2001, Adnan et al. 2010).

RpoS is important in biofilms for global gene regulation. Ito et al. (2009) suggest that expression of rpoS varies throughout the bacterial cell cycle and that it suppresses energy metabolism and flagella-mediated motility after micro-colony formation allowing biofilm maturation to continue (Ito et al. 2009).

Ito et al. (2009) used a mutant of E. coli with a chromosomal rpoS-GFP transcription fusion strain in flow cells. After 24 hours the biofilm showed a high fluorescence signal (signifying high rpoS expression) at the outside of the biofilm and no detectable expression inside. There was a constant signal of DsRed (red fluorescent protein) in the biofilm showing oxygen was diffusing throughout the biofilm. The expression of rpoS was found to be four times higher on the outside of the biofilm than the inside; this was accompanied by 717 genes upregulated and 117 genes downregulated in this region. The upregulated genes include 37 involved in adhesion, 28 genes involved in efflux systems and 7 genes involved in the stress response. Out of these, 103 genes have been reported to positively regulated by rpoS and are thought to be involved in adhesion, colanic acid synthesis, stress responses, multidrug resistance and efflux pumps. This expression at the outside of a biofilm is thought to contribute towards maturation and stress so RpoS is required for adjusting cell physiology (Ito et al. 2009).

1.5.2. CsrA

CsrA is a global regulator protein involved in biofilm formation and the stationary phase. CsrA represses metabolic pathways such as those involved in glycogen biosynthesis and catabolism and gluconeogenesis, which are activated in the stationary phase. It activates glycolysis, acetate metabolism and motility. It regulates these through posttranscriptionally binding to mRNA molecules to increase or decrease decay rates of target genes. CsrA is antagonised by the activity of CsrB RNA which binds to it (Jackson et al. 2001). CsrA is involved in glycogen biosynthesis, thought to be important for biofilm formation. If glycogen biosynthesis is increased there is an improvement in biofilm formation (Jackson et al. 2001). CsrA mutant strains have been found after attachment to form biofilms rapidly and extensively, generating a normal biofilm (Jackson et al. 2001).

CsrA is postulated to act as a signal for biofilm dispersal, as after induction of csrA there is dispersal within 4-6 hours. In biofilm formation it appears to increase carbon uptake through a redirection of the central carbon flux. So if glucose is added to the medium then csrA is expressed which represses biofilm formation (Jackson et al. 2001). CsrA represses the synthesis of the adhesion poly-β 1, 6-N-acetyl-D-glucosamine also involved in biofilm formation (Ma et al. 2011).

1.5.3. YcfR/BhsA

YcfR or BhsA is thought to be involved in metabolite transport in the cell and is one of the most induced genes in E. coli biofilms. It is involved in self identification, cell organisation via cell-cell contacts and intracellular signalling, is expressed in the cells stress response and also impedes cell aggregation. It is a putative membrane protein and its promoter has binding sites for CRP and SoxS. In biofilms it decreases formation by affecting glucose uptake and metabolism. YcfR induces a global stress response by inducing osmY, osmB, ompX, sodC, uspB, bssS and dnaK (Zhang et al. 2007). It is thought that deletion of YcfR results in a decrease of indole and in LB glucose medium an increase in biofilm mass. Mutant ycfR strains are more effective in coping with stress, resulting in the induction of stress tolerance genes, such as induction of acid resistant genes, gadABC so cells can survive at lower pH. The stress genes induced include rpoE which represses transcription of tnaA which in turn decreases the concentration of indole. So the loss of the ycfR gene results in greater biofilm formation in LB glucose medium (Zhang et al. 2007).

Deletion of ycfR functions to increase biofilm formation at liquid-solid interfaces by the induction of genes for cell surface proteins affecting the cells properties, increasing cell hydrophobicity and aggregation (Zhang et al. 2007).

1.5.4 BolA

bolA is an E. coli stress gene for the adaption into stationary phase, although its function is not clearly understood. When there is high expression of bolA mRNA it may result in formation of biofilms. bolA Is thought to effect cell envelopes and gives cells a round morphology with a reduced surface area which is the morphology found in cells in a biofilm. BolA regulates the transcription of the D, D-carboxypeptidases: PBP5, PBP6 and β-lactamase AmpC, all of which are involved in peptidoglycan metabolism. Expression of bolA is controlled by two promoters P1 and P2; P1 is controlled by RpoS and P2 under control of σ^D and transcribed by BolA (Adnan et al. 2010).

1.5.5. H-NS

H-NS is a global regulator in E. coli that represses transcription by recognising curved DNA sequences and has been found to reduce biofilm formation (Wood et al. 2011).

1.5.6. Reduced genome studies

May and Okabe (2011) found that an E. coli Δ13 mutant, lacking 17.6% of the genome of the MG1655 strain and lacking the genes for type I fimbriae, curli, exopolysaccharide polymers and AI-2, could still generate biofilms. From reduced genome mutants, they found the genes marR, mcbR, entB and yahK could be important for biofilm development.

entB Codes for enterobactin, an iron acquisition system which may act as a signalling molecule and is upregulated in early biofilm development. Some relevance can be drawn from low iron concentrations in the medium favouring biofilm formation, which decreases with increasing iron concentration. yahK Appears to be involved in aerobic to slightly aerobic conditions. It may have a role in biofilm regulation and is hypothesised to be a zinc type oxidoreductase like protein. dosC Encodes a diguanylate synthase which acts as an oxygen sensor, it is c-di-GMP dependent, involved in a pathway for RNA degradation and its expression accelerates early biofilm development. marR Is expressed in biofilm maturation in deeper parts of the structure where growth is suspended. It acts as a repressor to multiple antibiotic resistance by interacting with efflux pump AcrAB. mcbR Is also involved in biofilm maturation and regulates colonic acid expression as well as repressing the periplasmic protein YbiM which is involved in biofilm formation. It controls indole production, a major signal for biofilms to switch from attachment to maturation (May and Okabe 2011).

1.6. Quorum Sensing

Quorum sensing (QS) is a phenomenon of cell-cell communication which research is only in the early days of deciphering. It is thought to influence biofilm development through controlling different factors (Rosche et al. 2009, Jackson et al. 2001). QS signals work by being secreted by individual bacteria and building to an extracellular concentration; they are then either internalised or detected to result in a coordinated gene expression. The changing concentrations of the QS signals result in regulated signal responses depending on cell population density and this can act as cell-cell communication (Wood et al. 2011, Jackson et al. 2001). The ability to QS allows a cell to sense conditions and communicate this within a small population so there is a coordinated a response in the population (Tsao et al. 2011).

For a signal to be considered a QS signal it should:

- Be produced at a specific growth stage of the cells;

- Be able to accumulate extracellularly and be recognised by a specific receptor
- Be able to accumulate and generate a response throughout a population of cells; and
- Have a response that is beyond the physical changes required for the signals metabolism and detoxification.

Another two requirements put forward by Lee and Lee (2010) are:

- That the concentration of the signal required for the change in phenotype is not toxic to the cell; and
- That the signals network is adapted to the level of the community (Lee and Lee 2010).

1.6.1. QS in biofilm formation

1.6.1.1. AI-2

Autoinducer-2 (AI-2) is a QS signalling molecule in E. coli biofilms, it is normally expressed in exponential phase and also acts as an inter species signalling molecule (May and Okabe 2011, Lee and Lee 2010). It is generated from cleavage of S-ribosylhomocysteine (SRH) by LuxS to give homocysteine and 4, 5-dihydroxy-2, 3-pentanedione (DPD) which is then cyclised to AI-2. AI-2 functions by being internalised by the LsrABC transporter and its function depends on its state of phosphorylation, affecting which genes it regulates. AI-2 communicates the metabolism potential of E. coli especially when recombinant proteins are being expressed by the cell; the extracellular concentration of AI-2 decreases on increasing expression of recombinant proteins (Tsao et al. 2011). AI-2 accumulates when there is glucose present and reaches a maximum concentration at low pH. It is not heat stable, but at 37°C exogenous AI-2 increases biofilm formation (Lee and Lee 2010).

MqsR is known to regulate biofilm formation via AI-2, TqsA is involved in transporting AI-2 and BssR/BssS influences signalling through AI-2 and indole (Zhang et al. 2007).

1.6.1.2. Indole

Indole is an interspecies QS signal, with effects on biofilm formation, repressing formation through SdiA (Zhang et al. 2007). Indole is produced in stationary phase and indole in indole producing bacteria increases plasmid stability and drug resistance so the bacteria may use indole to survive against other non-indole producing bacteria and eukaryotes where indole has the opposite effect. It is classified as a QS signal as it is synthesised in stationary phase, is transported by AcrEF and Mtr, and it has a role in the control of biofilm

virulence, plasmid stability and in drug resistance. Its other effects include acting as a chemorepellent, decreasing cell motility and cell adherence to epithelial cells (Lee and Lee 2010).

In E. Coli, indole is generated in a reversible reaction from tryptophan to give indole, pyruvate and ammonia catalysed by tryptophanase (TnaA). TnaA can perform the reverse reaction, using indole as a carbon source to produce tryptophan, but the equilibrium is more favourable in the tryptophan to indole direction (Zhang et al. 2007, Lee and Lee 2010).

The gene tnaA encoding tryptophanase is induced at stationary phase and at high pH. Transcription of the tnaAB operon is controlled by cAMP and cAMP receptor protein (CRP) complex which regulate in response to glucose concentration (Zhang et al. 2007, Lee and Lee 2010). When E. coli has a large population and their carbon source is dwindling, a large quantity of indole is produced, so a glucose being present results in a lack of indole biosynthesis (Lee and Lee 2010).

Extracellular tryptophan concentration and other amino acids also influence the production of indole, and the extracellular concentration of indole is cell population density dependent (Lee and Lee 2010).

Low pH conditions, approximately below 4.3, lead to inhibition of indole production and a reduction in tnaA transcription (Lee and Lee 2010).

Indole signalling mainly occurs at low temperatures, with its effects on biofilms being more pronounced at 30°C then at 37°C, however it is not heat labile. At 30°C but not 37°C indole represses genes related to uracil biosynthesis (carAB, pyrLBI, pyrC, pyrD, pyrF, upp, and uraA)and if indole is added to an indole deficient mutant then it leads to differences in gene expression at the different temperatures leaving them less capable of surviving under stress(Lee and Lee 2010).

Some species which produces indole have been shown not to degrade it, but it is known that other bacterial species can. Many non-indole producing bacteria use dioxygenases and monoxygenases to metabolise indole, e.g. toluene-O-monoxygenase decreases levels of indole. This results in oxidised indole derivatives that affect biofilm formation, cell motility and the gene expression of pathogenic E. coli O157:H7 (Lee and Lee 2010).

Three permeases, Mtr, TnaB and AroP are thought to have a role in indole transport in different conditions: in addition indole can diffuse freely through the cell membrane. Mtr is thought to be primarily responsible for indole transport into the cell and TnaB is thought to mainly transport tryptophan (Lee and Lee 2010). Mtr is a high affinity tryptophan permease, however Pinero-Fernandez et al. (2011) found that loss of the Mtr transporter only made a cell slightly more sensitive to indole which is contradictory. They believed that Mtr could play a role in scavenging indole when the medium has low concentrations of indole (Pinero-Fernandez et al. 2011).

Indole is believed to be partly exported by multidrug exporter AcrEF, as deletion of the exporter results in an increase of the intracellular concentration. Several multidrug exporters may be involved as transcription of their genes increases on indole exposure. Indole import and export have been found to be very rapid (Zhang et al. 2007, Pinero-Fernandez et al. 2011): cells can export up to 0.6mM of indole within a rich medium (Lee and Lee 2010).

To determine the extent of indole diffusion, Pinero-Fernandez et al. 2011 used an artificial lipid membrane to see how well indole could move through an E. coli membrane unaided and found uniform movement in and out without protein transporters. However this diffusion of indole through the lipid membrane does alter the physical structure of the membrane (Pinero-Fernandez et al. 2011).

Indole is an intracellular signal for E. coli and is important to cell physiology and the transition of the cell into stationary phase. It is known to promote resistance to toxins due to its ability to induce xenobiotic exporters. Indole is also involved in preventing plasmid instability and is associated with accumulation of plasmid multimers. Indole has a role in biofilm formation, expression of virulence factors and interspecies signalling (Pinero-Fernandez et al. 2011, Lee and Lee 2010).

Indole acts upon sensor kinases BaeSR and CpxAR through their induction of mdtEF to increase drug resistance through, xenobiotic exporter genes, as well as interacting with GadX to control drug resistance (Lee and Lee 2010). Other genes it regulates are gabT, astD, cysK and tnaB when cell produced indole accumulates (Zhang et al. 2007, Lee and Lee 2010).

Indole has a role in biofilm formation through its ability to control groups of cells resulting in coordinated behaviour and it decreases biofilms by reducing motility, thought to be through interference to cell division, repressing acid resistance and reducing attachment (Lee and Lee 2010).

When indole is added to the growth medium at 1-6mM there is no increase in cell density due to toxicity. High concentrations of indole above 2mM decrease cell growth, possibly through blocking cell division, disruption of the cell envelope or oxidant toxicity. High doses affect cell metabolism and lead to a pleiotropic effect (Lee and Lee 2010).

Indole is also hypothesized to act as a signal for a non specific export response that could aid in cell survival through induction of multiple stress responses (Pinero-Fernandez et al. 2011).

1.6.1.3. SdiA

SdiA is an E. coli LuxR protein homologue that is a QS protein. It detects Acylated Homoserine Lactones (AHLs) which E. coli can sense but not generate and is required for indole sensing, but there is no proof of direct binding. SdiA variants have been shown to have a reduced ability at forming biofilms and indole has no effect on them (Lee and Lee 2010, Wood et al. 2011).

1.7. c-di-GMP as a master regulator of biofilm formation

Cyclic-di-GMP (c-di-GMP) regulates the transition between motile and sessile bacteria lifestyles based on environmental factors; it is part of QS signalling and other global signalling networks (Figure 4). Knowledge of c-di-GMP signalling is ill-defined but is thought to be regulated spatially and temporally by enzymes and downstream effectors at the transcription, translation and post-translation levels (Jenal and Malone 2006).

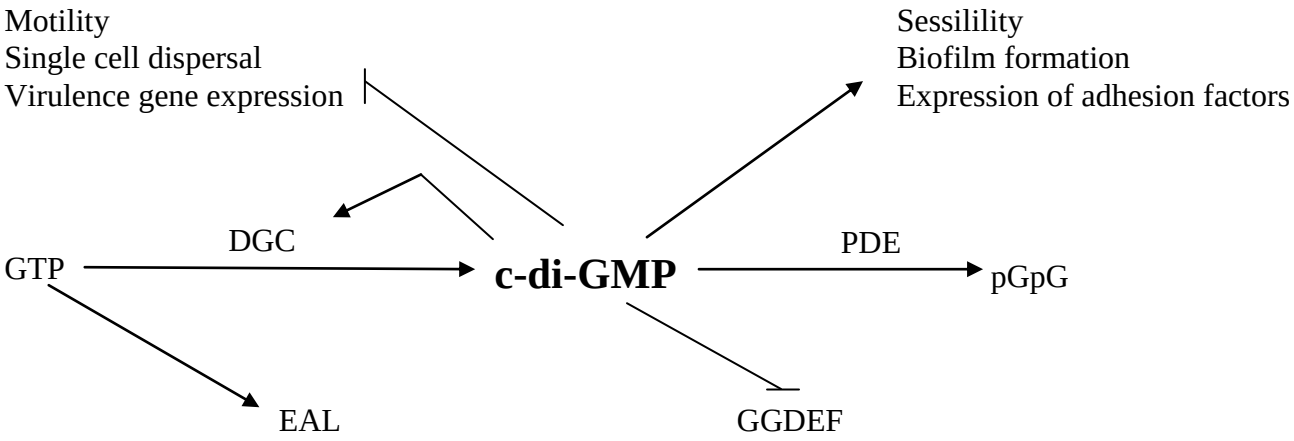


Figure 1.4: How c-di-GMP affects cell mobility
GTP is condensed by the enzyme diguanylate cyclase (DGC) to c-di-GMP which is then degraded to pGpG by the enzyme phosphodiesterase (PDE). The EAL holds the activity of PDE enzymes and is activated by GTP molecules, whereas the GGDEF domain holds the activity DGC enzymes and is inhibited by c-di-GMP (Jenal and Malone 2006).

1.7.1. Production

C-di-GMP is formed from two GTP molecules condensed by diguanylate cyclase (DGC) activity found in GGDEF domains. C-di-GMP is degraded to GMP with a transition of pGpG by phosphodiesterase (PDE) activity in EAL and HD-GYP domains. These domains are found within many bacteria and their activity controls intracellular concentration of c-di-GMP, which acts as a bacterial secondary messenger (Jenal and Malone 2006).

The balance of EAL and GGDEF domains is dependent on growth phase, cell cycle stage and adaption to surface growth. This controls the levels of c-di-GMP which controls flagellar motility through control of gene expression, motor function and organelle assembly (Figure 4) (Jenal and Malone 2006).

Proteins involved in the biosynthesis and turnover of c-di-GMP are expressed in higher numbers in Gram negative bacteria (Tagliabue et al. 2010).

C-di-GMP is important in biofilm formation in stimulating production of EPS and adhesion factors such as curli and cellulose (Tagliabue et al.. 2010) and c-di-GMP levels are inversely related to the amount of eDNA which is a component of the EPS (Wood et al.. 2011). Chemical and physical stress is known to increase biofilm formation and it has been found that in Gram negative bacteria this changes the concentration of intracellular c-

di-GMP. During biofilm formation, there is low motility and a high intracellular concentration of c-di-GMP, partly the result of inactivated DGCs (YeaI, YedQ and YfiN in E. coli). Conversely, low intracellular c-di-GMP concentrations result in increased motility and surface detachment. Thus high concentrations of c-di-GMP are required for biofilm formation and low concentrations occur later for the maturation and dispersal stages (Wood et al. 2011).

1.7.2. DGC activities in biofilm formation

Most GGDEF domains are associated, or are thought to be associated, with a signal input domain so they act in response to environmental stimuli (Jenal and Malone 2006). Many DGCs are expressed at growth temperatures below 30°C. DGCs act to increase production of c-di-GMP, but their relationship is not proportional (Tagliabue et al. 2010).

Many DGCs are important for biofilm formation. AdrA is a protein with an active GGDEF domain, synthesised in the presence of c-di-GMP and is involved in the biosynthesis of cellulose, a part of the EPS (Brombacher et al. 2006). AdrA stimulates production of cellulose through allosteric activation of the cellulose synthase protein machinery (Tagliabue et al. 2010).

YddV is one of the most highly expressed DGCs in E. coli, with its GGDEF domain in the C terminal, and helps regulate the curli operons, but YddV can also stimulate biofilm formation independently of curli. YddV activates of the csgBA operon in curli production in response to c-di-GMP levels (Tagliabue et al. 2010).

Several other E. coli DGCs are thought to be involved in biofilm regulation. YcdT is co-regulated with Poly-N-acetylglucosamine (PNAG) biosynthesis genes, another part of the EPS. Overexpression of YcdT results in a higher intracellular concentration of c-di-GMP affecting cell motility and colony size. YdaM is required for synthesis of curli and controls expression of cellulose through the expression of csgD. These DGC activities result in a reduction in cell motility (Tagliabue et al. 2010).

c-di-GMP can regulate gene expression through direct binding to riboswitch elements in mRNA (Tagliabue et al. 2010).

1.7.3. Modulation of c-di-GMP

The yddV-dos operon is the most expressed of the RpoS dependent genes related to c-di-GMP metabolism. Dos is a direct oxygen sensor protein with a heme prosthetic group and YddV is a heme binding oxygen sensor. Both YddV and Dos interact to form a stable protein complex which is highly expressed and possesses both DGC and PDE activity and YddV overexpression can stimulate biofilm formation. Dos has been found to have a negative role on curli production, its overexpression results in decreased biofilm production and a possible role in dispersal. YddV and Dos control curli production, biofilm formation and dispersal via modulation of intracellular c-di-GMP concentration (Tagliabue et al. 2010).

1.8. Industrial relevance of biofilms

Biocatalysis reactions can utilise enzymes and cells to catalyse reactions of high specificity under certain conditions that are more environmentally friendly than industrial chemical processes. For biocatalysis a whole cell can be used or the enzyme of interest can be immobilised. When a series of reactions are to be performed, this can be done within a microbe, however this is normally performed in batch or fed-batch reactors that are limited in how long they can be used. Use of immobilised enzymes is also limited by immobilisation as enzyme immobilisation is intricate and there are no universal ways to perform it (Rosche et al. 2009). Biofilms could provide an answer as they are cells immobilised in a self produced matrix and the community of bacteria could be engineered to perform the reactions required.

Biofilms have been successfully used in industry before, the first engineered biofilm was a consortium grown to prevent biocorrosion (Wood et al. 2011). They have been used in industry where substrates or reaction products affect cell viability such as in the conversion of agricultural products to alcohols or organic acids. In the food industry biofilms are used in production of vinegar using acetic acid bacteria. (Rosche et al. 2009).

Other interests for industry are due to the resistance provided by the physical barrier that the secreted EPS provides, the heterogeneity in the biofilm, the concentration of gradients found, mutations and the environmentally induced changes in gene expression (Wood et al.. 2011).

1.9. Requirements of engineered biofilms

Biofilms are used in reactors resulting in low cost operation, due to their robust and self immobilising nature, but they are little understood and complex due to intricacies in cell-cell communication and control. For use in

industry a biofilm species is required that can be easily genetically manipulated for the desired biocatalysis. This would have to be a non pathogenic species which is genetically well defined that would easily form a biofilm in a controllable way. The biofilm should be able to grow in a cheap medium and any cofactors required should be readily available or engineered to be produced by the bacteria. Further work is required to assess the cost benefit and efficiency of running long term continuous biofilm reactors. Other relevant work would be to look at the extracellular secretion of enzymes by biofilms for catalysis such as hydrolases for the production of biofuels from agricultural products and their use to produce pharmaceuticals so an enzyme cascade can be used to produce chiral compounds. (Rosche et al. 2009).

Further considerations for industry would be the production of a biofilms EPS, which would have to be controlled and monitored as overproduction would affect diffusion of substrates and products, reduce the amount of active cells for the reaction or could produce problems downstream. There is a possibility that there would be limitations with biofilms as well due to the different growth states of cells, as some will be metabolically inactive, slowing product production (Rosche et al.. 2009).

Biofilms could be controlled if there was a fuller knowledge of their genetics. Knowing this it would possible to engineer secretion of QS signals and compounds to affect biofilm phenotype, control biofilm formation and improve recombinant protein production (Wood et al.. 2011, Tsao et al.. 2011, Ma et al. 2011).

A problem with use in industry is the amount of time for a biofilm to form a certain amount of mass per volume for production. Previous biofilm reactors including fluidised bed biofilm reactors and packed bed reactors have suffered from this. The use of spin coated engineered biocatalysts (SCEBs) described in Tsoligkas et al. (2012), have overcome the time for initial attachment and monolayer formation, and allow the biofilm to go straight to maturation and formation of a 3D structure. Tsoligkas et al. (2012) used an E. coli K12 strain PHL644 which has a hyperadhesion phenotype due to a mutation in the gene encoding the osmolarity response regulator ompR which results in an increase in CsgD expression thus activating expression of curli genes csgBA generating curli. These factors allowed for extensive, heterogeneous EPS by day 6 with a transition period to this at days 5-7 to form the biofilm (Tsoligkas et al. 2012).

1.10. Genetics and Reaction

To produce a useful engineered biofilm biocatalyst, gene expression in formation, structure and function of the engineered biofilm must be understood, so they can be manipulated for efficient biocatalysis. This work initially focuses on E. coli K-12 strains with an ompR234 mutation which upregulates curli, and the biotransformation of haloindoles and serine to L-halotryptophans using TrpBA expressed from plasmid pSTB7. Understanding the cells' gene expression in the biofilm and biotransformation will enable design of conditions to optimise the processes.

The protein OmpR responds to changes in envelope pressure due to osmolarity changes detected by EnvZ, a sensor kinase. Curli is a protein component of EPS, a bacterial amyloid that promotes adherence, and thus biofilm formation and OmpR is involved in curli expression through positively regulating csgD (Barnhart and Chapman 2006). Importantly curli production has been linked to synthesis of cellulose, another component of EPS, leading to the formation of a biofilm EPS with tight cell-cell and cell-surface interactions (Brombacher et al. 2006).

The plasmid pSTB7 is a high copy number plasmid which produces tryptophan synthase from Salmonella enteric sv Typhimurium (Kawasaki et al. 1987). In this project TrpBA is being used for the biotransformation reaction of haloindoles and serine to L-halotryptophans, which are starting compound for the synthesis of bioactive products, such as the anticancer agents rebeccamycin and diazonamide (Tsoligkas et al. 2012).

Indole is the normal substrate of TrpBA and is known to affect E. coli cells by acting as an interspecies quorum sensing (QS) signal, with effects on biofilm formation (Zhang et al. 2007). Indole is synthesised in stationary phase, transported by AcrEF and Mtr, and has a role in the control of biofilms virulence, plasmid stability and drug resistance, leading to the robust and resistant nature of a biofilm. Other effects include acting as a chemo repellent, decreasing cell motility and cell adherence to epithelial cells (Lee and Lee 2010). It will be seen if halo-indoles have similar effects upon cells.

The pT7-CsgD plasmid is also used in this work, which expresses csgD and results in increased surface attachment (Brombacher et al. 2006). Expression of CsgD from the plasmid pT7-CsgD results in a functional

curli phenotype that is independent of activation of the csgDEFG promoter (Brombacher et al. 2006), thereby offering two pathways to enhanced curli production: ompR234; and csgD overproduction.

1.11. Objectives:

The project aims to use engineered biofilm catalysts for biotransformations due to their robust nature. The biotransformation currently investigated is L-haloindoles to L-halotryptophans, starting compounds in the synthesis of some anticancer agents, using the enzyme TrpBA. To optimise the use of a biofilm as a biocatalyst, gene expression of biofilms will be examined so conditions can be designed to increase the speed of biofilm formation and efficiency of the biotransformation.

This will be performed through:

- Continuing the work from Tsoligkas et al. (2012) to understand the reaction in an engineered biofilm as a biocatalyst and why it works better than cells in a planktonic state.
- Understanding the biofilms performs differently to planktonic cells in the biotransformation reaction.
- Tailoring conditions to optimise formation, maturation and biotransformation within the biofilm.

Chapter 2. Materials and Methods

Methods used to study viability of cells were flow cytometry, a laser based technology where a stream of cells in liquid is passed through a laser and scatter and fluorescence detection can be used to work out physical characteristics, such as forward scatter created by cells correlates with cell size and side scatter correlates with cell granularity Use of the dyes bis-oxanol (BOX) which binds to cells with a depolarised membrane potential and propidium iodide (PI) which binds to DNA allows estimations on population health so dead and injured cells can be detected. This was checked by performing colony forming units (CFU) to see how well cells could proliferate.

The Hoechst assay was also used to look at efflux of cells performing the biotransformation where the dye Hoechst is incubated with cells and its fluorescence is detected over a time period. Hoechst fluoresces when it binds with DNA and is a substrate for efflux pumps so fluorescence decreases as efflux increases.

2.1. Media and Strains

Four strains of E. coli were used:

- PHL628 (MG1655 malA-kan ompR234) which has a mutation in the ompR gene, a substitution at position 43 from leucine to arginine giving the ompR234 allele (Vidal et al. 1998), resulting in the strains ability to form a biofilm.
- PHL565R (MG1655), the paternal strain of PHL628 without the ompR234 allele which does not readily form a biofilm (Vidal et al. 1998).
- MC4100 (araD139 D(argF-lac)U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR) (Vidal et al. 1998).
- PHL644 (MC4100 malA-kan ompR234) comprising of MC4100 with the ompR234 allele (Vidal et al. 1998).

Strains were transformed using the heat shock method (Froger and Hall 2007) Three plasmid were used:

- pT7-csgD (Brombacher et al. 2006) a plasmid which over produces CsgD under the control of the T7 promoter to improve biofilm formation. Encodes ampicillin resistance.
- pT7-7 (Brombacher et al. 2006) an empty control plasmid. Encodes ampicillin resistance.

- pSTB7 containing the Salmonella enterica serovar Typhimurium TB1533 trpA and trpB genes under the control of the trp promoter containing a deletion eliminating tryptophan repression and encoding ampicillin resistance (Kawasaki et al., 1987).

The different strains were grown in Luria-Bertani (LB) broth ($10gL^{-1}$ tryptone, $5gL^{-1}$ yeast extract, $10gL^{-1}$ NaCl) or half LB ($5gL^{-1}$ tryptone, $2.5gL^{-1}$ yeast extract, $5gL^{-1}$ NaCl).

Biofilms were matured in M63 medium (100 mM KH_2PO_4, 15 mM $(NH_4)2SO_4$, 0.8 mM $MgSO_4.7H_2O$, 9 mM $FeSO_4$.2H2O, 17 mM K-succinate, 1 mM glucose, adjusted to pH 7.0)

2.2. Biofilm

All strains were grown as spin coated biofilms as described in Tsoligkas et al. (2012). The biofilms were formed on VWR glass slides 75mm by 25mm which had been coated with 4 ml of 0.1% Poly-L-lysine (Sigma) and dried for 24 hours in a 60°C oven. 200ml of ½ LB was inoculated with the appropriate strain and incubated for 24 hours in an orbital shaker at 30°C, 180rpm and with a throw of 19mm. The cultures were then transferred to 750ml polypropylene centrifuge bottles (Beckman Coulter UK Ltd) with the poly-L-lysine coated glass slides supported on glass beads at the bottom. This was then centrifuged for 10 minutes at 1811g to form a layer of cells on the slides. The slides were then aseptically transferred to 500ml wide necked conical flasks containing 70ml of M63 medium (100 mM KH_2PO_4, 15 mM $(NH_4)2SO_4$, 0.8 mM $MgSO_4.7H_2O$, 9 mM $FeSO_4$.2H2O, 17 mM K-succinate, 1 mM glucose, adjusted to pH 7.0) and left to mature for one week incubated in an orbital shaker at 30°C, at 70rpm with a throw of 19mm (figure 2.1.).

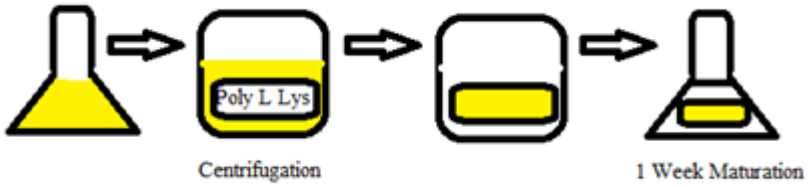


Figure 2.1: Diagrammatic Representation of Producing an Engineered Biofilm Biocatalyst
Strain were grown in a 200ml of ½ LB in a conical flask before being transferred to a centrifuge tube with a poly-L-lysine coated slide at the bottom. This was then centrifuged for 10 minutes at 180rpm to form a cell layer of the slide. This was then incubated in M63 to mature for a week.

2.3. Biotransformation

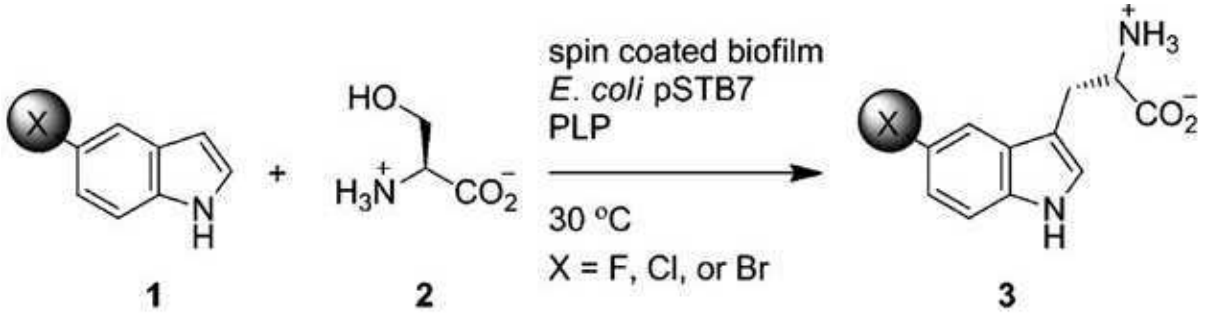


Figure 2.2: The biotransformation performed by tryptophan synthase from the plasmid pSTB7.
Schematic representation of the biotransformation of 5-haloindole (1) and serine (2) to 5-halotryptophan by tryptophan synthase as performed in the planktonic and biofilm reaction (Tsoligkas et al. 2012).

To investigate the different variables within the biotransformation of L-haloindoles to L-halotryptophans (figure 2.2.), the various strains were grown in 150ml of LB to OD_{650} >1, centrifuged for 10 minutes at 1811g and resuspended in PBS (tablets from Sigma-Aldrich) or biotransformation reaction buffer (0.1 M KH_2PO_4, 7 mM Serine, 0.1 mM PLP, adjusted to pH 7.0) supplemented with 5% DMSO and 2mM chloroindole (for chlorotryptophan) or 2mM indole (for tryptophan). Biotransformation reactions were incubated at 30°C and shaken at 2g.

Samples were taken after 0, 1, 2 and 24 hours: the number of viable cells was determined using CFU by performing serial dilutions of samples with PBS to get approximately 100-150 colonies per nutrient agar plate after incubation at 37°C overnight. Live/dead staining with flow cytometry to check was also performed.

2.4. Flow Cytometry

To determine the viability of cells in biofilms, they were detached from the glass slides, either by using an aseptic scraper or by using a pipette to agitate liquid on the biofilm and forcing the cells off, and resuspended in 5ml of PBS. Resuspended cells were analysed by flow cytometry (Accuri C6) using bis-oxanol (BOX) and propidium iodide (PI) stains.

Propidium iodide (PI) and Bis (1,3-dibarbituric acid) trimethine oxanol (BOX) were obtained from Sigma Aldrich. PI was made in water to a stock concentration of 200µg ml^{-1} and filtered and stored at 4°C and used at a working concentration of 5 µg ml^{-1}. BOX was made in DMSO to a stock concentration of 10mg ml^{-1}, 5µl of

this stock was added to 4765µl of filter-sterilised PBS and 200µl of 0.1M EDTA and thus used at a final concentration of 100ng ml-1.

For flow cytometry cells were diluted in 500µl of PBS to get a flow rate of 1000-2500 events per second. PI was used at 5 µg ml-1 to stain DNA of bacteria cells which have lost membrane integrity to show dead cells and BOX was used at 100ng ml^{-1} to stain cells with a collapsed membrane potential to show cells which were viable but injured. Red and green fluorescence was recorded as well as forward and side scatter.

Cells were killed to act as a dead control: cells from 500µl of culture were harvested by centrifugation and resuspended in 1 ml of 70% ethanol for 10 minutes, then pelleted and resuspended in 500µl of PBS to be run on the flow cytometer.

2.5. The Hoechst Assay

The Hoechst assay was used to try to establish the influx and efflux of the molecules involved in the biotransformation. This was adapted from Webber and Coldham (2010) and performed by comparing the fluorescence intensity of the efflux substrate 2.5M Hoechst H33342 by different E. coli strains suspended in PBS, reaction buffer and reaction buffer in the absence or presence of, 5%DMSO and indole, 5-chloroindole, 5-bromoindole or 5-fluoroindole. Hoechst H33342 is a substrate for major efflux pumps (e.g. AcrAB-TolC) and its fluorescence increases when bound to DNA (excitation maximum 350nm, emission maximum 461nm). Therefore if there is more efflux from the cell as a result of environmental conditions then the fluorescence of Hoechst will decrease more quickly over time.

PHL644 and PHL644 pSTB7 were grown in triplicate in 5ml of LB overnight. The next day 2ml of this was added to 50ml of LB and grown at 30°C in a 250ml conical flask to mid log phase. From each culture 3ml was harvested by centrifugation and resuspended in PBS to act as a control. 1ml of each culture was harvested by centrifugation and resuspended in 70% ethanol to act as a dead cell control. The remaining 47ml was harvested by centrifugation and resuspended in 47ml of reaction buffer and the OD_{600} adjusted to approximately 0.1 using reaction buffer. 5ml aliquots of cells suspended in reaction buffer were added to 5ml of each of the following:

- Reaction buffer
- Reaction buffer plus 5% DMSO

- Reaction buffer, 5% DMSO plus 2mM indole
- Reaction buffer, 5% DMSO plus 2mM chloroindole
- Reaction buffer, 5% DMSO plus 2mM bromoindole
- Reaction buffer, 5% DMSO plus 2mM fluoroindole

200µl of each suspension, dead control cells and control cells in PBS were added in triplicate to wells of a black plastic 96 well plate to act as control. 180µl of each suspension was added to wells as "test" samples which would have the Hoechst added. The assay was started by adding 20µl of 25µM Hoechst to the test suspensions before the plate was inserted into the Fluorstar plate reader which incubated the samples at 30°C and measured fluorescence (λ_{ex} 355nm and λ_{em} 465nm) every minute for 30 minutes. The data were then transferred to a Microsoft excel sheet, the triplicate values of fluorescence averaged and plotted against time to show the accumulation of Hoechst.

Chapter 3. Results and Discussion

3.1. Biotransformation

The biotransformation used in this study was tryptophan synthase converting 5-chloroindole plus serine to L-chlorotryptophan. For the reaction to take place in the biofilms formed, the plasmid pSTB7 (encoding tryptophan synthase) was transformed into E. coli cells using the heat shock method. To examine the effects the biotransformation has on the cells in the engineered biofilms as biocatalysts, planktonic counterparts were used and prepared as described in Tsoligkas et al. (2012) but at 50ml, a tenth of the volume of that in the referenced study.

Whether haloindoles have the same effect as indole on cells has bearing on the biotransformation, so the biotransformation reaction buffer with chloroindole was used to test its effect on cells and to try to optimise reaction conditions. Different variables of the biotransformation were identified and investigated to start understanding gene expression and start optimisation. The behaviour of cells with and without plasmid was looked at using PHL628 pT7-7, PHL644 and PHL644 pSTB7. The metabolic burden of a plasmid was investigated to see whether this leaves the cell susceptible to different conditions using PHL644 and PHL644 pSTB7. The metabolic burden of carrying out the biotransformation using PHL644 pSTB7 was investigated. Growth phase was investigated to see if this affected the biotransformation or the cells using early stationary and late stationary phase. The reaction buffer was looked at to see if the serine component is required for cell survival. Viability was investigated to find out what was affecting viability, the indole or halogen component of the molecule.

Previous studies showed the importance of the ompR234 allele for increasing curli production and thereby increasing biofilm formation. Since ompR234 increases curli production via activating synthesis of the CsgD regulator protein, the effect of overproduction of CsgD from plasmid pT7-csgD was tested in strains PHL565R (wild type $ompR^{+}$) and PHL628 (the isogenic ompR234 strain).

3.2. Growth Curve

To test if the plasmids used in this study exerted a metabolic strain affecting the growth of E. coli, absorbance at 650nm was measured every hour for cultures of PHL628 pT7-7 (an empty vector), PHL628 pT7-csgD (the same

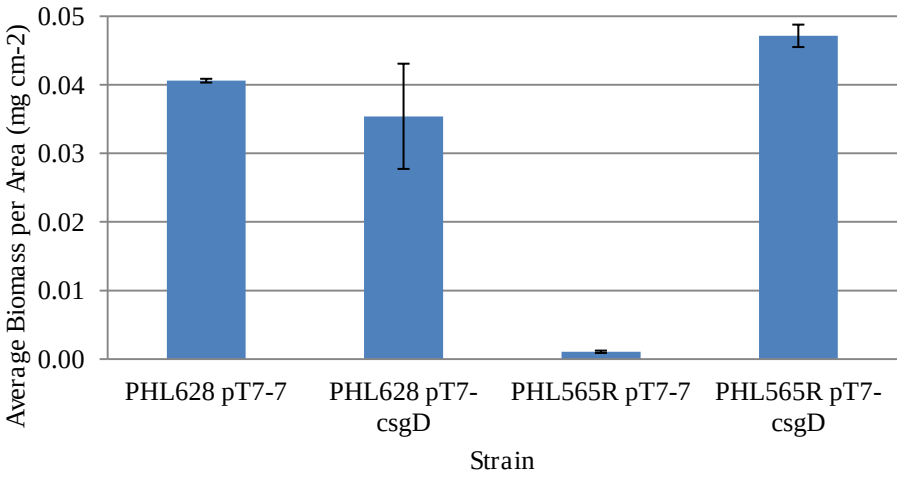
vector but containing the csgD gene), PHL565R pT7-7 and PHL565R pT7-csgD grown in LB. There was no noticeable difference in growth between different strains, so no strain is at a disadvantage in these growth conditions.

3.3 Initial Biofilm Tests

To test the ability of strains PHL565R and PHL628 transformed with either pT7-7 (empty vector) or pT7-csgD (overproducing the regulator of biofilm formation CsgD) to form biofilms, the method of Tsoligkas et al. (2012) was used to centrifugally attach the bacteria to glass slides. The glass slides were matured for a week in M63 medium at 30°C and 100rpm, and then the biomass attached to the glass slide and accumulated in the maturation medium were estimated using absorbance at 650nm, assuming that 1ml of culture with an OD_{650} of 1 is equal to approximately 0.4mg of dry mass (Figure 3.1.). The biofilm biomass per unit area (Figure 3.1.A.) and biomass of planktonic and biofilm cells per culture (Figure 3.1.B.) were calculated.

PHL628 pT7-7 biofilm had an average biomass of 0.041 mg cm^{-2}, slightly higher than the PHL628 pT7-csgD biofilms with an average biomass of 0.036 mg cm^{-2} (Figure 3.1A). PHL565R pT7-7 had a biomass per area of less than 0.001 mg cm^{-2}, demonstrating that without the ompR234 allele, biofilm generation is very poor. PHL565R pT7--csgD had a much higher average biofilm biomass of 0.047 mg cm^{-2}, demonstrating that overproduction of CsgD can improve biofilm production in a similar manner to the ompR234 mutation. The similar biomass per area of biofilms of PHL628 pT7-7 and PHL628 pT7-csgD demonstrate that the effects of the ompR234 allele and CsgD overproduction are not additive, probably since they both regulate biofilm production via the same regulatory pathway. In the M63 maturation medium, PHL628 transformed with each plasmid showed similar planktonic biomass, but PHL565R strains showed more, PHL565R + pT7-7 showing the most at 6.28mg average. All strains had greater biomass in the planktonic phase than in the biofilm (Figure 3.1.B).

3.1.A.



3.1.B.

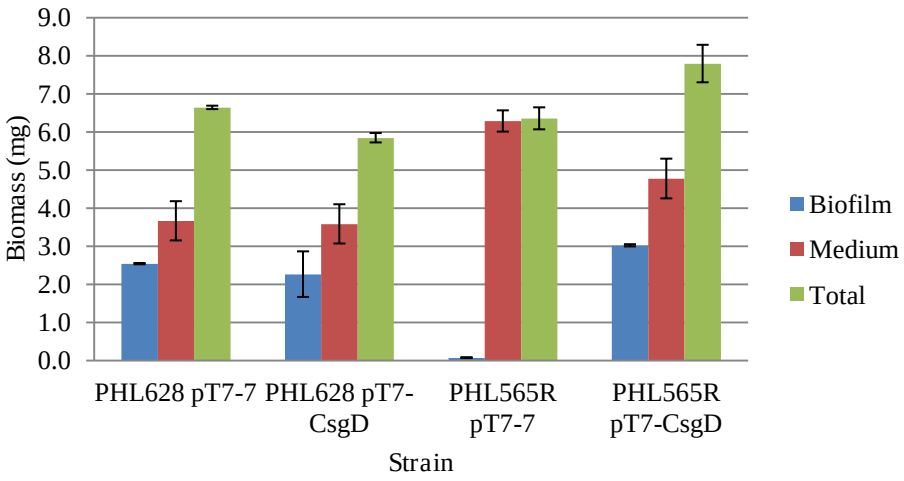


Figure 3.1. Biomass per unit biofilm area (A) and per culture (B) for strains PHL628 and PHL565R transformed with either pT7-7 or pT7-csgD.
Biofilms were generated using the spin-down method and matured for 7 days in M63 medium. Biomass was determined by scraping the biofilm from the glass slide and resuspending it in 5ml of PBS, drying 1ml of the suspension at 60°C for 48 hours (in duplicate) and measuring the resultant mass.

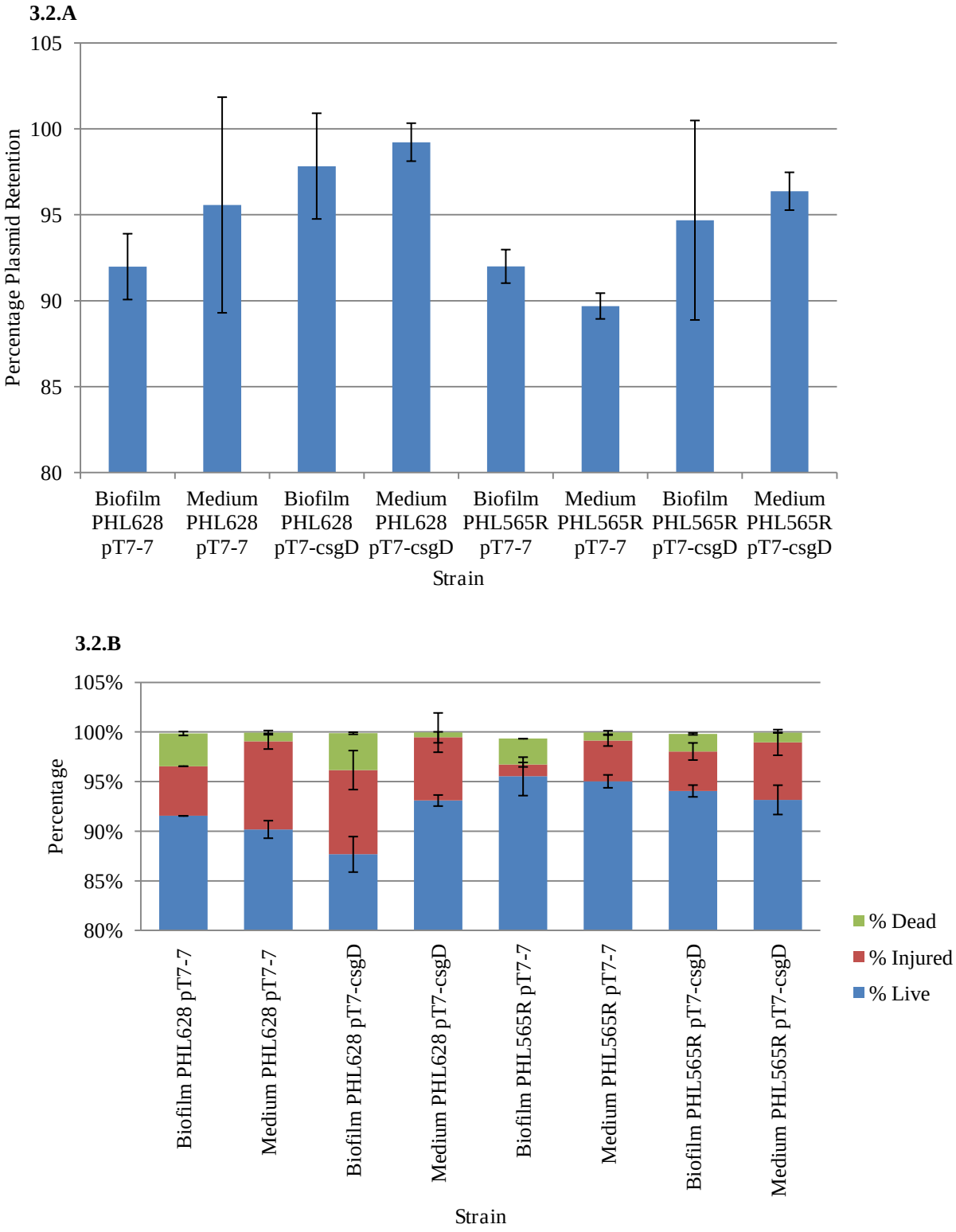


Figure 3.2. Plasmid retention (A) and flow cytometric determination of viability (B) for planktonic and biofilm cells. Calculated through CFU and replica plating in duplicate.

CFU determination and replica plating were performed to test the plasmid retention of biofilm and planktonic cells (Figure 3.2.A). All strains showed a high percentage of plasmid retention in both biofilms (>90%) and planktonically (>89% Figure 3.2.A). Flow cytometry was used to determine the viability of bacteria from both biofilm and planktonic cells (Figure 3.2.B). Over 87% of biofilm and planktonic cells were alive after their week-long incubation, but PHL628 pT7-csgD biofilm showed the largest proportion of injured cells in a biofilm at 8% of cells, however this group also had the most error. Also noted from forward scatter data was that PHL628 cells were smaller than PHL565R. In the medium there appeared to be little difference in cell viability.

These results tally with initial observations of the slides that showed that PHL565R pT7-7 produced no biofilm and remained as planktonic cells in the medium, whereas all other strains produced a biofilm on the glass slides.

For biofilms to be used in industry they would need to have a low cost of operation, use a non-pathogenic species which can generate biofilms in a controllable way, be genetically manipulated for the desired biocatalysis and grow in a cheap medium with readily available cofactors (Rosche et al. 2009). The E. coli used here is a good choice as it is a good genetic model, however laboratory strains do not readily form biofilms. Previously, Tsoligkas et al. (2012) used E. coli K-12 strain PHL644, resulting in heterogeneous EPS formation by day 6 and biofilm formation during days 5-7, due to increases in csgD expression caused by the ompR234 allele (Tsoligkas et al. 2012). E. coli PHL628 was obtained, which also has the ompR234 mutation. The OmpR234 mutation increases transcription from the csgD promoter and increased levels of CsgD increasing curli and cellulose biosynthesis for biofilm formation. On the pT7-csgD plasmid expression of csgD results in a functional curli phenotype that is independent of the csgDEFG promoter, resulting in increased surface attachment (Brombacher et al. 2006). So both ompR234 allele and pT7-csgD should increase biofilm formation, which corresponds with the results of biofilm testing (Figure 3.1). So for a biofilm to form in an E. coli K12 strain, either the plasmid pT7-csgD had to be present or the strain requires an ompR234 mutation (PHL628) as PHL565R failed to produce a biofilm when it had an empty plasmid (pT7-7) but formed one with pT7-csgD.

This demonstrates the importance of CsgD as a regulator for biofilm formation, as without an increase in cellular concentration, E. coli cells failed to attach. Other studies have found that E. coli biofilm attachment and adherence normally requires flagella, type I pili and curli fimbriae. In attachment flagellar mediated motility is required to create primary cell surface contacts then type I pili are used to stabilise these (May and Okabe 2011,

Jackson et al. 2001, Danese et al. 2000). However this data and others suggests that the formation of curli could be the most important determinant (Barnhart and Chapman 2006, Zhang et al. 2007).

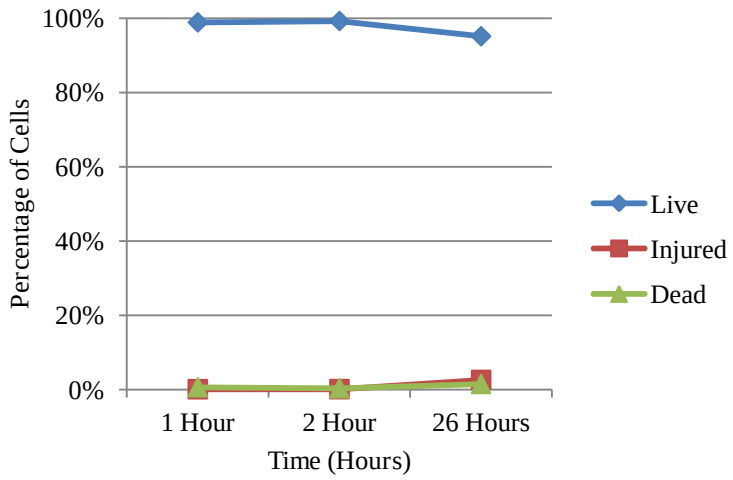
3.4 Flow cytometry analysis of bacterial viability during planktonic biotransformations

How cells respond to the biotransformation conditions required for the synthesis of 5-chlorotryptophan from 5-chloroindole was investigated by measuring the viability of planktonic cells during biotransformation reactions. Cell viability was assessed using flow cytometry live/dead staining and CFU determinations. E. coli strain PHL628 was initially used, grown in duplicate overnight in 3 ml of LB at 37°C and 2g, then 0.5ml of each overnight culture was added to 50ml LB and incubated at 37°C and 2g until the OD_{650} was greater than 1. The viability was measured following incubation in PBS, PBS with 5% DMSO or PBS, 5% DMSO and 2mM 5-chloroindole. Flow cytometry data could not be used due to an error in dye dilution, but CFU analysis showed that the number of viable cells was fairly constant after 0, 1 and 2 hours (data not shown) at approximately 1-2 $x10^{10}$ CFU ml^{-1}.

Strain PHL628 pT7-7 was incubated under the same three conditions as those used to determine whether the metabolic burden of a plasmid would make cells less viable. After 1 or 2 hours incubation, over 90% of cells were alive according to flow cytometry, but after 26 hours the cultures incubated in PBS plus 5% DMSO and 5-chloroindole contained a significant percentage of injured or dead cells (an average of 39.3% Figure 3.3.A-C) but with no measurements between 2 and 26 hours it is not known when death started to occur. The CFU data shows a fairly similar CFU ml^{-1} for 0, 1 and 2 hours for samples incubated without 5-chloroindole, but with 5-chloroindole there is a slightly larger decrease in CFU ml^{-1} after 2 hours incubation (Figure 3.3.D). These data indicate that the plasmid does put a metabolic burden on the cell and carrying the plasmid leaves it more susceptible to damage and death under the conditions of the biotransformation.

There was a change from using E. coli PHL628 to PHL644, which was shown through crystal violet staining and AFM data to be more robust in coping with the conditions of the biotransformation and able to form biofilms better than PHL628. PHL644 was grown in duplicate overnight in 3 ml of LB incubated at 30°C and 2g, then 0.5ml of each overnight culture was added to 50ml of LB and incubated at 30°C and 2g until an OD_{650} greater than 1 was reached. This was centrifuged at 1811g for 10 minutes and resuspended and

3.3.A.



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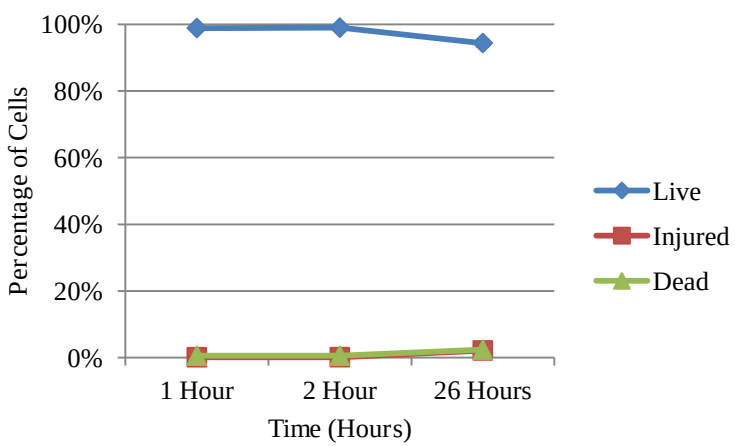

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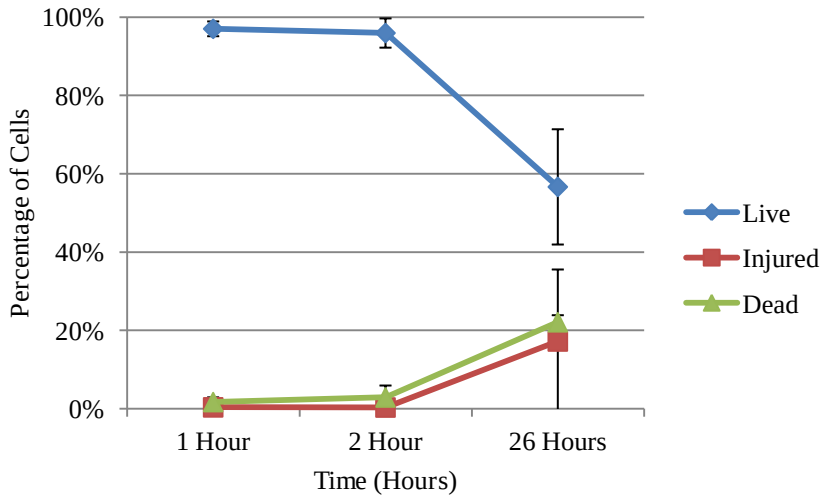


3.3.D.

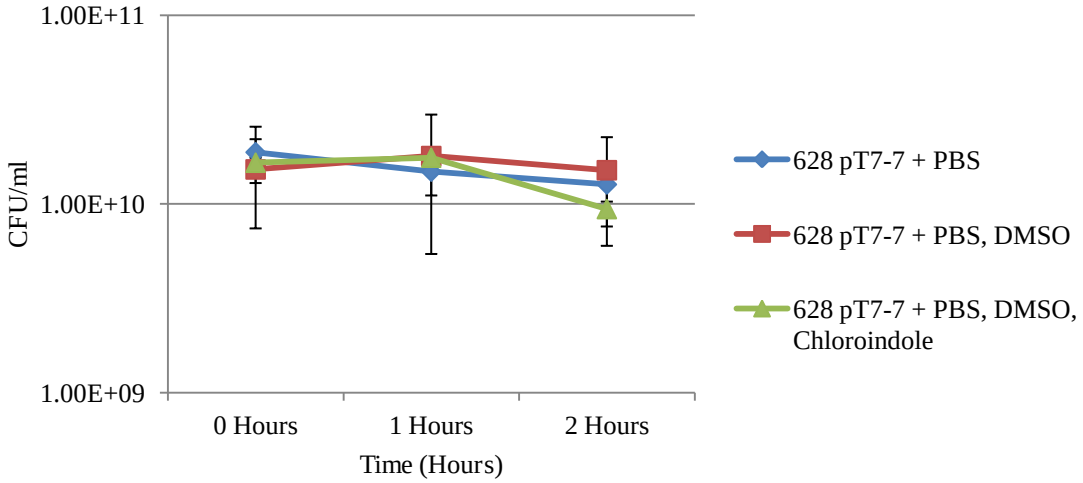
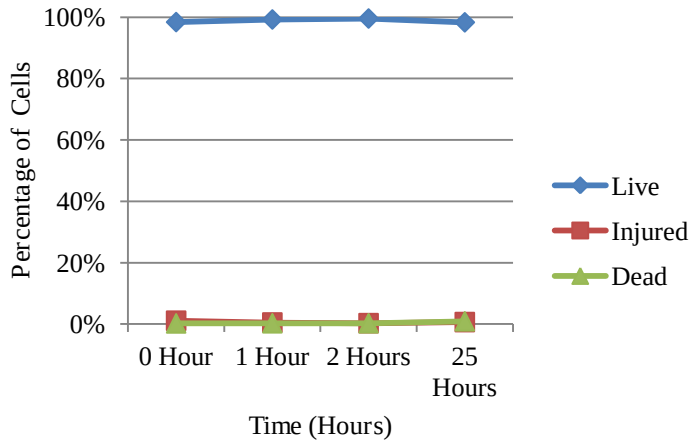
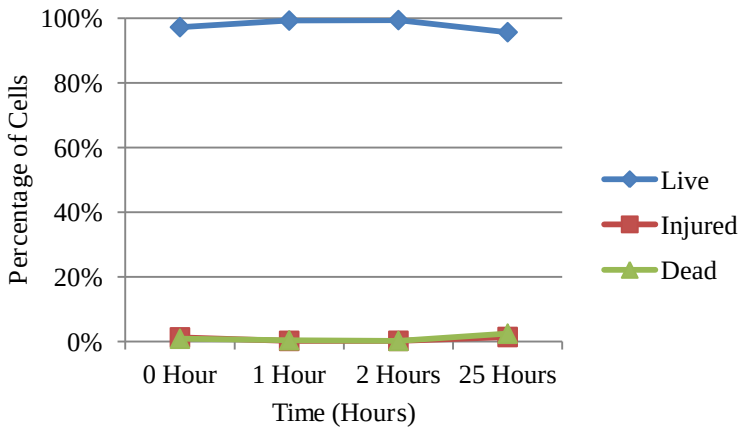


Figure 3.3. Determination of viability of exponential phase PHL628 pT7-7 in biotransformation conditions
E. coli strain PHL628 with plasmid pT7-7 were incubated in either PBS (A), PBS plus 5% DMSO (B) or PBS, 5% DMSO and 2 mM 5-chloroindole (C) and viability was determined using flow cytometry with dual propidium iodide – bisoxanol staining. Viability was also assessed using determination of colony forming units on nutrient agar plus 100µg ml^{-1} ampicillin plates (D). Data plotted are the mean of 2 independent experiments, error bars indicate standard deviation.

3.4.A.



3.4.B.



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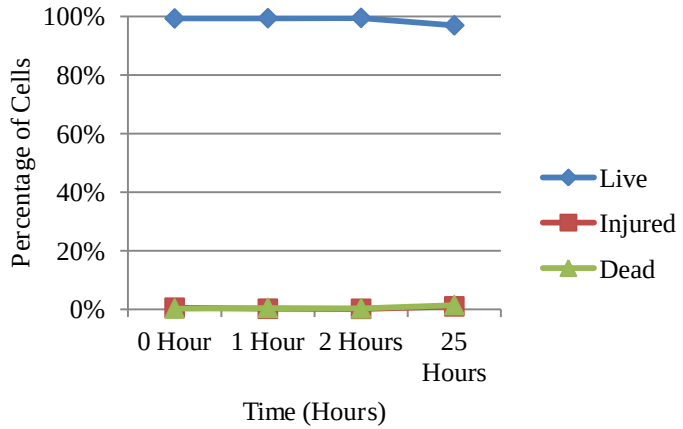


3.4.D.

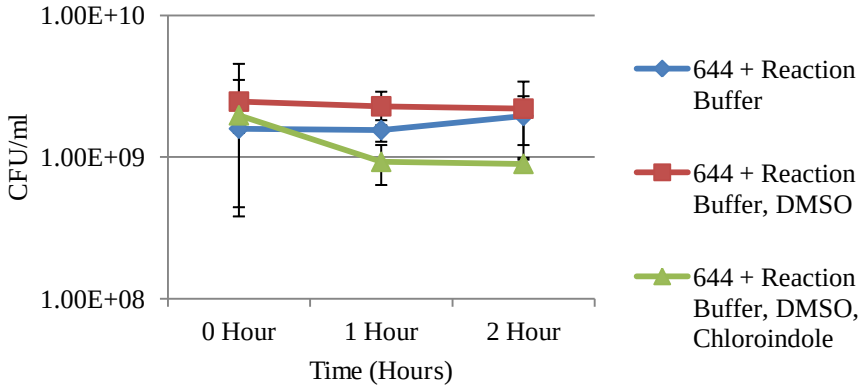


Figure 3.4. Determination of viability of exponential phase PHL644 in biotransformation conditions with chloroindole.
E. coli strain PHL644 was incubated in either biotransformation reaction buffer (A), reaction buffer plus 5% DMSO (B) or reaction buffer supplemented with 5% DMSO and 2 mM 5-chloroindole (C) and viability was determined using flow cytometry with dual propidium iodide – bisoxanol staining. Viability was also assessed using determination of colony forming units on nutrient agar plus 100µg ml^{-1} ampicillin plates (D). Data plotted are the mean of 2 independent experiments, error bars indicate standard deviation.

incubated in 3 different conditions: biotransformation reaction buffer; reaction buffer plus 5%DMSO, and reaction buffer supplemented with 5% DMSO and 2mM 5-chloroindole (Figure 3.4). The initial experiment determined the viability of PHL644 cells incubated at 30°C in the 3 different conditions. Even after 25 hours, flow cytometry showed little difference in the number of dead and injured cells in the three conditions, with the majority of cells still being healthy (Figure 3.4.A- C). CFU data also showed little difference between 0, 1, 2 and 25 hours except cells treated with chloroindole had a larger decrease after 2 hours (Figure 3.4.D). These data indicate that PHL644 is robust enough to tolerate the conditions required for the biotransformation.

PHL644 pSTB7 was used in similar reaction conditions to check the metabolic burden of the plasmid and the biotransformation of 5-chloroindole to 5-chlrorotryptophan (Figure 3.5). Flow cytometry, shown in Figure 3.5.A-C, demonstrates that at 0 hours approximately 90% of cells were alive under all conditions, but at 1 hour cells in just reaction buffer or reaction buffer and DMSO, over 93% of cells were alive, and in reaction buffer, DMSO and 5-chloroindole there was an average of 9.40% dead and injured cells. The same is true at 2 hours with the majority of cells without 5-chloroindole being viable but cells exposed to 5-chloroindole showed significant proportions of injured and dead cells. At 28 hours the majority of cells exposed to 5-chloroindole were dead (approximately 75% dead, 1.5% injured and 11% alive). With reaction buffer and reaction buffer and DMSO over 95% were still alive (Figure 3.5.A-B). CFU showed a dramatic decreased viability in cells exposed to 5-chloroindole with far fewer CFU ml^{-1} at 0 hour compared to the other conditions and at time points after that $<1 \times 10^7$ CFU (Figure 3.5.D).

The same experiment was performed using cells grown until late stationary phase (OD_{650} >4) which have a lower metabolic rate and higher tolerance to stress. Flow cytometry and CFU data (Figure 3.6.A-D) showed cells were viable at 0, 1 and 2 and 28 hours with flow cytometry showing 98% live cells at these times under all incubation conditions. Plasmid retention was also tested at 0 and 28 hours (Figure 3.6.E): at 0 hours in just reaction buffer 99% of cells were plasmid positive, cells with reaction buffer and DMSO 92% plasmid positive and cells with reaction buffer, DMSO and 5-chloroindole 87% plasmid positive. At 28 hours both the cells in reaction buffer and reaction buffer plus DMSO had dropped their plasmid retention to approximately 82% whereas the cells exposed to 5-chloroindole had a similar retention to 0 hours of 86%.

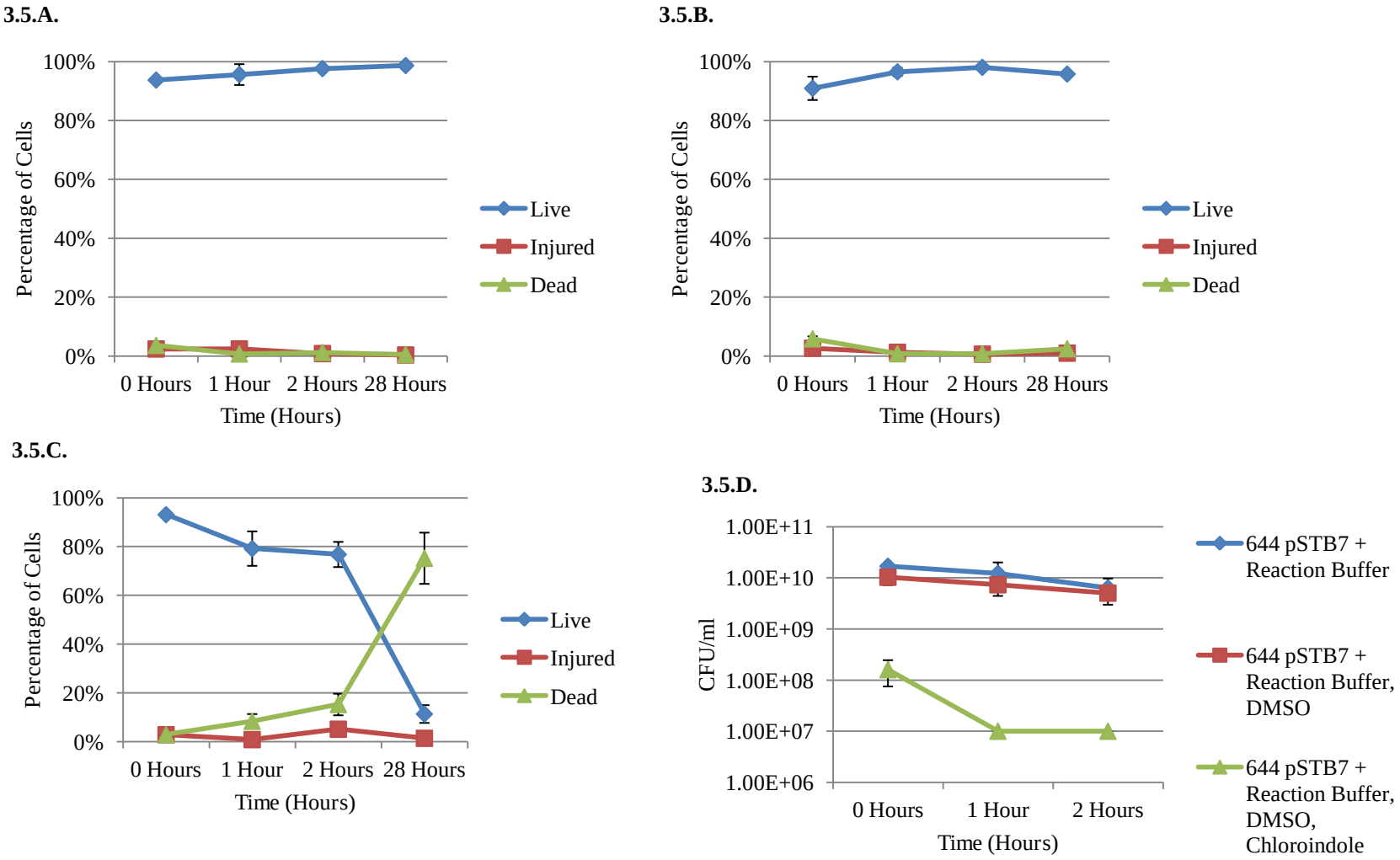


Figure 3.5. Determination of viability of exponential phase PHL644 pSTB7 in biotransformation conditions with chloroindole.
E. coli strain PHL644 pSTB7 was incubated in either biotransformation reaction buffer (A), reaction buffer plus 5% DMSO (B) or reaction buffer supplemented with 5% DMSO and 2 mM 5-chloroindole (C) and viability was determined using flow cytometry with dual propidium iodide – bisoxanol staining. Viability was also assessed using determination of colony forming units on nutrient agar plus 100µg ml^{-1} ampicillin plates (D). Data plotted are the mean of 2 independent experiments, error bars indicate standard deviation.

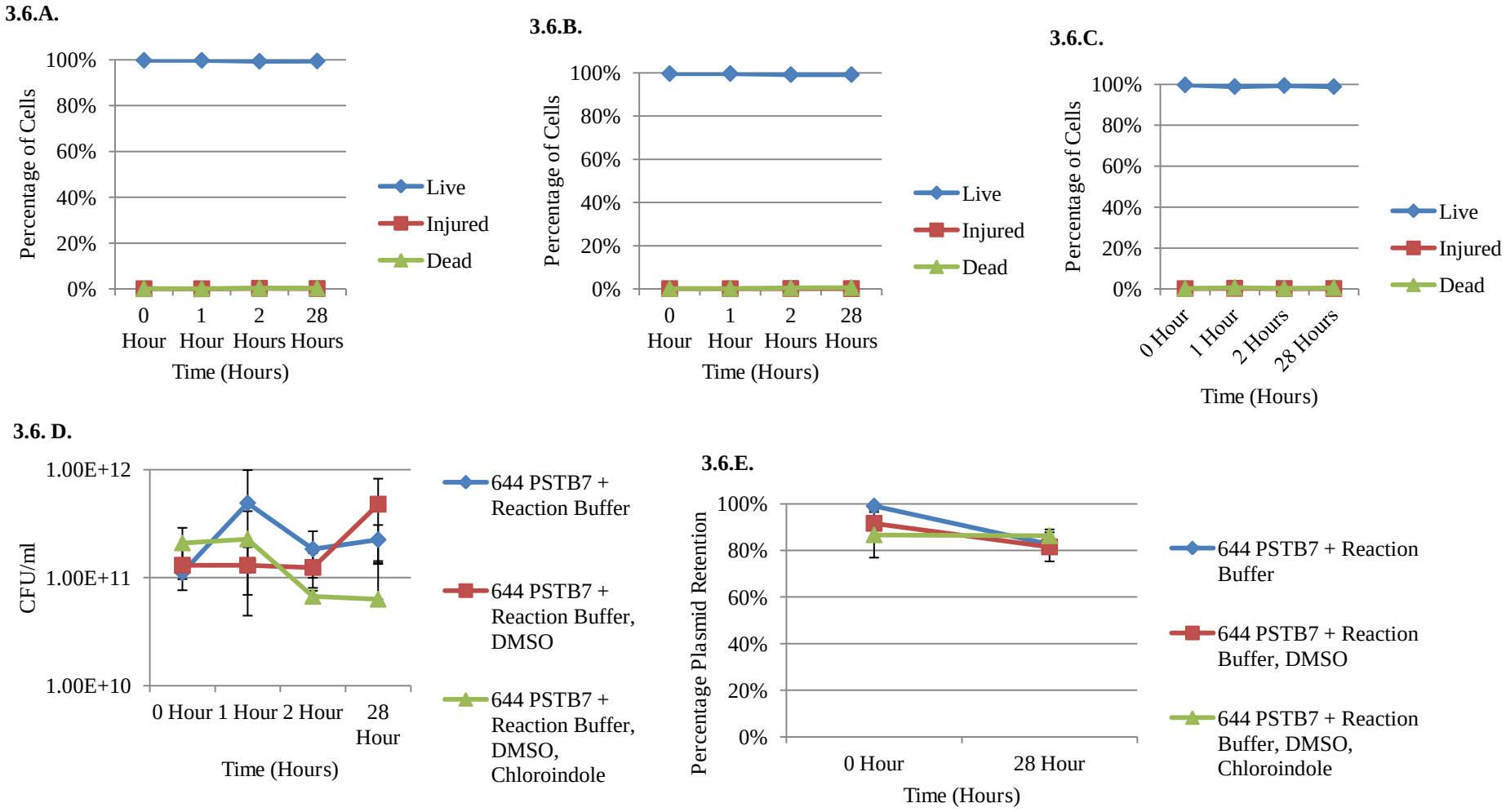


Figure 3.6. Determination of viability of PHL644 pSTB7 grown to stationary phase in biotransformation conditions with chloroindole.
E. coli strain PHL644 pSTB7 was grown to late stationary phase and was then incubated in either biotransformation reaction buffer (A), reaction buffer plus 5% DMSO (B) or reaction buffer supplemented with 5% DMSO and 2 mM 5-chloroindole (C) and viability was determined using flow cytometry with dual propidium iodide – bisoxanol staining. Viability was also assessed using determination of colony forming units on nutrient agar plus 100µg ml^{-1} ampicillin plates (D). Plasmid retention was determined in the biotransformation through replica plating from nutrient agar plates to nutrient agar plus 100µg ml^{-1} ampicillin plates (E). Data plotted are the mean of 2 independent experiments, error bars indicate standard deviation.

From looking at the planktonic data, 5-chloroindole has shown to have a toxic effect on exponential phase E. coli PHL644 pSTB7 which decreases their viability over 24 hours; this would be likely to decrease their ability to perform the biotransformation. The same toxicity is not seen with just PHL644 suggesting the biotransformation products are toxic, or the extra metabolic burden is leaving the cells susceptible to the toxicity of chloroindole. This may tie in with indole causing toxicity when added to growth medium at 1-6mM resulting in no increase in cell density (Lee and Lee 2010), so chloroindole may be producing the same effect. High concentrations of indole, above 2mM, the concentration being used here, decrease cell growth, possibly through blocking cell division, disruption of the cell envelope or oxidant toxicity and affect cell metabolism leading to a pleiotropic effect (Lee and Lee 2010). So to optimise the biotransformation, the source of chloroindole toxicity should be found to minimise its effect. The fact that stationary phase cells do not appear to be affected by chloroindole (Figure 3.6) suggests that viability is most affected when cells are most actively metabolising and toxicity occurs when chloroindole is metabolised.

Reaction buffer components may also affect cell viability during the biotransformation. In all experiments the addition of 5% DMSO showed little difference to cell viability (Figure 3.3.B-3.7.B), so DMSO concentration could potentially be increased to increase haloindole solubility without interfering with cell viability and preventing haloindole being a limiting factor.

If the serine present in the biotransformation reaction is respired by the cells it may affect viability during the biotransformation. To test this, the reaction buffer was made with serine omitted. PHL644 pSTB7 was incubated in 3 different conditions: reaction buffer without serine; reaction buffer without serine plus 5% DMSO; and reaction buffer without serine supplemented with 5% DMSO and 2mM 5-chloroindole. CFU and flow cytometry showed very similar results at 0, 1 and 2 hours (Figure 3.7.A-D) to the same experiment with serine (Figure 3.6), so the cells were still able to respire and react. These results are similar to ones with serine, indicating that serine is not used as a major energy source for respiration and is not required for cell survival but is probably just used in the biotransformation reaction.

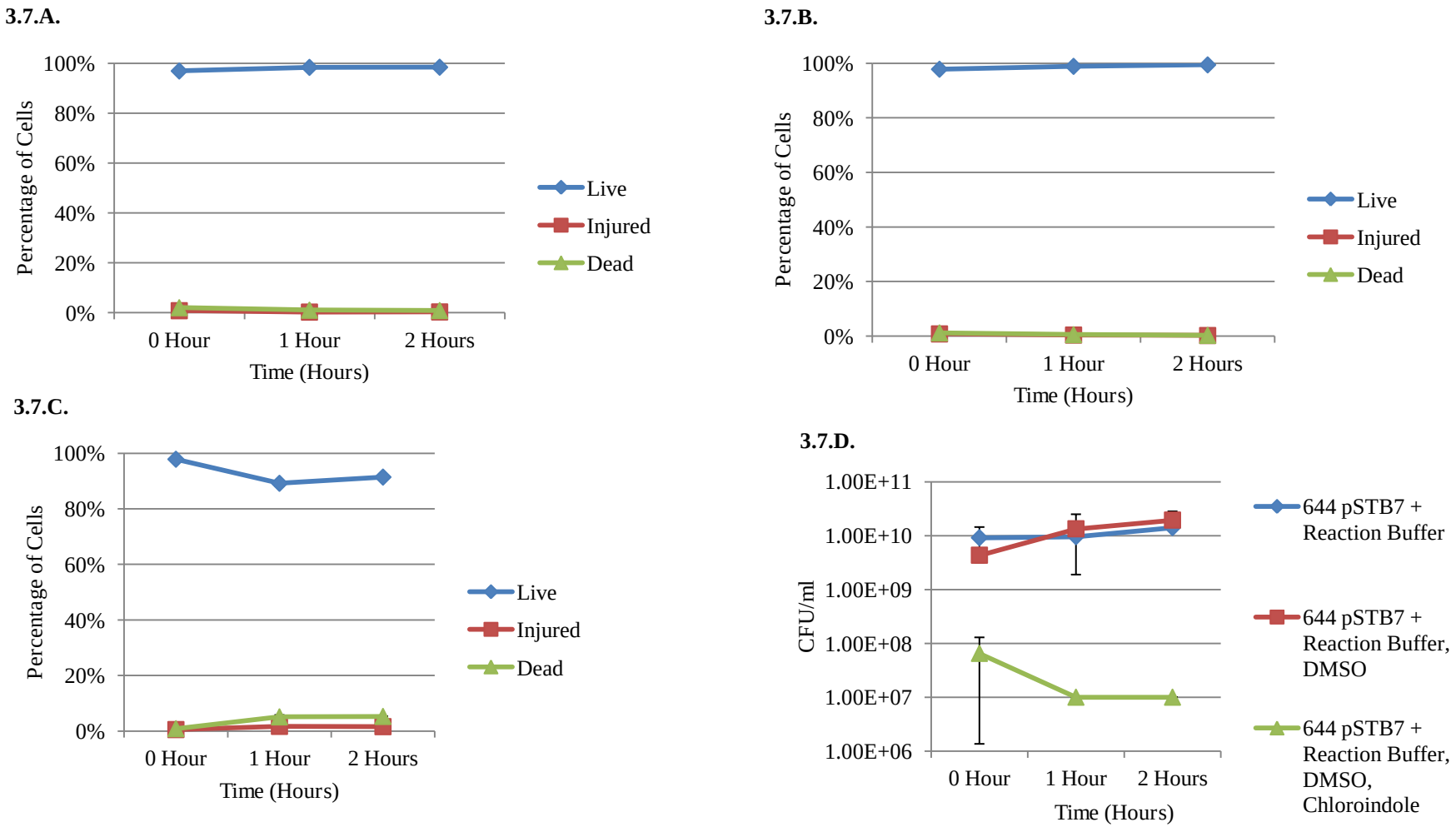


Figure 3.7. Determination of viability of PHL644 pSTB7 grown to stationary phase in biotransformation conditions without serine and with chloroindole.
E. coli strain PHL644 pSTB7 was incubated in either biotransformation reaction buffer without serine (A), reaction buffer without serine plus 5% DMSO (B) or reaction buffer without serine supplemented with 5% DMSO and 2 mM 5-chloroindole (C) and viability was determined using flow cytometry with dual propidium iodide – bisoxanol staining. Viability was also assessed using determination of colony forming units on nutrient agar plus 100µg ml^{-1} ampicillin plates (D). Data plotted are the mean of 2 independent experiments, error bars indicate standard deviation.

To determine whether chloroindole is more toxic than indole, the same biotransformation experiment was performed with stationary phase cells treated with 5-chloroindole or indole in the reaction buffer in the presence of DMSO (Figure 3.8). Cells treated with indole flow cytometry showed little loss of viability over the first 2 hours with approximately 98% alive but at 22 hours there was a small loss of viability with an average of 91% of cells alive (Figure 3.8.A). Cells treated with 5-chloroindole (Figure 3.8.B) showed a decrease in live cells over the first 2 hours with a drop from approximately 98% to 95% live cells from 0- 2 hours, with a further decrease at 25 hours to approximately 71% live cells. CFU data in Figure 3.8.C, mirrored flow cytometry data with declines in magnitude in CFU for 5-chloroindole over 2 hours, unfortunately the 25 hour CFU data was contaminated, but a CFU ml^{-1} of around $1x10^{10}$ for indole. This correlates with indole not having the same effect on viability as chloroindole, indicating the halogen part of the molecule increases the toxicity. It could be that a combination of chlorotryptophan and chloroindole is causing the toxicity or chloroindole becoming trapped in the membrane of the cells and blocking transporters due to its size or chlorotryptophan being produced than incorporated into proteins resulting in misfolded proteins. Another possibility is that chloroindole is causing oxidant toxicity.

To investigate all biotransformations flow cytometry was performed on stationary phase planktonic cells under all conditions; PBS; biotransformation reaction buffer; reaction buffer plus 5% DMSO; reaction buffer supplemented with 5% DMSO and 2mM indole; reaction buffer supplemented with 5% DMSO and 2mM 5-chloroindole; reaction buffer supplemented with 5% DMSO and 2mM 5-bromoindole; and reaction buffer supplemented with 5% DMSO and 2mM 5-fluroindole (Figure 3.9 and 3.10) all the possible haloindoles that could be used in the biotransformation. After both 2 and 24 hours the majority of cells under all possible biotransformation conditions (indole, chloroindole, bromoindole and fluoroindole) were alive, this suggests that as stationary phase cells show high viability in the biotransformation that biofilms would also display this high viability.

To test this, flow cytometry was used to determine the viability of PHL644 pSTB7 biofilm cells that had been incubated at 30°C at 70rpm in biotransformation reaction buffers with or without the addition of DMSO, indole and haloindoles for 24 hours then shaken free and resuspended in PBS (Figure 3.11). The flow cytometric data after 24 hours shows that approximately 90% of cells were alive under all conditions, so they should be able to

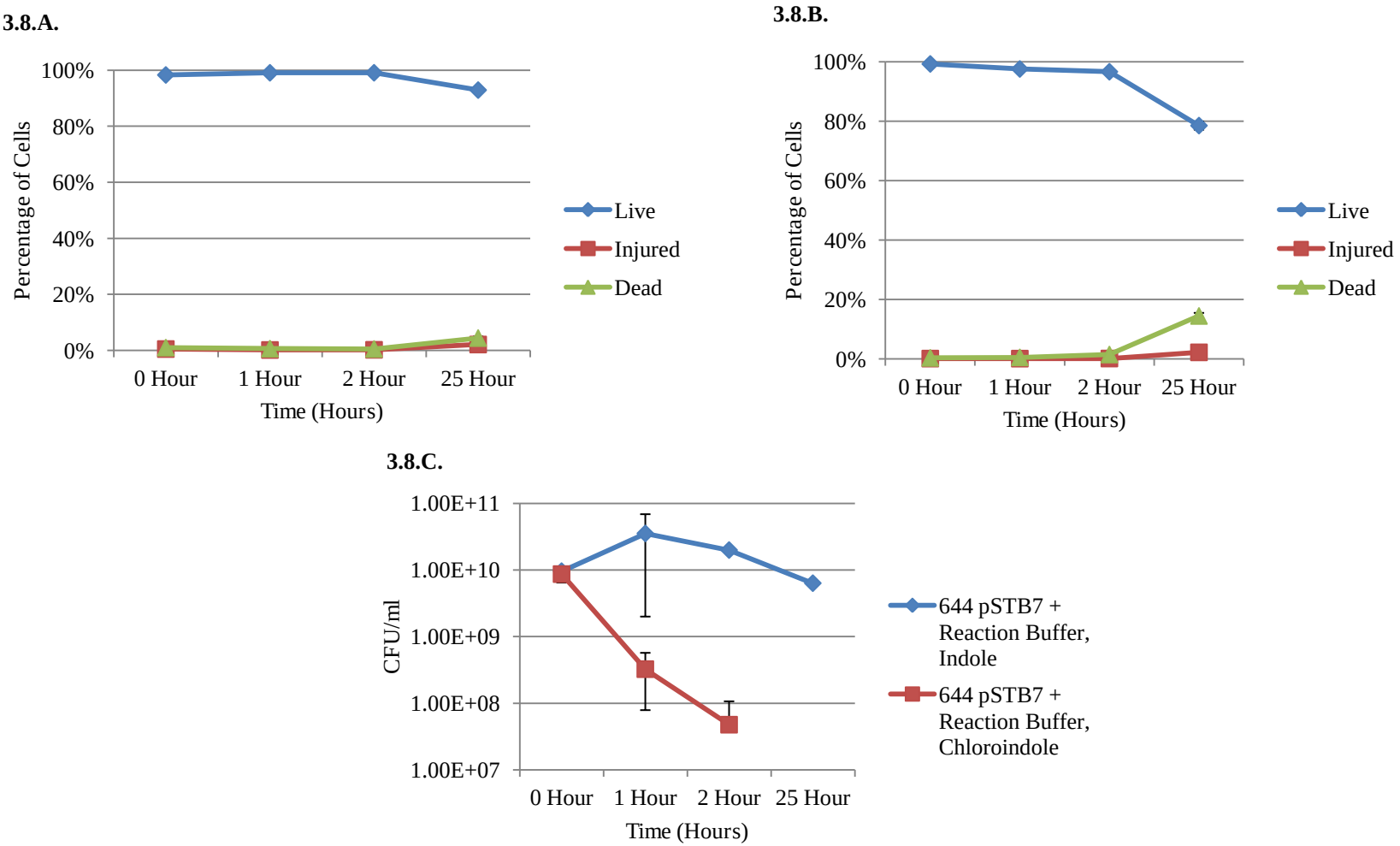


Figure 3.8. Determination of viability of stationary phase PHL644 pSTB7 in biotransformation conditions with chloroindole or indole.
E. coli strain PHL644 pSTB7 was incubated in either biotransformation reaction buffer supplemented with 5% DMSO and 2 mM indole (A), or reaction buffer supplemented with 5% DMSO and 2 mM 5-chloroindole (B) and viability was determined using flow cytometry with dual propidium iodide – bisoxanol staining. Viability was also assessed using determination of colony forming units on nutrient agar plus 100µg ml^{-1} ampicillin plates (C). Data plotted are the mean of 2 independent experiments, error bars indicate standard deviation.

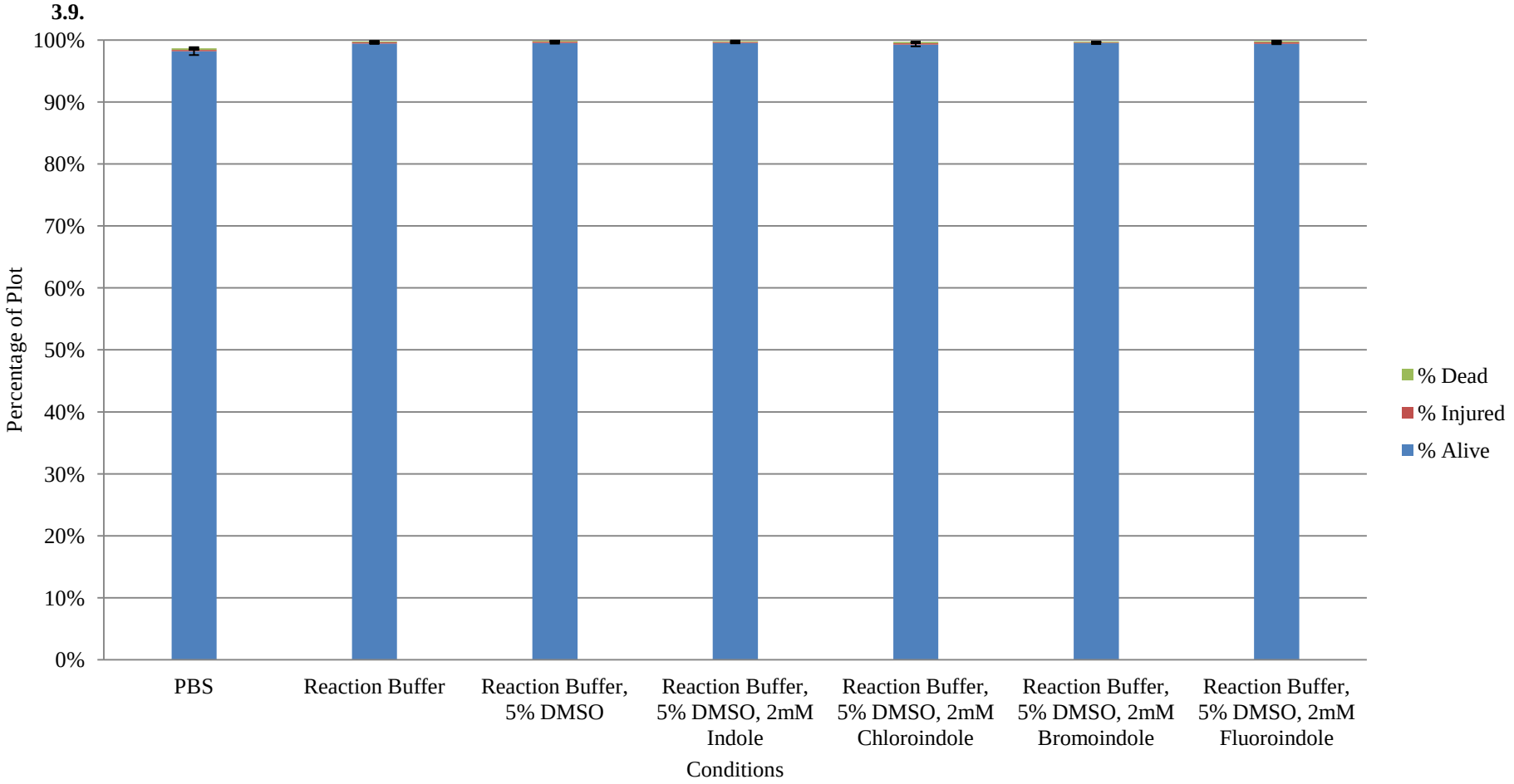


Figure 3.9. Flow cytometric determination of viability for stationary phase PHL644 pSTB7planktonic cells under all reaction conditions after 2 hours.
E. coli strain PHL644 pSTB7 was incubated in either PBS, biotransformation reaction buffer, reaction buffer supplemented with 5% DMSO, reaction buffer supplemented with 5% DMSO and 2 mM indole, reaction buffer supplemented with 5% DMSO and 2 mM 5-chloroindole, reaction buffer supplemented with 5% DMSO and 2 mM 5- bromoindole, reaction buffer supplemented with 5% DMSO and 2 mM 5-fluoroindole and viability was determined after 2 hours using flow cytometry with dual propidium iodide – bisoxanol staining. Data plotted are the mean of 2 independent experiments, error bars indicate standard deviation.

3.10.

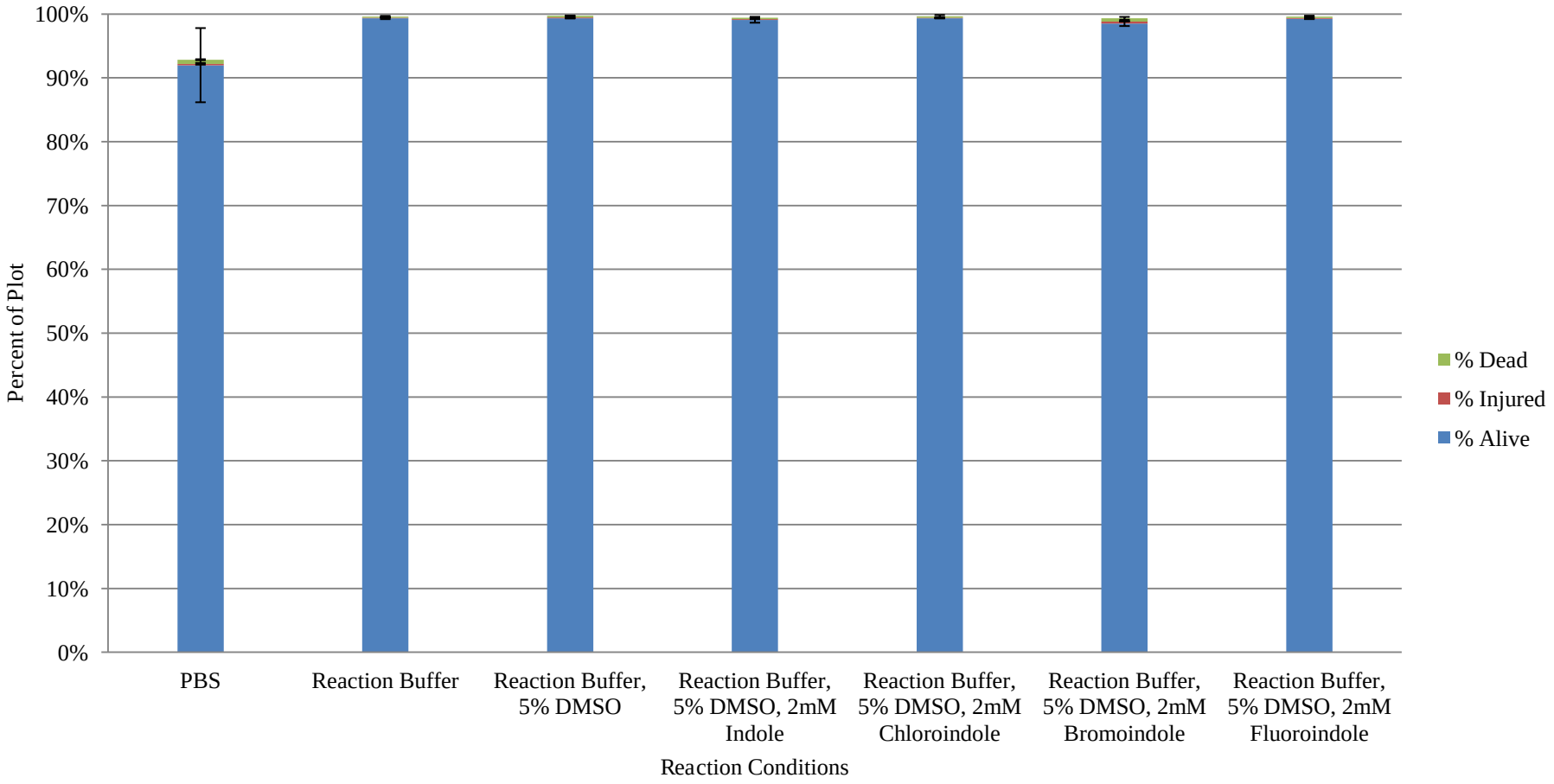


Figure 3.10. Flow cytometric determination of viability for stationary phase PHL644 pSTB7planktonic cells under all reaction conditions after 24 hours.
E. coli strain PHL644 pSTB7 was incubated in either PBS, biotransformation reaction buffer, reaction buffer supplemented with 5% DMSO, reaction buffer supplemented with 5% DMSO and 2 mM indole, reaction buffer supplemented with 5% DMSO and 2 mM 5-chloroindole, reaction buffer supplemented with 5% DMSO and 2 mM 5- bromoindole, reaction buffer supplemented with 5% DMSO and 2 mM 5-fluoroindole and viability was determined after 24 hours using flow cytometry with dual propidium iodide – bisoxanol staining. Data plotted are the mean of 2 independent experiments, error bars indicate standard deviation.

3.11.

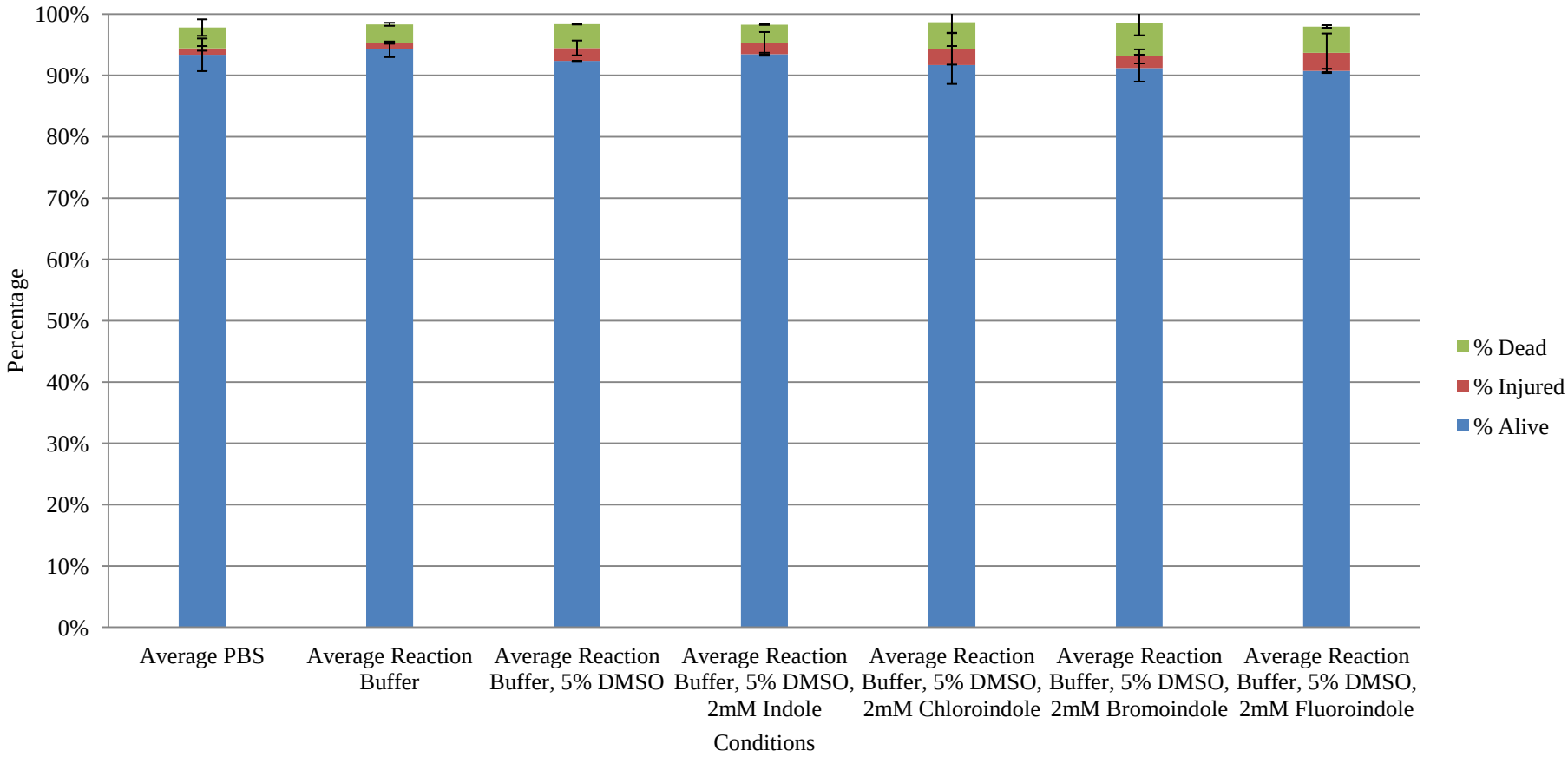


Figure 3.11. Flow cytometric determination of viability for PHL644 pSTB7biofilm under all reaction conditions after 24 hours.
E. coli strain PHL644 pSTB7 biofilm was incubated in either PBS, biotransformation reaction buffer, reaction buffer supplemented with 5% DMSO, reaction buffer supplemented with 5% DMSO and 2 mM indole, reaction buffer supplemented with 5% DMSO and 2 mM 5-chloroindole, reaction buffer supplemented with 5% DMSO and 2 mM 5- bromoindole, reaction buffer supplemented with 5% DMSO and 2 mM 5-fluoroindole and viability was determined after 24 hours using flow cytometry with dual propidium iodide – bisoxanol staining. Data plotted are the mean of 2 independent experiments, error bars indicate standard deviation.

perform the biotransformation. The slightly higher proportion of dead cells in the biofilm than the stationary could be due to the effect of removing the biofilms from the slide and putting them into suspension. A better test for biofilm viability would be staining a biofilm directly with PI and BOX and viewing them under a confocal microscope, this will be an aim of future work.

3.5. Determination of rates of efflux using the Hoechst assay

3.12.

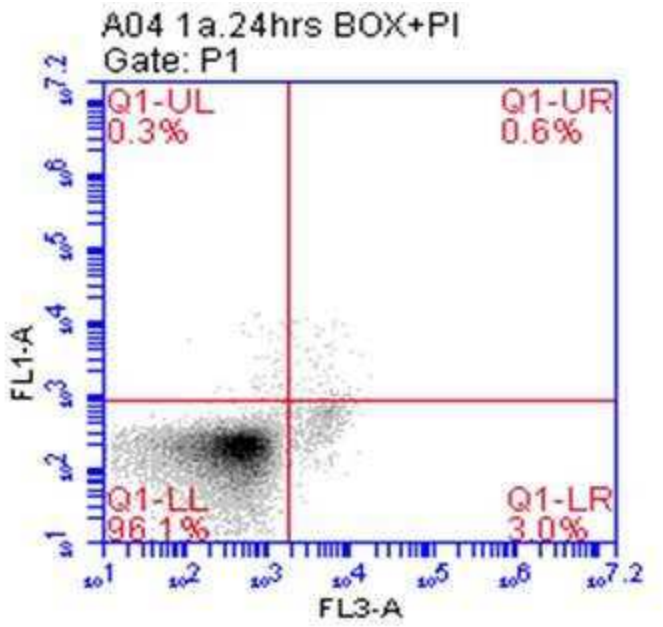


Figure 3.12: Flow cytometric chart of PHL644 pSTB7
The diagram shows the fluorescence of PHL644 pSTB7 cells in all four quadrants: live, in the lower left quadrant unable to take up PI or BOX; injured in the upper left quadrant able to take up BOX due to their collapsed membrane potential; dead cells in the upper right quadrant able to take up both BOX and PI due to their ruptured membrane; and the unknown lower right quadrant, theoretically impossible, since PI positive cells with ruptured membranes cannot have a membrane potential and thus be BOX negative.

During flow cytometry analysis it was noted that cells were present in all four quadrants of the PI/BOX plots (Figure 3.12): lower left (live cells, unable to take up PI or BOX); upper left (injured cells, able to take up BOX due to their collapsed membrane potential); upper right (dead cells, able to take up both BOX and PI due to their ruptured membrane); and lower right (theoretically impossible, since PI positive cells with ruptured membranes cannot have a membrane potential and thus be BOX negative). It was hypothesised that the cells in the lower right quadrant could have altered efflux properties that led to them effluxing the Hoechst dye, resulting in their unusual fluorescence properties. Efflux and influx are also important properties of the cells since the biotransformation reaction would be impeded if the reactants were rapidly effluxed from the cells in particular, indole is known to be an efflux substrate in E. coli (Lee and Lee 2010). To test the efflux properties of the cells in biotransformation conditions, the Hoechst efflux assay was used to monitor cells under different reaction

conditions and how they efflux the Hoechst dye which fluoresces when bound to DNA. Increased rates of efflux lead to a decrease in observed Hoechst fluorescence.

The first Hoechst experiment performed was on mid log phase planktonic PHL644 pSTB7 cells under different reaction conditions. 5ml Overnight culture were grown in triplicate at 30°C and 150 rpm, from each of these 2ml was added to 50 ml of fresh LB and grown to mid log phase. 3ml of each resultant culture was taken for a dead cell control by being centrifuged at 1811g for 10 minutes to be resuspended in 70% ethanol for 10 minutes then centrifuged at 1811g for 10 minutes then resuspended in 3ml of PBS. Another 3 ml was centrifuged at 1811g for 10 minutes resuspended in 3ml of PBS as a live cell control. The rest of the culture was also centrifuged at 1811g for 10 minutes and was resuspended in reaction buffer, before being diluted so the OD_{650} was approximately 0.2. This was then divided into the different reaction conditions: reaction buffer; reaction buffer plus 5% DMSO; reaction buffer supplemented with 5% DMSO and 2 mM indole; reaction buffer supplemented with 5% DMSO and 2 mM 5-chloroindole; reaction buffer supplemented with 5% DMSO and 2 mM 5-bromoindole; and reaction buffer supplemented with 5% DMSO and 2 mM 5-fluoroindole. The reaction was started with the addition of 20µl of Hoechst and the fluorescence (λ_{ex}=350 nm, λ_{em}=460 nm) measured every minute (Figure 3.13). It was found that dead cells had the highest fluorescence, caused by their inability to efflux the Hoechst dye, which is an ATP requiring process. The fluorescence gradually decreased over time, which may be caused by DNA degradation.

The live cells had lower fluorescence than the dead cells, indicating ATP-dependent efflux of Hoechst. Cells incubated in reaction buffer had similar fluorescence profiles to that of dead cells, with equilibrium fluorescence values in the order 5-chloroindole > 5-bromoindole > 5-fluoroindole. Cells incubated with indole, reaction buffer only or reaction buffer supplemented with DMSO had equivalent, low, fluorescence values. Cells suspended in PBS showed a slower initial increase in fluorescence so were difficult to compare to reaction buffer incubated cells.

The Hoechst assay was also performed with E. coli PHL644 without the pSTB7 biotransformation plasmid to determine whether the biotransformation of haloindole to halotryptophans was affecting the rates of efflux (Figure 3.14). Comparable fluorescence versus time curves was found in PHL644 (Figure 3.14.) compared to

PHL644 pSTB7 (Figure 3.13.) these data indicate that the biotransformation is not causing a difference in efflux.

Since the curves for PHL644 pSTB7 in the various reaction buffers had a similar shape to the dead cells, repeats of this assay were performed with the addition of flow cytometric analysis of the cells' viability (Figure 3.15). This showed that the majority of cells incubated with chloroindole and bromoindole were dead, explaining why curves in Figures 3.13. and 3.14 look very similar to the dead cell controls. It can be inferred that the cells grown for the Hoechst assay were more susceptible to the toxic effects of chloroindole and bromoindole due to only being grown to mid log phase. However the comparable level of dead cells in reactions containing reaction buffer supplemented with DMSO, indole and fluoroindole suggests that these data may be compared.

3.13.

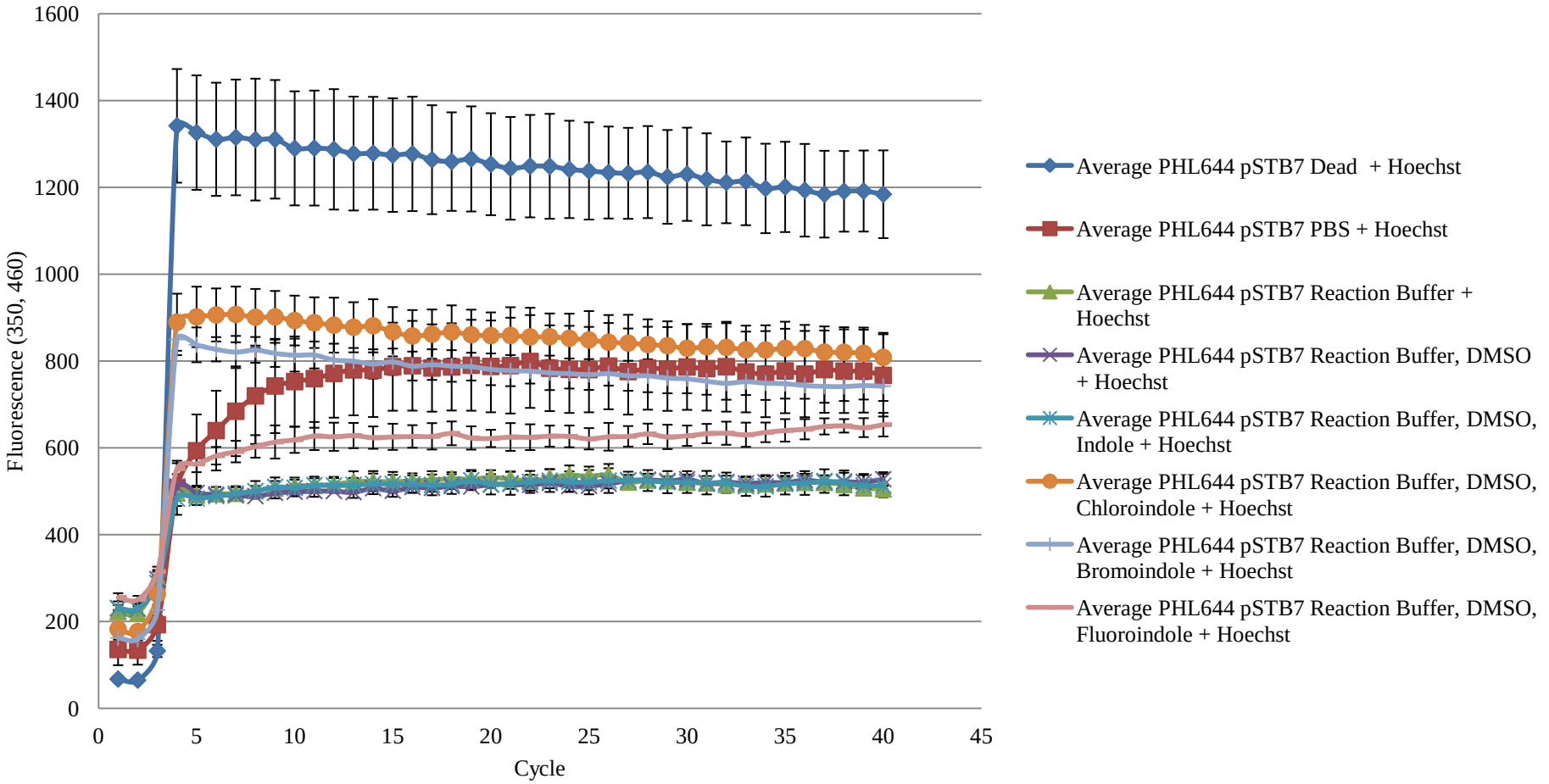


Figure 3.13: Accumulation of Hoechst by PHL 644 pSTB7 incubated in different reaction conditions over time.
Planktonic E. coli strain PHL644 pSTB7 was incubated in either PBS, reaction buffer, reaction buffer plus 5% DMSO, reaction buffer supplemented with 5% DMSO and 2 mM indole, reaction buffer supplemented with 5% DMSO and 2 mM 5-chloroindole, reaction buffer supplemented with 5% DMSO and 2 mM 5-bromoindole, and reaction buffer supplemented with 5% DMSO and 2 mM 5-fluoroindole and efflux and influx were examined by monitoring the fluorescence of level of Hoechst dye (λ_{ex}=350 nm, λ_{em}=460 nm) over time. Data plotted are the mean of 2 independent experiments performed in triplicate, error bars indicate standard deviation.

3.14.

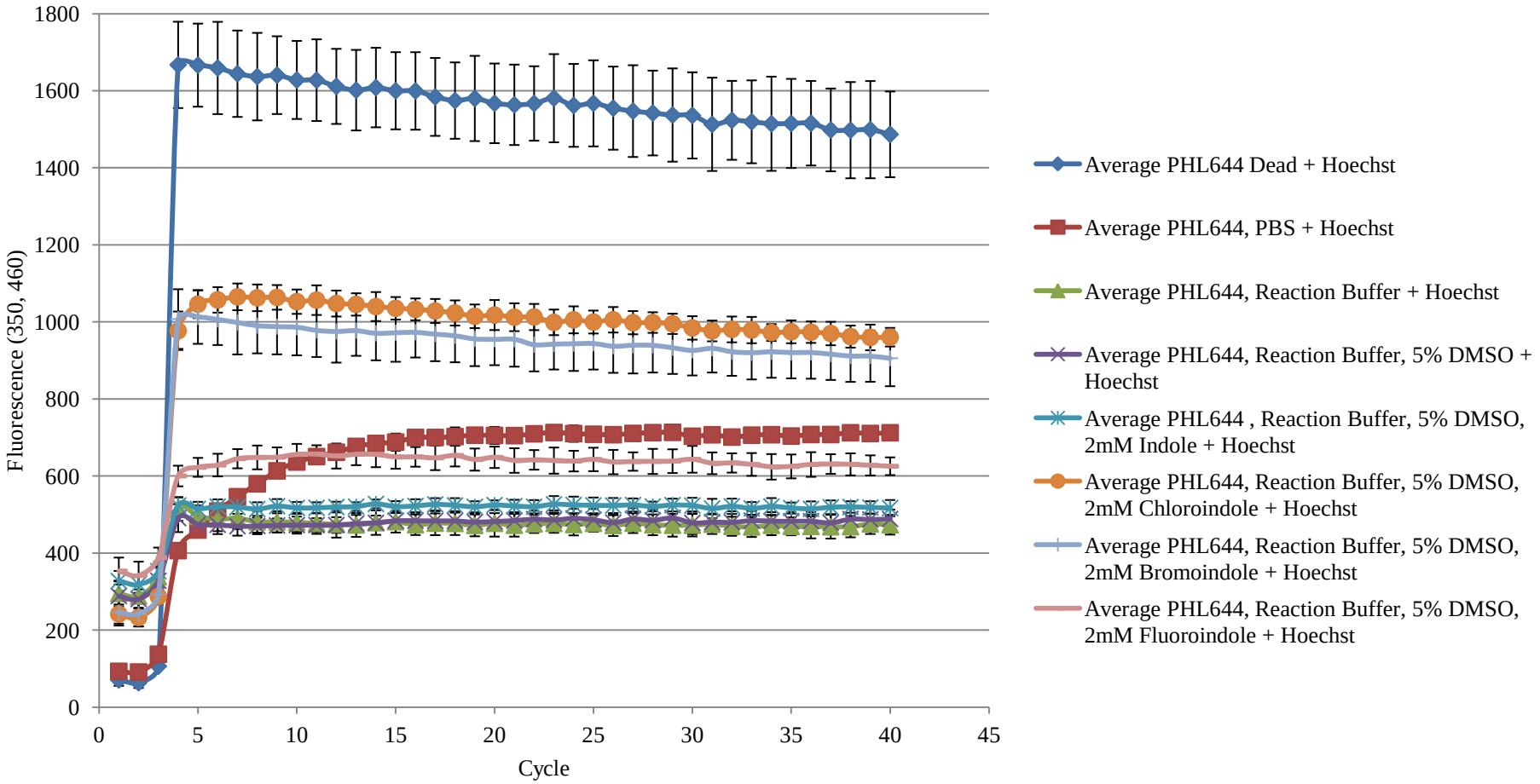
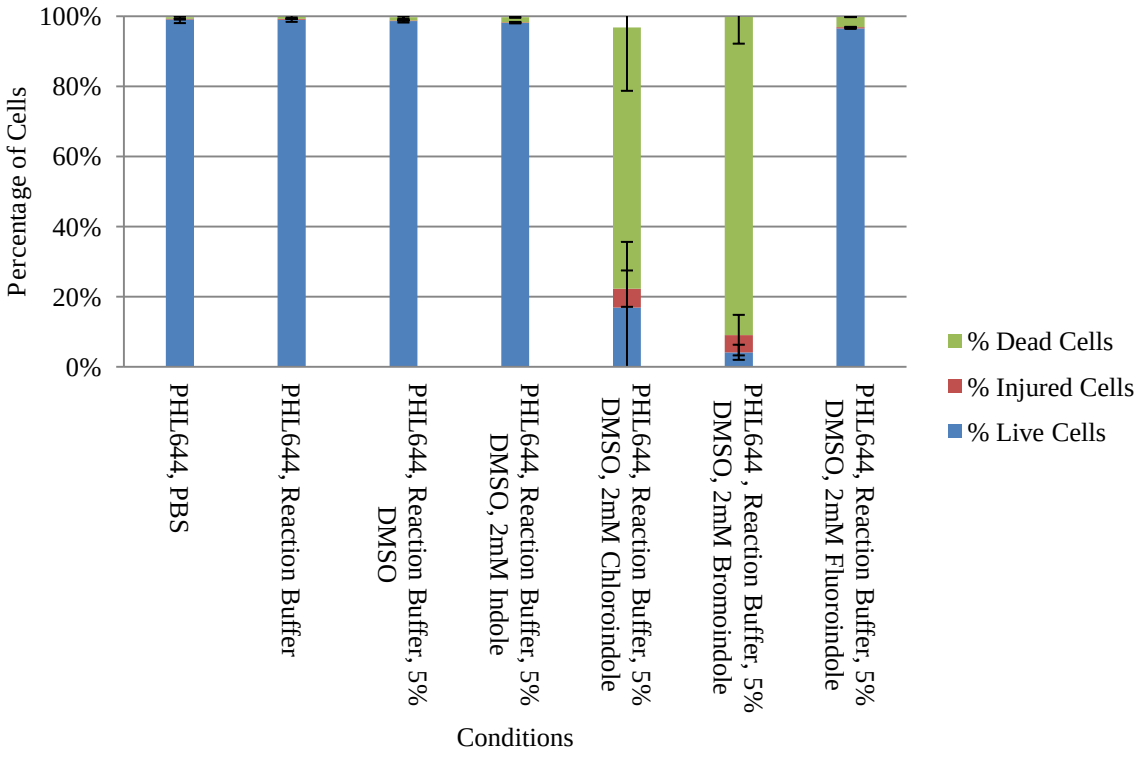


Figure 3.14: Accumulation of Hoechst by PHL644 incubated in different reaction conditions over time.
Planktonic E. coli strain PHL644 was incubated in either PBS, reaction buffer, reaction buffer plus 5% DMSO, reaction buffer supplemented with 5% DMSO and 2 mM indole, reaction buffer supplemented with 5% DMSO and 2 mM 5-chloroindole, reaction buffer supplemented with 5% DMSO and 2 mM 5-bromoindole, and reaction buffer supplemented with 5% DMSO and 2 mM 5-fluoroindole and efflux and influx were examined by monitoring the fluorescence of level of Hoechst dye (λ_{ex}=350 nm, λ_{em}=460 nm) over time. Data plotted are the mean of 2 independent experiments performed in triplicate, error bars indicate standard deviation.

3.15.A.



3.15.B.

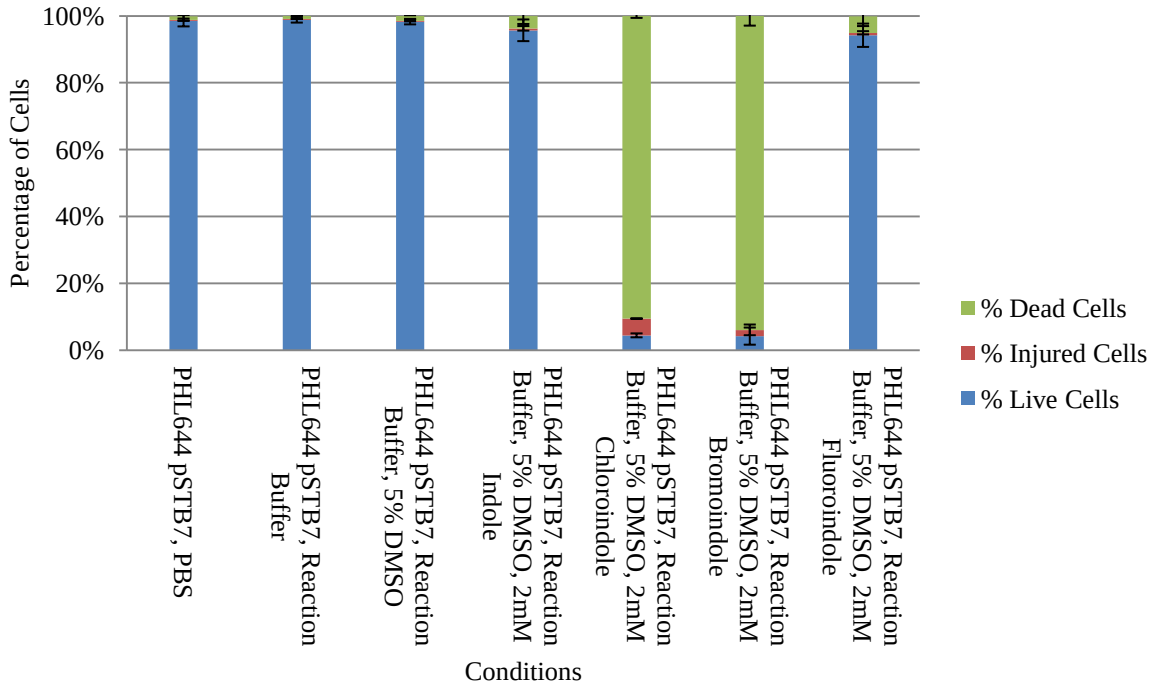


Figure 3.15.: Flow cytometric analysis of PHL644(a)and PHL 644 pSTB7 (b) incubated in different reaction conditions for the Hoechst assay

Planktonic E. coli strain PHL644 (a) or PHL644 pSTB7 (b) were incubated in either PBS, reaction buffer, reaction buffer plus 5% DMSO, reaction buffer supplemented with 5% DMSO and 2 mM indole, reaction buffer supplemented with 5% DMSO and 2 mM 5-chloroindole, reaction buffer supplemented with 5% DMSO and 2 mM 5-bromoindole, or reaction buffer supplemented with 5% DMSO and 2 mM 5-fluoroindole for the Hoechst assay. Viability was determined using flow cytometry with dual propidium iodide – bisoxanol staining.

To test for any difference in efflux abilities in each condition the Hoechst assay with PHL644 and PHL644 pSTB7 were performed in triplicate as above with the addition of the efflux pump inhibitor (EPI) carbonyl cyanide m-chlorophenylhydrazone (CCCP) at a final concentration of 100µM. CCCP acts by dissipating the proton motive force to prevent efflux of Hoechst so that it accumulates more within the cells, which should result in a larger fluorescence that increases over time.

The profiles for reaction buffer supplemented with 5% DMSO and 2 mM 5-chloroindole or 2 mM 5-bromoindole with and without CCCP have been discounted as these cells were dead as shown in Figure 3.15. Cells incubated in all other conditions were mainly alive as determined by flow cytometry.

Adding CCCP to each of these reactions had an effect on their efflux (Figure 3.16). For E. coli PHL644 and PHL644 pSTB7 there is a dramatic increase in fluorescence in the cells treated with CCCP. These data indicate that under these conditions PHL644 and PHL644 pSTB7 are able to efflux normally.

There is also only a slight difference in the accumulation of Hoechst between PHL644 and PHL644 pSTB7 (Figure 3.16) which indicates that the plasmid and thus the ability to perform the biotransformation has no negative effect on efflux.

Comparing PHL644 and PHL644 pSTB7 in PBS (Figure 3.16.A.) and reaction buffer (Figure 3.16.B.) without the addition of the EPI the curves have a slightly different shape as seen in previous Hoechst assays (Figure 3.13 and 3.14.) showing the cells incubated in PBS either have decreased influx of the Hoechst or have increased efflux compared to cells incubated in reaction buffer. However when the EPI is added the curves become very similar. The cells incubated in PBS (Figure 3.16.A.) do reach a higher fluorescence than the cells incubated in reaction buffer (Figure 3.16.B.) showing that efflux and influx are different between these conditions with more Hoechst being brought into the cells incubated in PBS.

DMSO is used primarily to increase the solubility of the haloindoles but might also increase the solubility of Hoechst, making it easier for the cells to import it and giving higher fluorescence, which can be checked by performing the same experiments but without DMSO. Comparing PHL644 and PHL644 pSTB7 incubated in reaction buffer (Figure3.16.B.) and reaction buffer plus 5% DMSO (Figure 3.16.C.) there appears to be little

difference in the curves both with and without EPI suggesting that DMSO has little effect on efflux and likely does not increase Hoechst's solubility. Again when the EPI is added the cells incubated in reaction buffer plus 5% DMSO act as expected with an increase in fluorescence.

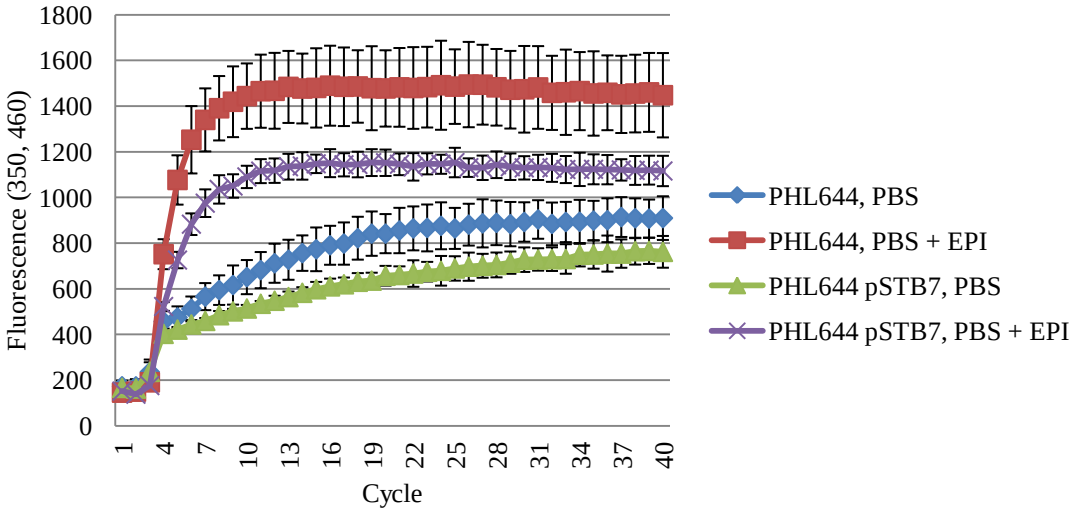
When 2mM indole is added to reaction buffer plus 5% DMSO (Figure 3.16.D.) there is a slight increase in fluorescence compared to reaction buffer plus 5% DMSO (Figure 3.16.C.) suggesting that either the influx is increasing or the efflux is decreasing in the presence of indole; either of these scenarios is advantageous for the biotransformation. Studies have been done looking at indole transport into and out of the cell; it is exported by AcrEF, a multi drug exporter and its efflux and influx have been found to be rapid through the diffusion through the cell membrane and transported in by Mtr (Zhang et al. 2007, Pinero-Fernandez et al. 2011). So when the EPI is added with cells incubated with indole there should be rapid transport into the cell still but no export out, which may explain the slightly higher fluorescence.

When the cells are incubated with reaction buffer plus 5% DMSO and 2mM 5-fluoroindole (Figure 3.16.E.), in the absence of EPI the fluorescence is significantly higher compared to reaction buffer plus 5% DMSO and 2mM indole. This indicates that either fluoroindole is increasing influx or decreasing efflux of Hoechst. When the EPI is added the fluorescence becomes similar, indicating that fluoroindole is inhibiting the efflux of Hoechst rather than the increasing influx. It is possible that the fluoride on the indole is difficult for the cells to efflux and is competing with Hoechst for efflux pump activity.

The next stage was to see if biofilm cells act in the same as planktonic PHL644 and PHL644 pSTB7. Biofilms were generated using the spin-down method and matured for 7 days in 70ml ofM63 medium in wide necked conical flasks. The M63 was removed and the flask and biofilms each washed with reaction buffer before being submerged in 70ml of either: PBS; reaction buffer; reaction buffer plus 5% DMSO; reaction buffer supplemented with 5% DMSO and 2 mM indole; reaction buffer supplemented with 5% DMSO and 2 mM 5-chloroindole; reaction buffer supplemented with 5% DMSO and 2 mM 5-bromoindole; or reaction buffer supplemented with 5% DMSO and 2 mM 5-fluoroindole for and incubated at 30°C at 70 rpm for 2 hours. The biofilms were then removed through vigorous shaking or pipetting the reaction buffer at the slide. The OD_{650} was measured for each and adjusted with reaction buffer to give an OD_{650} of approximately 0.2 and this was used in triplicate for the Hoechst assay. Unfortunately no replicable results were produced from these

experiments, possibly due to the cells in a biofilm not being able to take up Hoechst well enough, due to EPS or the physical state of the cells

3.16.A.



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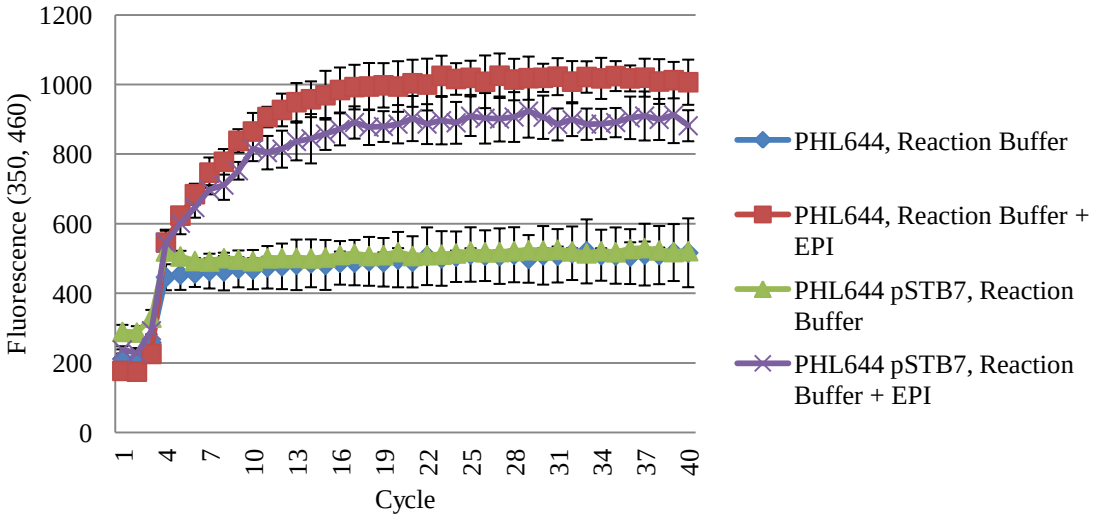

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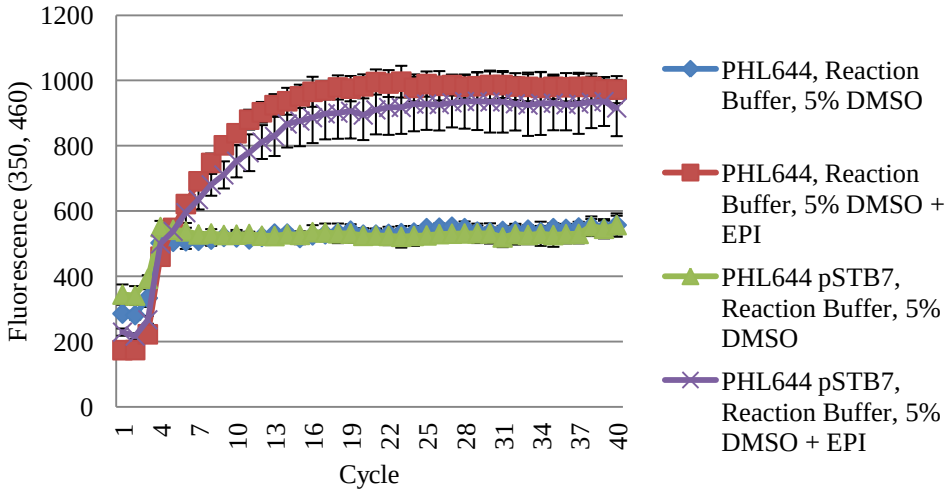

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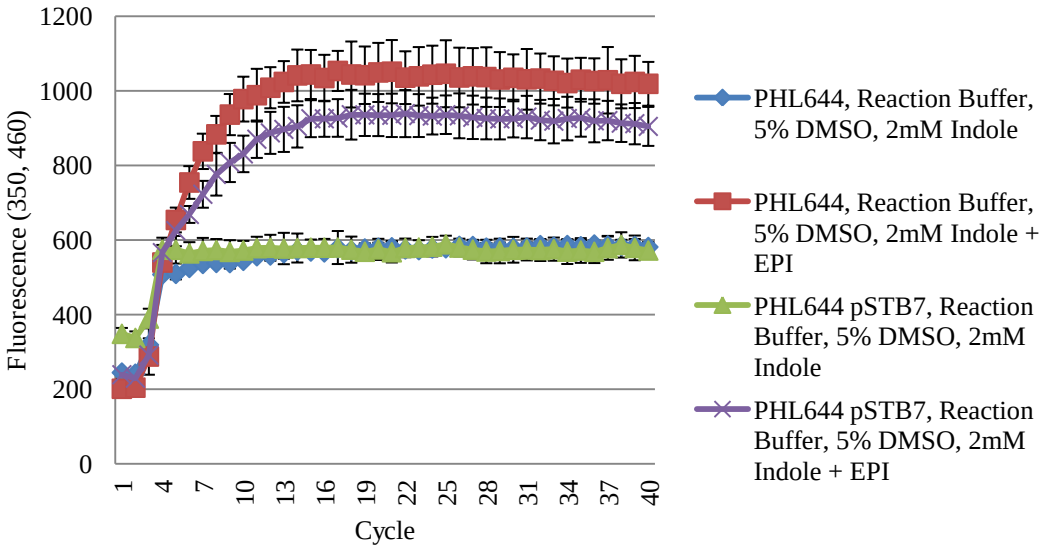

3.16.E.

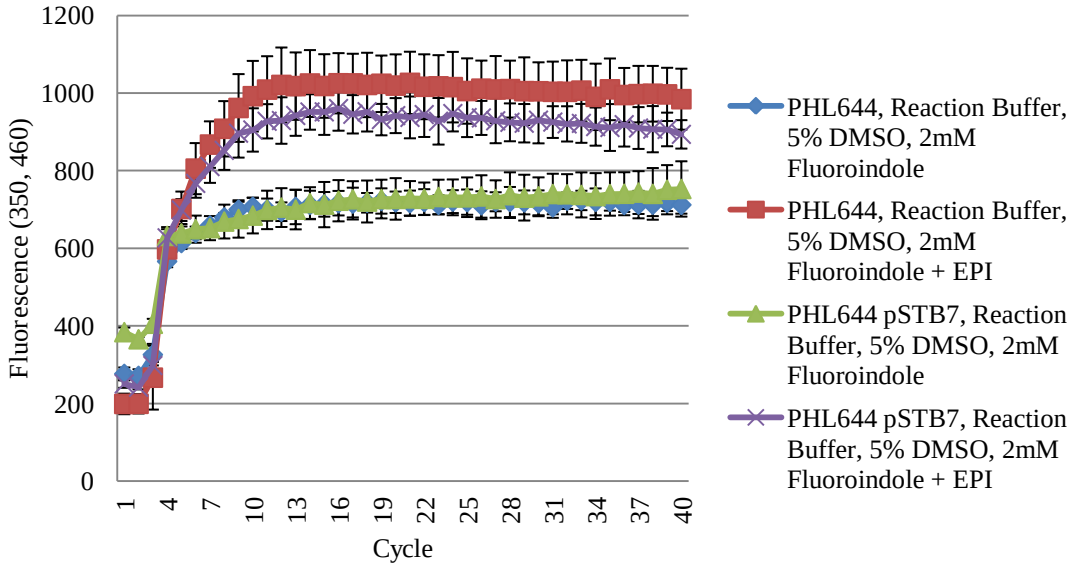

Figure 3.16: Accumulation of Hoechst by PHL644 and PHL644 pSTB7 incubated in different reaction conditions over time with the efflux pump inhibitor carbonyl cyanide m-chlorophenylhydrazone.
Planktonic E. coli strains PHL644 and PHL644 pSTB7 were incubated in either PBS (A), reaction buffer (B), reaction buffer plus 5% DMSO (C), reaction buffer supplemented with 5% DMSO and 2 mM indole (D), or reaction buffer supplemented with 5% DMSO and 2 mM fluoroindole (E) and efflux and influx were examined by monitoring the fluorescence of level of Hoechst dye (λ_{ex}=350 nm, λ_{em}=460 nm) over time. An efflux pump inhibitor (EPI) carbonyl cyanide m-chlorophenylhydrazone (CCCP) was added to half or the reactions as indicated..

3.6. Conclusions and future work

For a laboratory E. coli K12 strain to form a biofilm for use in industry as self-immobilising cells for biocatalysis, either the plasmid pT7-csgD is required or the strain needs an ompR234 mutation. This gives a hyperadhesive phenotype so cells produce an extensive EPS, with curli being upregulated, leaving cells immobilised to a surface to be immersed in reaction conditions for biocatalysis.

For the biocatalysis of converting haloindoles to halotryptophans, the plasmid pSTB7 is required for the expression of tryptophan synthase. It has been found that 5-chloroindole has a toxic effect, decreasing the viability of PHL644 pSTB7 over 24 hours and their ability to perform the biotransformation. The toxicity could be caused by reaction products or chloroindole being metabolised or the halotryptophans produced could be incorporated into cellular proteins. There is also indication that the toxicity is increased by the presence of the chloro group.

The results indicate that the concentration of serine and DMSO have little effect on the cells viability but other parts of the reaction buffer require testing to see if it affects the reaction.

Other results indicate that when in stationary phase or as a biofilm PHL644 pSTB7 in not affected by the toxicity of haloindoles and so should be able to perform the biotransformations without this being limiting.

The Hoechst experiments require more work so that live cells incubated with chloroindole and bromoindole can be tested to see if there is any difference in efflux compared to the other reactions and the same experiments needs to be done with biofilms. The results so far indicate that efflux may be decreased when cells are incubated with fluoroindole, an advantage for biotransformation.

Future work consists of investigating why planktonic E. coli dies when performing the biotransformation of 5-chloroindole to 5-chlorotryptophan whereas the engineered biofilm performs the conversion for over 30 hours (Tsoligkas et al. 2012). To do this continued investigation on the toxicity of 5-chloroindole will be performed through: using chloramphenicol to stop protein synthesis to see if toxicity still occurs. If the toxicity does not occur, it may be caused from 5-chloroindole being incorporated into proteins rather than inhibiting cells which can be checked by looking at the mass of the proteins produced; changing DMSO concentration in the

biotransformation to see its effect and if larger concentrations of haloindoles can be used; and performing the reactions with empty plasmids in PHL644 to see if the biotransformation products are causing toxicity or if the metabolic burden of a plasmid leads to susceptibility to toxicity. Experiments in future will have samples taken for HPLC measurement to find out the conversion rate of the biotransformation, which is previously not done. Planktonic E. coli will also be used to find out what the rate limiting step in the biotransformation is and confirm that planktonic cells are less robust than a biofilm.

Other future work includes more work on biofilms. With continuing with the Hoechst assay to get replicable data of biofilms performing the biotransformation to investigate whether cells in a biofilm are effluxing the haloindoles and/or halotryptophans rapidly differently to planktonic cells. Biofilm viability will also be investigated in-situ, confocal microscopy will measure fluorescence of PI and BOX (Live/dead) staining. This would be compared with flow cytometry, OD and CFU results; gene regulation in biofilm development will be investigated through plasmids with biofilm regulator promoter-GFP fusions to see when and where the regulators are expressed in the biofilm with particular interest in CsgD.

Further investigations on optimising the biotransformation will be performed by looking at different cell transporters, such as Mtr, an indole and tryptophan transporter and TdcC, a serine import transporter, to see if down or up-regulation by changing components of the growth media alters reaction rates.

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Appendix: Journal Article

Optimisation of engineered Escherichia coli biofilms for enzymatic biosynthesis of l-halotryptophans.

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Optimisation of engineered *Escherichia coli* biofilms for enzymatic biosynthesis of L-halotryptophans

Stefano Perni[1], Louise Hackett[1], Rebecca JM Goss[2], Mark J Simmons[1] and Tim W Overton[1*]

Abstract

Engineered biofilms comprising a single recombinant species have demonstrated remarkable activity as novel biocatalysts for a range of applications. In this work, we focused on the biotransformation of 5-haloindole into 5-halotryptophan, a pharmaceutical intermediate, using *Escherichia coli* expressing a recombinant tryptophan synthase enzyme encoded by plasmid pSTB7. To optimise the reaction we compared two *E. coli* K-12 strains (MC4100 and MG1655) and their *ompR234* mutants, which overproduce the adhesin curli (PHL644 and PHL628). The *ompR234* mutation increased the quantity of biofilm in both MG1655 and MC4100 backgrounds. In all cases, no conversion of 5-haloindoles was observed using cells without the pSTB7 plasmid. Engineered biofilms of strains PHL628 pSTB7 and PHL644 pSTB7 generated more 5-halotryptophan than their corresponding planktonic cells. Flow cytometry revealed that the vast majority of cells were alive after 24 hour biotransformation reactions, both in planktonic and biofilm forms, suggesting that cell viability was not a major factor in the greater performance of biofilm reactions. Monitoring 5-haloindole depletion, 5-halotryptophan synthesis and the percentage conversion of the biotransformation reaction suggested that there were inherent differences between strains MG1655 and MC4100, and between planktonic and biofilm cells, in terms of tryptophan and indole metabolism and transport. The study has reinforced the need to thoroughly investigate bacterial physiology and make informed strain selections when developing biotransformation reactions.

Keywords: *E. coli*; Biofilm; Biotransformation; Haloindole; Halotryptophan

Introduction

Bacterial biofilms are renowned for their enhanced resistance to environmental and chemical stresses such as antibiotics, metal ions and organic solvents when compared to planktonic bacteria. This property of biofilms is a cause of clinical concern, especially with implantable medical devices (such as catheters), since biofilm-mediated infections are frequently harder to treat than those caused by planktonic bacteria (Smith and Hunter, 2008). However, the increased robustness of biofilms can be exploited in bioprocesses where cells are exposed to harsh reaction conditions (Winn *et al.,* 2012). Biofilms, generally multi-species, have been used for waste water treatment (biofilters) (Purswani *et al.,* 2011; Iwamoto and Nasu, 2001; Cortes-Lorenzo *et al.,* 2012), air filters (Rene *et al.,* 2009) and in soil bioremediation (Zhang *et al.,* 1995; Singh and Cameotra, 2004). Most recently, single species biofilms have found applications in microbial fuel cells (Yuan *et al.,* 2011a; Yuan *et al.,* 2011b) and for specific biocatalytic reactions (Tsoligkas *et al.,* 2011; Gross *et al.,* 2010; Kunduru and Pometto, 1996). Recent examples of biotransformations catalysed by single-species biofilm include the conversion of benzaldehyde to benzyl alcohol (*Zymomonas mobilis*; Li *et al.,* 2006), ethanol production (*Z. mobilis* and *Saccharomyces cerevisiae*; Kunduru and Pometto, 1996), production of (*S*)-styrene oxide (*Pseudomonas* sp.; Halan *et al.,* 2011; Halan *et al.,* 2010) and dihydroxyacetone production (*Gluconobacter oxydans*; Hekmat *et al.,* 2007; Hu *et al.,* 2011).

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When compared to biotransformation reactions catalysed by purified enzymes, whole cell biocatalysis permits protection of the enzyme within the cell and also production of new enzyme molecules. Furthermore, it does not require the extraction, purification and immobilisation involved in the use of enzymes, often making it a more cost-effective approach, particularly upon scale-up (Winn *et al.,* 2012). Biofilm-mediated reactions extend these benefits by increasing protection of enzymes against harsh reaction conditions (such as extremes of pH or organic solvents) and offering simplified downstream processing since the bacteria are immobilised and do not require separating from reaction products. These factors often result in higher conversions when biotransformations are carried out using biofilms when compared to purified enzymes (Winn *et al.,* 2012; Halan *et al.,* 2012; Gross *et al.,* 2012).

To generate a biofilm biocatalyst, bacteria must be deposited on a substrate, either by natural or artificial means, then allowed to mature into a biofilm. Deposition and maturation determine the structure of the biofilm and thus the mass transfer of chemical species through the biofilm extracellular matrix, therefore defining its overall performance as a biocatalyst (Tsoligkas *et al.,* 2011; 2012). We have recently developed methods to generate engineered biofilms, utilising centrifugation of recombinant *E. coli* onto poly-L-lysine coated glass supports instead of waiting for natural attachment to occur (Tsoligkas *et al.,* 2011; 2012). These biofilms were used to catalyse the biotransformation of 5-haloindole plus serine to 5-halotryptophan (Figure 1a), an important class of pharmaceutical intermediates; this reaction is catalysed by a recombinant tryptophan synthase TrpBA expressed constitutively from plasmid pSTB7 (Tsoligkas *et al.,* 2011; 2012; Kawasaki et al. 1987). We previously demonstrated that these engineered biofilms are more efficient in converting haloindole to halotryptophan than either immobilised TrpBA enzyme or planktonic cells expressing recombinant TrpBA (Tsoligkas *et al.,* 2011).

In this study, we further optimised this biotransformation system by investigating the effect of using different strains to generate engineered biofilms and perform the biotransformation of 5-haloindoles to 5-halotryptophans. Engineered biofilm generation was tested for four *E. coli* strains: wild type K-12 strains MG1655 and MC4100; and their isogenic *ompR234* mutants, which overproduce curli (adhesive protein filaments) and thus accelerate biofilm formation (Vidal et al. 1998). Biofilms were generated using each strain with and without pSTB7 to assess whether the plasmid is required for these biotransformations as *E. coli* naturally produces a tryptophan synthase. The viability of bacteria during biotransformation reactions was monitored using flow cytometry. We also studied the biotransformation reaction with regard to substrate utilisation, product synthesis and conversion efficiency to allow optimisation of conversion and yield. This constitutes an essential step forward which will provide knowledge to future practitioners wishing to scale up this reaction.

Materials and Methods

Strains, biofilm generation and maturation

pSTB7, a pBR322-based plasmid containing the *Salmonella enterica* serovar Typhimurium TB1533 *trpBA* genes and encoding ampicillin resistance (Kawasaki *et al.,* 1987), was purchased from the American Type Culture Collection (ATCC 37845). *E. coli* K-12 strains MG1655 (λ – F – prototroph), PHL628 (MG1655 *malA-kan ompR234*; Vidal et al. 1998), MC4100 (*araD139Δ(argF-lac)U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*) and PHL644 (MC4100 *malA-kan ompR234*; Vidal et al. 1998) were employed in this study. All *E. coli* strains were transformed with pSTB7 using the heat-shock method. Transformants were selected on Luria-Bertani-agar (10 g L^{-1} tryptone, 5 g L^{-1}

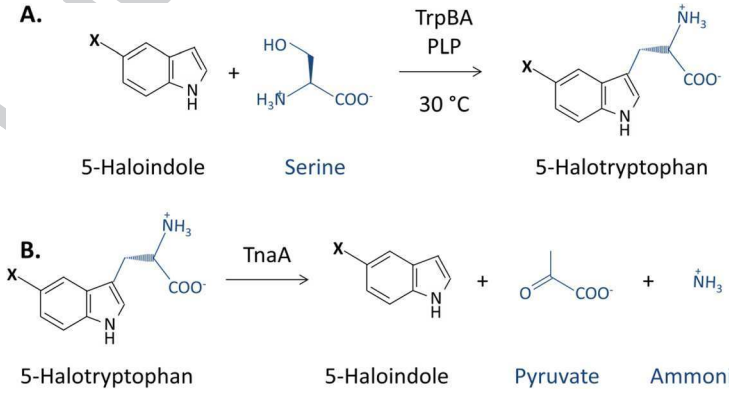

Figure 1 Formation and breakdown of 5-halotryptophan in *E. coli*. **(a)** Reaction scheme for biocatalytic conversion of 5-haloindole and serine to 5-halotryptophan, catalysed by tryptophan synthase TrpBA. **(b)** Reaction scheme for the reverse reaction, catalysed by tryptophanase TnaA.

yeast extract, 10 g L^{-1} NaCl, 15 g L^{-1} Bacteriological Agar; Sigma, UK) supplemented with ampicillin (100 μg mL^{-1}). All *E. coli* strains were grown in 200 mL half strength Luria-Bertani (LB) broth (5 g L^{-1} tryptone, 2.5 g L^{-1} yeast extract, 5 g L^{-1} NaCl; Sigma, UK), supplemented with ampicillin (100 μg mL^{-1}) for pSTB7 transformants, in an orbital shaker at 30°C, 70 rpm with a throw of 19 mm for 24 hours. Engineered biofilms were generated using the spin-down method described by Tsoligkas *et al.* (2011) and available in Additional file 1.

Biotransformations

Biotransformation reactions were carried out as previously described (Tsoligkas *et al.*, 2011; full details in Additional file 1) using either planktonic cells or engineered biofilms in a potassium phosphate reaction buffer (0.1 M KH_2PO_4, 7 mM Serine, 0.1 mM Pyridoxal 5′-phosphate (PLP), adjusted to pH 7.0) supplemented with 5% (v/v) DMSO and either 2 mM 5-fluoroindole (270 mg L^{-1}), 2 mM 5-chloroindone (303 mg L^{-1}), or 2 mM 5-bromoindole (392 mg L^{-1}). 5-chloroindole and 5-bromoindole are less soluble than 5-fluoroindole, so lower concentrations were present in the reaction buffer; around 0.7 mM for 5-chloroindole and 0.4 mM for 5-bromoindole (Additional file 1: Table S1). In each case, reaction buffer was made with an initial quantity of haloindole equivalent to 2 mM and decanted into biotransformation vessels, preventing any undissolved haloindole from entering the biotransformation. No attempt has been made to carry out the reactions at the same starting concentrations since an in-depth kinetic analysis was not the focus of this study. All biotransformations, irrespectively of the cells' physiological state, were conducted on two or three independent cultures. Since 5-fluoroindole biotransformations were the most active, biotransformations were performed with all strain combinations. Biotransformations with 5-chloroindole and 5-bromoindole were performed with selected strains to generate indicative data.

HPLC analysis

Haloindole and halotryptophan concentrations were measured in biotransformation samples by HPLC using a Shimadzu HPLC with a ZORBAX (SB-C18 4.6 mm × 15 cm) column resolved with methanol versus water at a rate of 0.7 mL min^{-1}; a UV detector at 280 nm was used throughout the analysis (Additional file 1: Figure S1). Both solvents were acidified with 0.1% formic acid and run using the gradient described in the supplementary data. Linear standard curves (Additional file 1: Figure S2; peak area versus concentration) were generated for 5-fluoro-, 5-chloro- and 5-bromoindole and each corresponding 5-halotryptophan using standards of known concentration (0.125 mM to 2 mM) in triplicate and used to correlate sample peak area to concentration. Biotransformation data are presented as three percentages of halotryptophan yield (Y), haloindole depletion (D) and selectivity of conversion (S) for each timepoint:

$$Y = \frac{\text{halotryptophan concentration}}{\text{initial haloindole concentration}} \times 100 \quad (1)$$

$$D = \frac{\text{initial haloindole concentration-haloindole concentration}}{\text{initial haloindole concentration}} \times 100 \quad (2)$$

$$S = \frac{Y}{D} \times 100 \quad (3)$$

Quantification of the dry cell biomass and Crystal Violet staining

The total biofilm biomass was determined for 5 slides that had been coated with *E. coli* biofilms and matured for 7 days. The glass slides were washed twice in phosphate buffer. In a pre-weighed centrifuge tube kept at 100°C overnight, the biofilm was disrupted in sterile water using a vortex mixer for 30 minutes; the glass slide was removed and the cells centrifuged at 1851 g for 10 minutes. The supernatant was removed and the biomass dried at 100°C for at least 24 hrs. The dry biomass was determined when the mass stopped decreasing.

The quantification of dry cell biomass of planktonic cells was performed directly on 10 mL of three independent cell suspensions in pre-weighed centrifuge tubes kept at 100°C overnight. Following centrifugation (1851 g for 10 minutes) and washing in sterile water, the cells were centrifuged again (1851 g for 10 minutes) and, after removing the liquid, allowed to dry at 100°C for at least 24 hours until a constant mass was reached.

Biofilms on glass slides were also quantified using Crystal Violet staining; after washing in sterile phosphate buffer the slides were coated with 1 mL of Crystal Violet solution (0.1% (w/v) for 15 min. The slides were washed in water three times and placed in Duran bottles with 20 mL of ethanol. The crystal violet on the glass slides was allow to dissolve for 1 hour and the optical density of the ethanol solution determined at 570 nm using a UV–vis spectrophotometer.

Flow cytometry

Cell membrane potential and membrane integrity were analysed by flow cytometry after 2 and 24 hours in each reaction condition using staining with 5 μg mL^{-1} propidium iodide (PI, which enters cells with compromised membrane integrity) and 0.1 mg mL^{-1} Bis (1,3-dibarbituric acid) trimethine oxanol (BOX, which enters cells with depolarised membranes) as previously described by Whitehead *et al.* (2011). Cells were analysed using an Accuri C6 flow cytometer (BD, UK) as described in the Additional file 1.

Results

Biofilm formation by different *E. coli* strains

Crystal Violet staining was used to compare the biomass within biofilms generated using the spin-down method with four *E. coli* strains: MG1655 and MC4100; and their *ompR234* derivatives PHL628 and PHL644 (Figure 2). MG1655 generated more biofilm than MC4100, and the *ompR234* mutation increased the amount of biofilm formed by both strains. The presence of pSTB7 decreased biofilm formation by PHL628 but did not significantly affect biofilm formation by the other strains. The corresponding dry mass of each biofilm was 1.5 ± 0.2 mg for PHL644 pSTB7 and 2.3 ± 0.3 mg for PHL628 pSTB7.

Biotransformation by planktonic cells

The ability of planktonic cells to convert 5-haloindoles to 5-halotryptophans was assessed by measuring 5-haloindole depletion, 5-halotryptophan synthesis and the selectivity of conversion of 5-haloindole to 5-halotryptophan as defined in equations 1–3. These three measurements are required since, although the conversion of haloindole plus serine to halotryptophan is catalysed by the TrpBA enzyme, halotryptophan is a potential substrate for tryptophanase (TnaA) which would convert it to haloindole, pyruvate and ammonium (Figure 1b). Alternatively, halotryptophans could be sequestered for protein synthesis (Crowley *et al.*, 2012). Thus, selectivity of conversion to halotryptophan is a critical parameter for the reaction to be considered as a

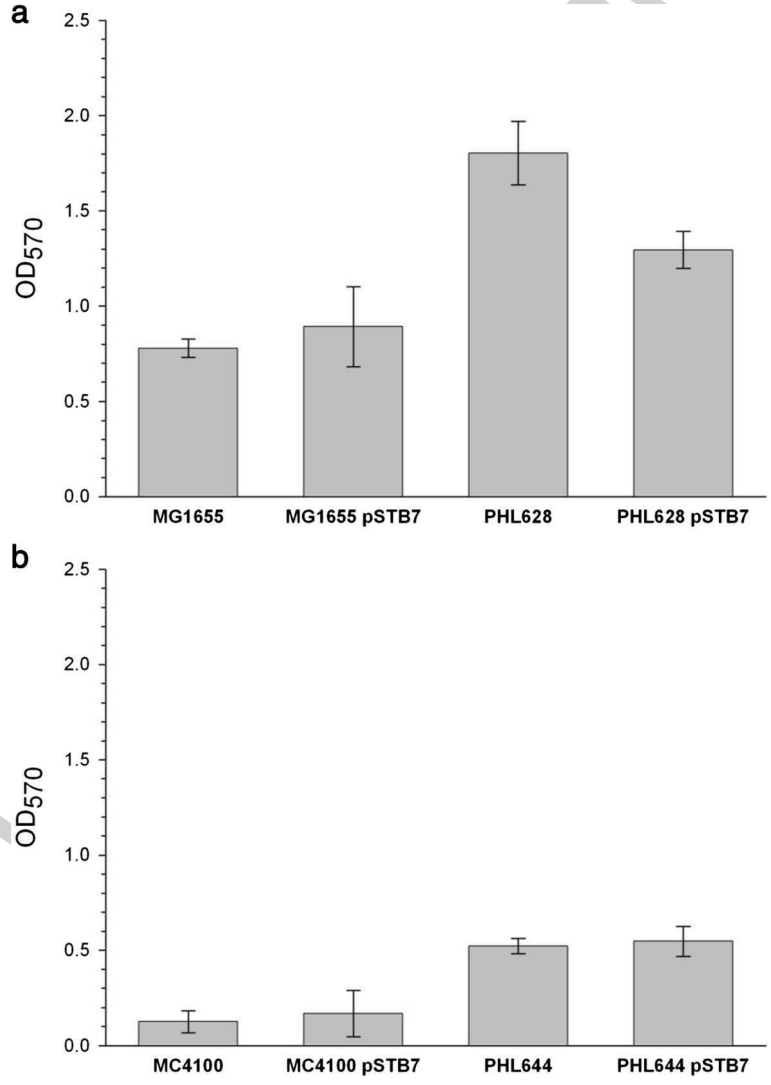

Figure 2 Crystal Violet staining of *E. coli* engineered biofilms. Biofilms were generated from strains MG1655 and PHL628 **(a)** or MC4100 and PHL644 **(b)** with and without pSTB7 using the spin-down method, matured for 7 days in M63 medium and biomass was estimated using crystal violet staining.

viable route for production of these compounds. Neither depletion of haloindole nor production of halotryptophan was detected when biotransformations were performed using bacteria without the pSTB7 plasmid, either planktonically or in biofilms, confirming that the constitutively expressed recombinant tryptophan synthase is required for the reaction (data not shown).

F3 Figure 3a shows that the concentrations of 5-fluorotryptophan increased over the reaction period with the rate of generation decreasing as the reaction proceeded. No significant difference was noticed in synthesis rate or overall yield between MG1655 pSTB7 and PHL628 pSTB7; the rate and yield were higher for MC4100 pSTB7, and higher still for PHL644 pSTB7. The profile of 5-fluoroindole depletion (Figure 3b) appeared similar to that of 5-fluorotryptophan generation in strains MG1655 pSTB7 and PHL628 pSTB7, but displayed a rapid increase (to nearly 20%) in MC4100 pSTB7 and PHL644 pSTB7 in the first hour of the reaction. This suggests that indole efflux is much more rapid in MC4100 than in MG1655, and reflects an inherent difference between the strains. Selectivity of conversion of 5-fluoroindole to 5-fluorotryptophan increased rapidly in PHL628 pSTB7, PHL644 pSTB7 and MG1655 pSTB7, although MG1655 pSTB7 selectivity was highest after 8 hours (Figure 3c). Planktonic biotransformation reactions (in 10 mL of culture volume) contained a dry mass of 1.1 ± 0.1 mg for PHL644 pSTB7 and 1.2 ± 0.2 mg for PHL628 pSTB7.

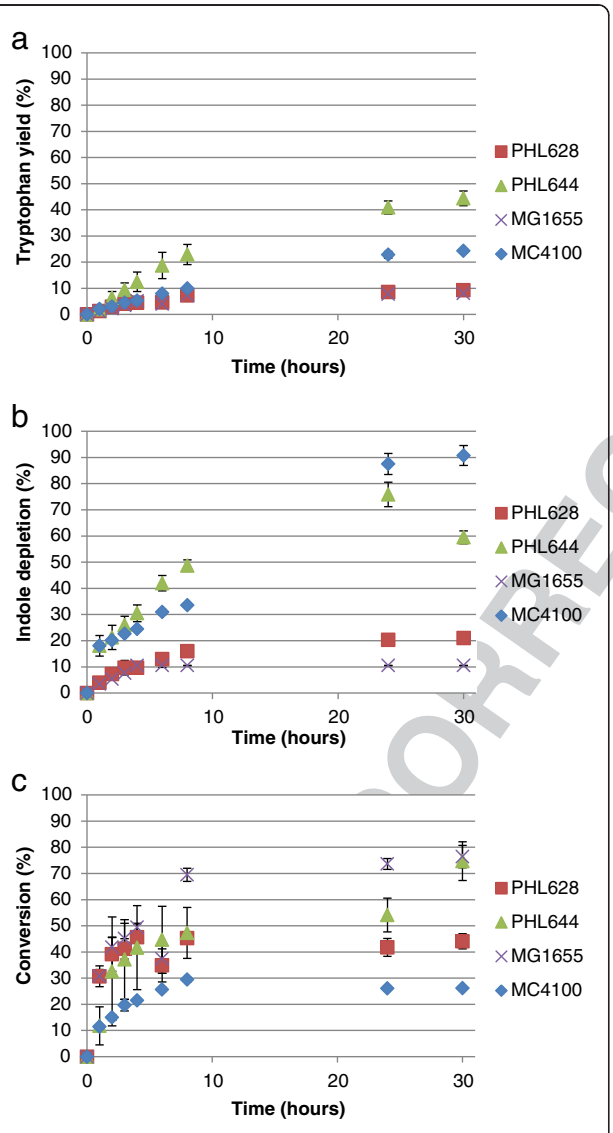

Figure 3 Biotransformation of 5-fluoroindole to 5-fluorotryptophan using planktonic cells of four strains. Concentrations of 5-fluorotryptophan and 5-fluoroindole were measured using HPLC and percentage 5-fluorotryptophan accumulation **(a)**, percentage 5-fluoroindole depletion **(b)** and the selectivity of the 5-fluoroindole to 5-fluorotryptophan reaction **(c)** were plotted against time.

The same parameters are shown for the biotransformation of 5-chloroindole to 5-chlorotryptophan in Figure 4. F4 Unlike the 5-fluoroindole reaction, strains PHL628, PHL644 and MG1655 showed similar overall percentage chlorotryptophan yields. As with the fluoroindole reactions (Figure 3), strains MC4100 pSTB7 and PHL644 pSTB7 both showed rapid chloroindole depletion in the first hour of the reaction whereas MG1655 pSTB7 and PHL628 pSTB7 displayed more gradual depletion. As a result, the selectivity of the reaction was initially higher in MG1655 pSTB7 and PHL628 pSTB7, peaking at around 75% at 4 hours, although the selectivity of these two strains decreased to around 50% over the course of the reaction. PHL644 pSTB7 selectivity increased over time to around 50% after 25 hours. As with the fluoroindole reaction, the selectivity of MC4100 pSTB7 was lowest throughout. Planktonic biotransformations yielded extremely low production of 5-bromotryptophan (>10%; Additional file 1: Figure S3). 5-bromoindole was depleted in these biotransformation reactions (although not to the same extent as fluorindole and chloroindole), but the rate of conversion to 5-bromotryptophan was very low. As with the 5-fluoroindole and 5-chloroindole reactions, 5-bromoindole was rapidly taken up by strains PHL644 and MC4100.

Biofilm-mediated biotransformation

Results for the biotransformation of 5-fluoroindole to 5-fluorotryptophan using engineered biofilms that had been matured for 7 days in M63 medium are shown in Figure 5. F5 Biofilm-mediated reactions were dramatically different to planktonic reactions, both in terms of each strain's relative activity but also in overall reaction kinetics. The rapid import of haloindole observed in planktonic MC4100 strains (Figures 3 and 4) was not observed in biofilm reactions, probably a consequence of the changes in indole transport and metabolism upon biofilm formation (Lee & Lee, 2010). Strains containing the *ompR234* mutation were all

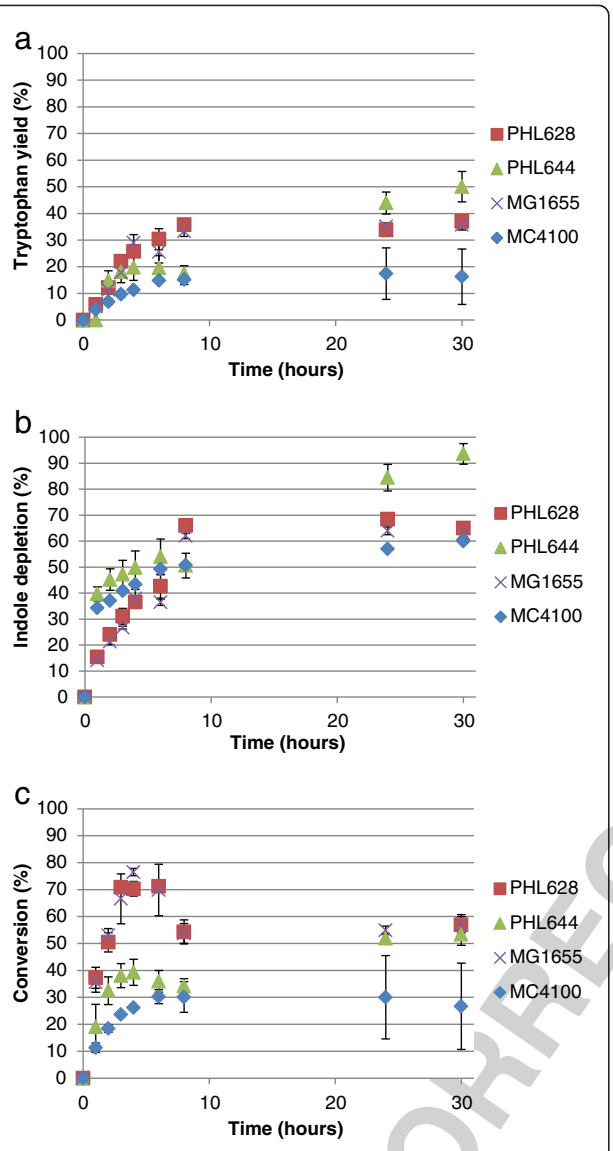

Figure 4 Biotransformation of 5-chloroindole to 5-chlorotryptophan using planktonic cells of four strains. Concentrations of 5-chlorotryptophan and 5-chloroindole were measured using HPLC and percentage 5-chlorotryptophan accumulation **(a)**, percentage 5-chloroindole depletion **(b)** and the selectivity of the 5-chloroindole to 5-chlorotryptophan reaction **(c)** were plotted against time.

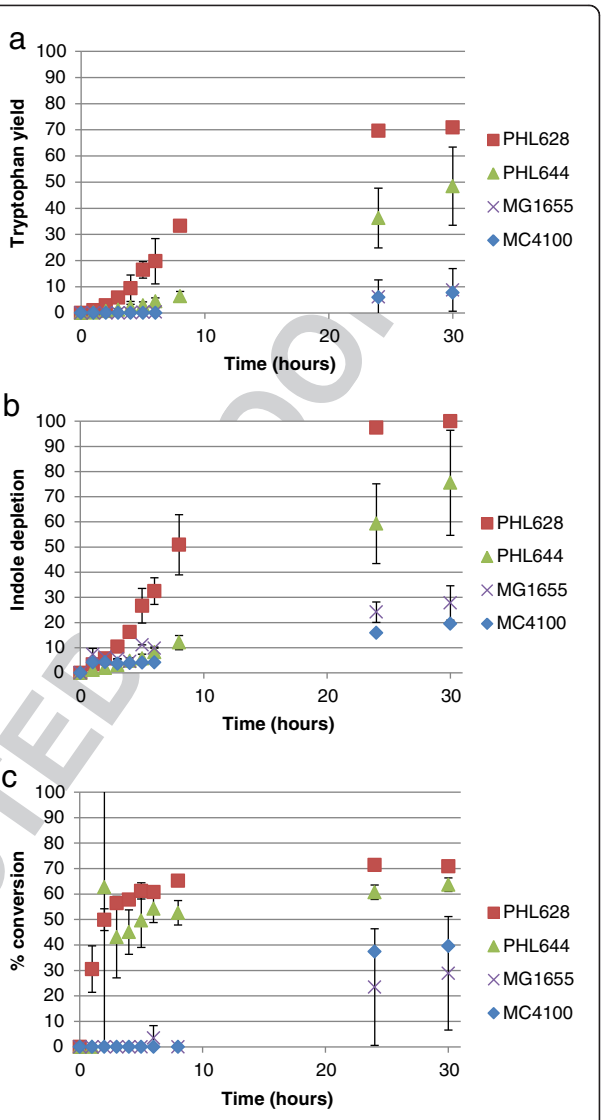

Figure 5 Biotransformation of 5-fluoroindole to 5-fluorotryptophan using engineered biofilms comprising four strains. Concentrations of 5-fluorotryptophan and 5-fluoroindole were measured using HPLC and percentage 5-fluorotryptophan accumulation **(a)**, percentage 5-fluoroindole depletion **(b)** and the selectivity of the 5-fluoroindole to 5-fluorotryptophan reaction **(c)** were plotted against time.

more catalytically active than their wild type counterparts; this is probably due in part to the lower entrapment of wild type cells (Figure 1). Unlike reactions performed with the cells in the planktonic state, the PHL628 pSTB7 biofilm outperformed PHL644 pSTB7 in terms of overall fluorotryptophan yield, rate of conversion and selectivity. MG1655 pSTB7 and MC4100 pSTB7 displayed minimal conversion of metabolised fluoroindole to fluorotryptophan until after 24 hours incubation (Figure 5c).

For the biofilm-mediated conversion of 5-chloroindole to 5-chlorotryptophan (Figure 6), PHL628 pSTB7 displayed rapid 5-chloroindole import (similar to MC4100 planktonic cells). Conversion was higher in PHL644 pSTB7 than PHL628 pSTB7, probably a consequence of the earlier exhaustion of 5-chloroindole in the latter strain. As with the planktonic 5-bromotryptophan reactions, the yields of biofilm-catalysed 5-bromotryptophan biotransformations were very low; 5-bromoindole was taken up by cells, but converted to 5-bromotryptophan at a very low rate (Additional file 1: Figure S4).

In order to compare the biotransformation reaction on an equivalent basis between different strains and

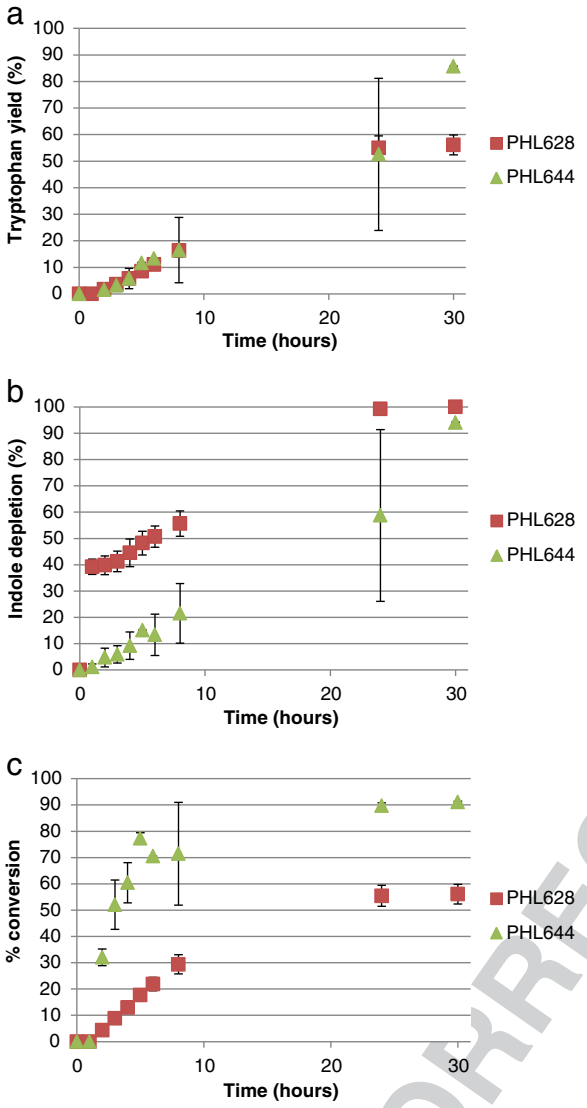

Figure 6 Biotransformation of 5-chloroindole to 5-chlorotryptophan using engineered biofilms comprising two strains. Concentrations of 5-chlorotryptophan and 5-chloroindole were measured using HPLC and percentage 5-chlorotryptophan accumulation **(a)**, percentage 5-chloroindole depletion **(b)** and the selectivity of the 5-chloroindole to 5-chlorotryptophan reaction **(c)** were plotted against time.

haloindoles, initial reaction rate data normalised by cell dry mass (expressed in units of μmol halotryptophan (mg dry cells)$^{-1}$ h^{-1}) are presented in Table 1. As previously observed (Tsoligkas *et al.*, 2011), reaction rates followed the trend fluoroindole > chloroindole > bromoindole. Biofilms and planktonic cells had very similar initial reaction rates except for MG1655 pSTB7 and PHL628 pSTB7 for fluoroindole when the initial conversion rate using biofilms was three to four times that of planktonic cells. It should be noted that initial rates do not necessarily relate to overall reaction yields, and these data should be consulted in conjunction with Figures 3, 4, 5 and 6.

Cell physiology during biotransformation reactions

To eliminate the possibility that differences in biotransformation yields were due to changes in bacterial viability or physiology, flow cytometry was used to determine the proportion of PHL644 pSTB7 cells with membrane potential and membrane integrity (i.e. live cells) after 2 and 24 hours of biotransformation reactions (Table 2). In all conditions, the vast majority of the cell population were live cells. Neither the presence of DMSO or any 5-haloindole had any detrimental effect on cell viability in planktonic biotransformations, even after 24 hours ($p < 0.05$). The presence of 5-haloindoles did not have a statistically significant effect on the percentage of biofilm cells alive after either 2 or 24 hours ($p < 0.05$); however, the proportion of live biofilm cells decreased between 2 and 24 hours ($p < 0.05$). Examples of plots obtained through flow cytometry are shown in Additional file 1: Figure S5.

Discussion

Biofilm formation

Biofilm formation is a complex process governed by many environmental cues, detected and coordinated through a complex regulatory network (Beloin *et al.*, 2008). The osmolarity-sensing two component regulatory system EnvZ-OmpR is crucial to the regulation of biofilm formation in *E. coli* (Shala *et al.*, 2011; Vidal *et al.*, 1998). OmpR transcriptionally activates the *csgDEFG* operon; CsgD in turn activates transcription of the *csgBAC* operon, encoding the curli structural proteins which enable initial attachment of bacteria to surfaces (Prigent-Combaret *et al.*, 2001; Ogasawara *et al.*, 2010; Brombacher *et al.*, 2003). In addition, CsgD also activates transcription of *adrA*, encoding a putative diguanylate cyclase which is predicted to generate c-di-GMP and thus activate cellulose production (Bhowmick *et al.*, 2011). The *ompR234* mutation carried in strains PHL628 and PHL644 comprises a point mutation (L43R) located within the receiver domain, which enhances activation of *csgDEFG* (Prigent-Combaret *et al.*, 2001; Prigent-Combaret *et al.*, 1999; Vidal et al. 1998). It was, therefore, expected that the *ompR234* strains would form biofilm more readily than MC4100 and MG1655 (Figure 2).

Indole has previously been shown capable of enhancing biofilm formation (Chu *et al.*, 2012; Pinero-Fernandez *et al.*, 2011), whereas tryptophan has been shown to decrease biofilm formation (Shimazaki *et al.*, 2012). Therefore the presence of pSTB7 could result in decreased biofilm formation since tryptophan concentrations (both intracellular and extracellular) could be predicted to be higher in cells containing pSTB7. *E. coli* MC4100 and MG1655 did not form substantial biofilms, hence the presence of pSTB7

t1.1 **Table 1 Summary of the initial rate of halotryptophan production expressed as μmol halotryptophan (mg dry cells)$^{-1}$ h^{-1}**

t1.2 Strain	5-fluoroindole		5-chloroindole		5-bromoindole	
t1.3	Planktonic	Biofilm	Planktonic	Biofilm	Planktonic	Biofilm
t1.4 MG1655 pSTB7	0.26	0.72	0.17	ND	0.13	ND
t1.5 PHL628 pSTB7	0.28	1.08	0.19	0.16	0.08	0.05
t1.6 MC4100 pSTB7	0.35	0.33	0.25	ND	0.05	ND
t1.7 PHL644 pSTB7	0.73	0.65	0.43	0.37	0.06	0.07

t1.8 *ND*, Not determined.

did not have a significant effect on these strains (Figure 2). pSTB7 decreased the biomass of PHL628 biofilms, although it did not decrease biofilm formation in PHL644. This was possibly a consequence of the higher activity of tryptophan synthase in biofilms of PHL628 pSTB7 compared to PHL644 pSTB7 (Table 1), which would deplete intracellular indole.

Biotransformation rates and efficiencies

As previously noted (Tsoligkas *et al.,* 2011), the initial rate of biotransformation reactions followed the trend 5-fluorotryptophan > 5-chlorotryptophan > 5-bromotryptophan, irrespective of strain (Table 1); this has been ascribed to steric hindrance of the TrpBA enzyme by bulky halogen adducts (Goss and Newill, 2006). The selectivity of the haloindole to halotryptophan reaction was not 100% in any of the cases studied. In most cases, the reaction stopped due to haloindole depletion. Since, in the absence of pSTB7, haloindole concentrations did not decrease over the course of 30-hour biotransformation reactions, it can be concluded that all haloindole consumed by pSTB7 transformants was initially converted to halotryptophan by the recombinant TrpBA, and that haloindole influx into cells was driven by this conversion. Indole is thought to predominantly enter bacteria via diffusion through the membrane, a process which would probably be aided by the presence of DMSO in the reaction buffer (Pinero-Fernandez *et al.,* 2011). Haloindole utilisation data (Figures 3b and 4b) reveal that MC4100 and its *ompR234* derivative PHL644 display an extremely rapid initial influx of haloindole within the first hour of planktonic reactions. This is not observed in planktonic reactions with MG1655 or PHL628, where indole influx is steadier. Initial halotryptophan production rates reflect these data (Table 1). Biofilm reactions display a different trend; rapid indole influx is only seen in PHL628 chloroindole reactions (Figure 6b), and indole influx is slower in PHL644 than PHL628. Again, this is probably due to the higher rate of halotryptophan production in biofilms of PHL628 than PHL644 (Table 1), driving haloindole influx via diffusion.

Since halotryptophan concentrations were measured here by HPLC in the cell-free extracellular buffer, all measured halotryptophan must have been released from the bacteria, either by active or passive processes. Therefore, conversion ratios of less than 100% must derive either from failure of halotryptophan to leave bacteria or alternative halotryptophan utilisation; the latter could be due to incorporation into proteins (Crowley *et al*., 2012) or degradation to haloindole, pyruvate and ammonia mediated by tryptophanase TnaA (Figure 1). Although regenerating haloindole, allowing the TrpBA-catalysed reaction to proceed again, this reaction would effectively deplete serine in the reaction buffer and so potentially limit total conversion. The concentration of serine could not be monitored and it was not possible to determine the influence of this reverse reaction. Deletion of *tnaA* would remove the reverse reaction, but since TnaA is required for biofilm production (Shimazaki *et al.,* 2012) this would unfortunately also eliminate biofilm formation so is not a remedy in this system.

Synthesis of TnaA is induced by tryptophan, which could explain the decrease in conversion selectivity over time observed in planktonic MG1655 and PHL628

t2.1 **Table 2 Percentage (mean ± S.D.) of *E. coli* PHL644 pSTB7 cells that were alive determined using flow cytometry**
t2.2 **during biotransformations performed with planktonic cells or biofilms**

t2.3 Reaction conditions	Cell type and time of sampling			
t2.4	Planktonic	Planktonic	Biofilm	Biofilm
t2.5	2 hours	24 hours	2 hours	24 hours
t2.6 Reaction Buffer, 5% DMSO	99.52 ± 0.14	99.32 ± 0.40	95.73 ± 2.98	92.34 ± 0.10
t2.7 Reaction Buffer, 5% DMSO, 2 mM 5-fluoroindole	99.38 ± 0.60	99.24 ± 0.80	96.44 ± 1.51	90.73 ± 0.35
t2.8 Reaction Buffer, 5% DMSO, 2 mM 5-chloroindole	99.27 ± 0.33	99.33 ± 0.20	95.98 ± 2.64	91.69 ± 3.09
t2.9 Reaction Buffer, 5% DMSO, 2 mM 5-bromoindole	99.50 ± 0.18	99.33 ± 0.20	96.15 ± 1.94	91.17 ± 2.19

chlorotryptophan reactions (Figure 4c); chlorotryptophan synthesis could potentially induce TnaA production and thus increase the rate of the reverse reaction. In other reactions, selectivity gradually increased over time to a plateau, suggesting that initial rates of halotryptophan synthesis and export were slower than that of conversion back to haloindole.

Taken together, these observations are likely due to underlying differences between strains MG1655 and MC4100 and between planktonic and biofilm cells in terms of: indole and tryptophan metabolism, mediated by TrpBA and TnaA; cell wall permeability to indole; and transport of tryptophan, which is imported and exported from the cell by means of transport proteins whose expression is regulated by several environmental stimuli. They underline the requirement to assess biotransformation effectiveness, both in terms of substrate utilisation and product formation, in multiple strains, in order that the optimal strain might be selected.

We had previously hypothesised that biofilms were better catalysts than planktonic cells for this reaction due to their enhanced viability in these reaction conditions, allowing the reaction to proceed for longer; however, flow cytometry reveals this to be untrue. Therefore, the reasons for extended reaction times in biofilms as compared to planktonic cells must be more complicated. A second possible reason for such behaviour could the higher plasmid retention of biofilm cells (O'Connell *et al.,* 2007) that could allow greater *trpBA* expression and thus more enzyme in biofilm cells. However, the initial rate of halotryptophan production per mass of dry cells were very similar in most of the cases apart from PHL628 pSTB7 and MG1655 pSTB7 for fluoroindole; therefore it appears that such hypothesis could be disregarded. Furthermore the similarity between the initial conversion rates between the two physiological states (biofilms and planktonic) suggests that mass transfer of haloindole through the biofilm was not the limiting step in the biotransformation because, if this was the case, lower initial conversion rates would have been found for biofilm reactions. Future studies will focus on the increased longevity of the reaction in biofilms when compared to planktonic cells, and the differences in tryptophan and indole metabolism in biofilms and planktonic cells.

In conclusion, in order to be used as engineered biofilms *E. coli* strains need to be able to readily generate biofilms, which can be achieved through the use of *ompR234* mutants. Despite the presence of native tryptophan synthase in *E. coli*, a plasmid carrying the *trpBA* genes under the control of a non tryptophan-repressed promoter was required to achieve detectable conversions of 5-haloindole to 5-halotryptophan. PHL644 pSTB7 returned the highest conversion when planktonic cells were employed in biotransformations but PHL628 pSTB7 gave the highest production of halotryptophan when biofilms were used. Higher viability is not the reason for biofilms' greater performance than planktonic cells; complex differences in indole and tryptophan metabolism and halotryptophan transport in biofilm and planktonic cells probably determine reaction efficiency. The results underline that biotransformation reactions need to be optimised in terms of host strain choice, recombinant enzyme production and method of growth for the chosen biocatalyst.

Additional file

Additional file 1: Supplemental methods, Figures S1-S5 and Table S1.

Competing interests
The authors declare that they have no competing interests.

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